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P21RAS and INSULIN SIGNAL TRANSDUCTION

P21RAS en INSULINE SIGNAAL TRANSDUCTIE
(Met een samenvatting in het Nederlands)

P21RAS e INSULINA SEGNALAZIONE
(Con una sintesi dei contenuti in Italiano)

Proefschrift

Ter verkrijging van de graad van doctor
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Voor mijn ouders

Aan Cristina en Susanna

Knowledge is not wisdom.

F.V. Zappa

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Abbreviations

ATP	adenosine 5'-triphosphate
cAMP	cyclic adenosine 3':5'-monophosphate
CAT	chloramphenicol acetyl transferase
CMV	cytomegalovirus
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMP	2,3-dimercaptopropanol
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
erk	extracellular signal-regulated kinase
FCS	fetal calf serum
GAP	GTPase activating protein
GDP	guanosine 5'-diphosphate
GNRP	guanine nucleotide releasing protein
GTP	guanosine 5'-triphosphate
IGF	insulin-like growth factor
IP_3	inositol triphosphate
IR	insulin receptor
PAO	phenylarsine oxide
PC	phosphatidylcholine
PCho	phosphocholine
PDGF	platelet-derived growth factor
PI	phosphatidylinositol
PI3K	phosphatidylinositol 3'-kinase
PIP_2	phosphatidylinositol 4,5-biphosphate
PKC	protein kinase C
PLC	phospholipase C
RSV	Rous sarcoma virus
RTK	receptor tyrosine kinase
SH	src homology
TCR	T cell receptor
TGF	transforming growth factor
TLC	thin layer chromatography
TPA	12-O-tetradecanoylphorbol-13-acetate
TRE	TPA responsive element

CHAPTER 1

Introduction

The role of p21ras in receptor tyrosine kinase signaling.

R.H. Medema and J.L. Bos, submitted for publication.

Abstract.

The notion that ras proteins are required for stimulation of mitogenesis by different receptor tyrosine kinases (RTKs) has urged researchers to investigate the precise role of p21ras in signal transduction. A large number of stimuli can drive p21ras in the active conformation and several proteins have been identified, which play an important role in regulating the GTP/GDP balance on p21ras. Indeed, activation of p21ras has been demonstrated to occur by stimulation of guanine nucleotide release proteins (GNRPs) or inhibition of GTPase activating proteins (GAPs). Moreover, a number of SH2-containing proteins have been implicated in this signaling pathway, such as shc and sem-5/grb2. On the other hand, downstream signaling from p21ras involves an important protein kinase cascade. This pathway seems to be conserved in evolution and analogous routes have been described in organisms such as yeast, nematodes and fruit flies. Nevertheless, the direct effector molecule of p21ras that could couple to this kinase cascade is still obscure. Some indications have been obtained that suggest that this function might partially be performed by p120GAP. This review will give an overview of the role of p21ras in signaling from diverse RTKs. Elucidation of this pathway will improve our understanding of mitogenic signaling pathways and the basis of cancer.

1. p21ras as molecular switch.

1.1. ras genes

Ras genes were initially identified as the transforming genes of the Harvey and Kirsten rat sarcoma virus strains (Harvey, 1964; Kirsten and Mayer, 1967). The cellular homologs of these viral ras genes were subsequently isolated as dominant oncogenes by their ability to transform NIH3T3 cells after DNA transfection (Chang *et al.*, 1982; Parada *et al.*, 1982; Shih and Weinberg, 1982). In addition, a third ras-oncogene, called N-ras, was isolated from a neuroblastoma cell line (Hall *et al.*, 1983; Shimizu *et al.*, 1983). The transforming genes turned out to be mutated alleles of the normal cellular ras genes (proto-oncogenes) (Reddy *et al.*, 1982; Tabin *et al.*, 1982). Oncogenic forms of p21ras have been detected in a large variety of human tumors and the transforming potential of the ras oncogenes can be attributed to a single amino acid substitution at position 12, 13 or 61 of the ras protein (reviewed by Bos, 1989).

The ras genes constitute a family of highly conserved genes, which code for 21 kDa proteins that are localized at the inner side of the plasma membrane (for review see Barbacid, 1987). The ras proteins can bind GTP and GDP with high affinity (Scolnick *et al.*, 1979; Shih *et al.*, 1980) and have intrinsic GTPase activity (McGrath *et al.*, 1984; Sweet *et al.*, 1984). By analogy with other small GTP-binding proteins, such as EF-Tu (Jurnak, 1985), the ras proteins were proposed to function as molecular switches, cycling between an inactive GDP-bound state, and an active GTP-bound state (reviewed by Bourne *et al.*, 1990a). Based on the radical changes in cellular growth that occur upon expression of

oncogenic mutants of p21ras, the GTP-bound protein was most likely to have a positive effect on cellular growth and differentiation. This hypothetical function was substantiated by a number of experimental observations. First, oncogenic p21ras can induce morphological transformation and proliferation of NIH3T3 cells (Feramisco *et al.*, 1984; Stacey and Kung, 1984), morphological differentiation of rat pheochromocytoma cells (PC12) (Bar-Sagi and Feramisco, 1985) and maturation of *Xenopus laevis* oocytes (Birchmeier *et al.*, 1985). The GTP-bound, but not the GDP-bound form of p21ras seems to be responsible for the observed effects (Satoh *et al.*, 1987; Trahey and McCormick, 1987). Second, oncogenic mutations in the ras proto-oncogenes invariably cause a loss of GTPase activity (McGrath *et al.*, 1984; Sweet *et al.*, 1984), or an increased rate of GTP/GDP exchange (Feig and Cooper, 1988b; Sigal *et al.*, 1986b), indicating that the transforming potential is a result of a higher steady-state level of the GTP-bound form of p21ras in the cell.

Mutational analysis of oncogenic forms of p21ras indicated that apart from the regions essential for nucleotide binding, two other domains are indispensable for transformation; the so-called "effector region" (residues 32-40) (Sigal *et al.*, 1986a; Willumsen *et al.*, 1986) and an intact CAAX-motif at the C-terminus of the protein, which is essential for membrane localization (Willumsen *et al.*, 1984). Mutations in the effector region destroy the transforming ability of oncogenic ras mutants without altering the cellular localization, stability, and nucleotide binding properties of the protein. Therefore, it has been proposed that the cellular effector of p21ras binds to this region. Crystal structures of the GDP- and the GTP-bound form of p21ras have learned that the most significant conformational differences that occur upon GTP hydrolysis are restricted to two regions of the protein that are exposed on the molecular surface (Milburn *et al.*, 1990; Schlichting *et al.*, 1990). One of these regions is the effector region, and it is plausible that the effector of p21ras binds specifically to GTP-bound p21ras by recognition of these two regions. In addition, analysis of the crystal structure of p21ras has provided more insight in the mechanism of the GTPase reaction of p21ras. A water molecule is hydrogen-bound to the carbonyl of Thr35, which positions it directly opposite of the β-phosphate of the guanine nucleotide, ideal for a nucleophilic attack on the γ-phosphate (Pai *et al.*, 1990). Oncogenic mutations at position 12 interfere with the binding of this water molecule, resulting in a protein unable to hydrolyze GTP, which is therefore locked in the active conformation (Krengel *et al.*, 1990).

Membrane-association of p21ras requires post-translational processing, starting with farnesylation of the cysteine residue of the CAAX-motif (Casey *et al.*, 1989; Hancock *et al.*, 1989), followed by a proteolytic cleavage of the last three amino acids (Gutierrez *et al.*, 1989) and methylation of the newly formed α-carboxyl group of the cysteine residue (Clarke *et al.*, 1988; Gutierrez *et al.*, 1989). These modifications are stable and allow some low avidity membrane binding and biological activity (Hancock *et al.*, 1989). In addition, p21ras requires palmityolation at cysteine residues located immediately upstream of the CAAX-motif (H-ras, N-ras, or K-ras(A)) (Buss and Sefton, 1986; Magee *et al.*, 1987), or a polybasic domain (K-ras(B)) (Hancock *et al.*, 1991a; Hancock *et al.*, 1991b) for efficient membrane-binding.

Palmityolation increases the avidity of membrane binding, but the palmityl group has a half-life of ~20 min (Magee *et al.*, 1987), indicating that high-affinity membrane binding of p21ras is unstable, which suggests that the localization of p21ras is subject to some kind of control mechanism.

Since membrane binding is a prerequisite for the transforming potential of oncogenic forms of p21ras, investigators have aimed at pharmacological approaches to control membrane binding of p21ras (reviewed by Gibbs, 1991). Drugs that inhibit biosynthesis of mevalonate, a precursor of sterols and other isoprenes such as farnesyl pyrophosphate, were shown to block the effects of oncogenic forms of p21ras in *Xenopus laevis* oocytes (Schafer *et al.*, 1989) and PC12 cells (Mendola and Backer, 1990). However, mevalonate is an important precursor for many other isoprenoid derivatives (Goldstein and Brown, 1990), and it seems unlikely that general inhibitors of mevalonate synthesis will provide fruitful anticancer reagents. Indeed, lovastatin, which can block the isoprenylation of p21ras, inhibits cell growth, but does so independent of the function of p21ras (DeClue *et al.*, 1991a). A more selective approach would be to inhibit the enzyme(s) required for farnesylation of p21ras. A protein-farnesyltransferase has been purified from rat brain (Reiss *et al.*, 1990), and specific inhibitors have been designed (Goldstein *et al.*, 1991; Reiss *et al.*, 1990). Still, this approach does not distinguish active oncogenic p21ras from normal p21ras or other proteins that are farnesylated, which is a major drawback for a possible drug therapy. Nevertheless, the finding that certain oncogenic forms of p21ras that no longer have the CAAX-motif can function as dominant negative mutants (Gibbs *et al.*, 1989; Medema *et al.*, 1991b; Michaeli *et al.*, 1989) is reason for optimism. These dominant negative mutants presumably couple efficiently to the effector molecule and can trap it in the cytosol, thereby blocking the formation of a functional complex at the membrane. Moreover, it has been shown that oncogenic forms of p21ras are more sensitive to this type of inhibition than normal p21ras (Farnsworth *et al.*, 1991; Stacey *et al.*, 1991). Thus, (a partial) inhibition of farnesylation is expected to have a profound effect on cells that express oncogenic p21ras, whereas normal cells would be less affected.

1.2. The ras-cycle.

The balance between GTP- and GDP-bound p21ras is controlled by its GTPase activity, as well as the exchange of GDP for GTP. This is exemplified by the transforming potential of mutations that affect either one of these characteristics (Feig and Cooper, 1988b; Sigal *et al.*, 1986b). However, the intrinsic GTPase activity of p21ras and the dissociation rate for GDP are very slow, which urged a search for factors that could accelerate these steps to ensure rapid cycling of p21ras in the cell. Two classes of regulatory proteins have been identified; GTPase activating proteins (GAPs), which accelerate GTP hydrolysis, and guanine nucleotide release proteins (GNRPs or exchange factors), which facilitate release of bound GDP, that under the conditions prevailing in the cytoplasm is most likely to result in the binding of GTP (for review see Bourne *et al.*, 1990b). Therefore, two control mechanisms for the activation state of p21ras exist, either through regulation of a GAP-activity, or through regulation of the activity of a GNRP (see Fig.1). The existence of these factors enables p21ras to respond rapidly to incoming

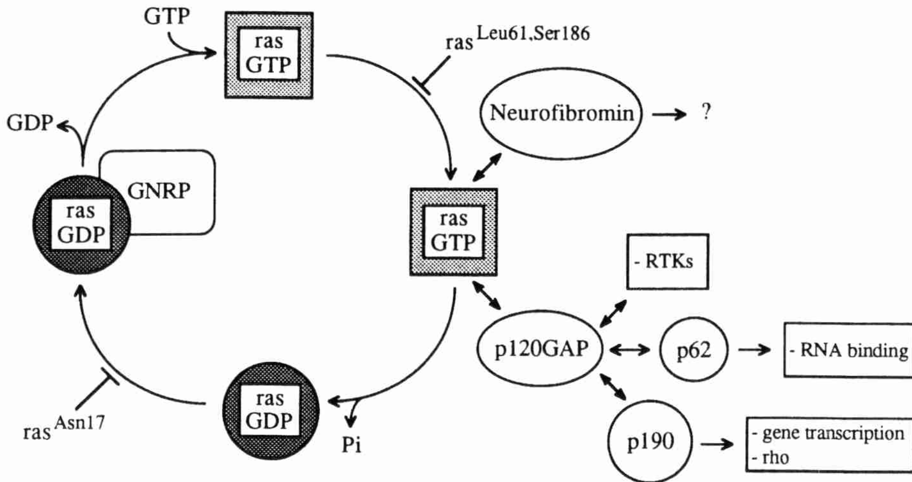

Fig.1. The p21ras-cycle. Schematic representation of the cycling of p21ras between the inactive GDP-bound conformation and the active GTP-bound conformation. GTP/GDP-exchange and GTP-hydrolysis on p21ras are relatively slow events. The exchange of GTP for GDP on p21ras is catalyzed by a GNRP (=guanine nucleotide releasing protein); and GTP-hydrolysis by p21ras is accelerated by two different GAPs (=GTPase activating proteins); p120GAP and neurofibromin. The ratio between p21ras-GDP and ras-GTP is therefore determined by the activity of the GNRPs compared to that of the GAPs. Accumulation of p21ras-GTP could be a consequence of an acceleration of GTP/GDP-exchange, as a result of activation of a GNRP. Alternatively, an inactivation of the GAPs would result in a slower GTP-hydrolysis, which would also amount to an increase in p21ras-GTP. The active GTP-bound conformation of p21ras interacts with the effector molecule, which could be p120GAP or NF-1. Potential targets of the p21ras-p120GAP complex are indicated, such as; the activated RTKs (shown to bind p120GAP), p62 (an RNA-binding protein), p190 (which has GAP-activity for the rho-family of small GTP-binding proteins and has homology with the glucocorticoid repressor).

signals and at the same time makes it possible for the signal to be quickly shut off, which is a prerequisite for efficient signal transduction.

Until now, two different proteins with GAP-activity for p21ras have been identified in mammalian cells, namely p120GAP (Gibbs *et al.*, 1988; Trahey *et al.*, 1988; Vogel *et al.*, 1988) and the product of the neurofibromatosis type 1 (NF-1) gene (Ballester *et al.*, 1990; Cawthon *et al.*, 1990; Martin *et al.*, 1990; Viskochil *et al.*, 1990; Wallace *et al.*, 1990; Xu *et al.*, 1990a). p120GAP was first identified as an abundant cytoplasmic factor from *Xenopus* oocytes with the ability to stimulate the GTPase activity of p21ras (Trahey and McCormick, 1987). The p120GAP protein fails to stimulate the hydrolysis of GTP on oncogenic mutants of p21ras, although the physical interaction between the two proteins was judged to be normal on the basis of competition experiments (Vogel *et al.*, 1988). The effect of p120GAP on p21ras suggests that it functions as a negative, upstream regulator of p21ras. The finding that overexpression of p120GAP can suppress transformation of NIH3T3 cells by normal Ha-ras supports this notion (Zhang *et al.*, 1990). Also, overexpression of p120GAP reduces the level of GTP-bound p21ras in unstimulated, as well as platelet-derived growth factor (PDGF)-stimulated cells (Gibbs *et al.*, 1990). In addition to this negative regulatory function, p120GAP may also be required for p21ras effector function, since it binds to the effector region (Adari *et al.*, 1988; Cales *et al.*, 1988).

The NF-1 gene was cloned by different groups as the gene affected in patients suffering from von Recklinghausen neurofibromatosis (Cawthon *et al.*, 1990; Viskochil *et al.*, 1990; Wallace *et al.*, 1990). The sequence showed significant similarity to p120GAP in the region known to be essential for interaction with ras proteins, and subsequent experiments with recombinant NF-1 protein (neurofibromin) showed it could also function as an upstream, negative regulator of p21ras (Ballester *et al.*, 1990; Martin *et al.*, 1990; Wallace *et al.*, 1990; Xu *et al.*, 1990b). Neurofibromin binds p21ras with a much higher affinity than p120GAP, indicating that it could be a more significant regulator of p21ras than p120GAP, considering the concentration of p21ras in the cell (Bollag and McCormick, 1991). Indeed, a reduction in the level of expression of neurofibromin in Schwannoma cells results in an elevation of the level of GTP-bound p21ras, although the expression of p120GAP is normal (Basu *et al.*, 1992; DeClue *et al.*, 1992). Low basal levels of p21ras-GTP in these cells could be restored by overexpression of the catalytic domain of p120GAP, indicating that p120GAP can also function as negative regulator when present in high amounts. Nevertheless, one could argue whether p120GAP functions as a true negative regulator of p21ras *in vivo*. The action of p120GAP *in vitro* is blocked by physiological salt concentrations (Gibbs *et al.*, 1988) and interference with p21ras signaling has only been observed when p120GAP is expressed at high levels (Bortner *et al.*, 1991; DeClue *et al.*, 1991b; Nori *et al.*, 1991). At this point a regulatory function for p120GAP should definitely not be excluded, nor should it be taken for granted.

Several groups have reported the identification and partial purification of exchange factors from mammalian cells with the ability to stimulate release of GDP from recombinant ras proteins. First, a cytosolic protein with a molecular size between 100 and 160 kDa, which is active on ras proteins but not on other small GTP-binding proteins, was purified from rat brain (Wolfman and Macara, 1990). Second, a cytosolic protein of about 60 kDa, which also specifically stimulates the rate of guanine nucleotide exchange of several ras proteins, was purified from human placenta (Downward *et al.*, 1990b). Third, a membrane-associated protein was purified from bovine brain with similar activity (West *et al.*, 1990). However, this membrane-associated protein of 35 kDa turned out to increase exchange of guanine nucleotides on all small GTP-binding proteins that were tested (Huang *et al.*, 1990).

More recently, several (putative) mammalian GNRPs for p21ras were cloned by homology to yeast *Saccharomyces cerevisiae* and *Drosophila melanogaster* ras-GNRPs. In *Saccharomyces cerevisiae* genetic and biochemical evidence indicate that the CDC25 gene product is the major GNRP for RAS (Broek *et al.*, 1987; Jones *et al.*, 1991), and in *Drosophila melanogaster* the son of sevenless (SOS) gene product has been identified genetically as the potential GNRP for *ras1* (Bonfini *et al.*, 1992; Rogge *et al.*, 1991; Simon *et al.*, 1991). The first mammalian GNRP shown to be specific for p21ras was cloned by homology to CDC25 from a rat brain library (Shou *et al.*, 1992). Antibodies against the protein product detected a protein of 140 kDa in brain cytosol, similar to the molecular weight of a mammalian protein that crossreacts with anti-CDC25 polyclonal antibodies (Gross *et al.*, 1992b). Other mammalian CDC25 homologs were cloned by

functional complementation of yeast CDC25 minus mutants with a mouse cDNA expression library (Martegani *et al.*, 1992). The predicted protein encoded by this partial cDNA contains a region of 234 amino acids with 34% homology to CDC25, and a recombinant protein that contains this region was later shown to stimulate guanine nucleotide exchange on p21ras (Jacquet *et al.*, 1992). Multiple full length cDNAs were subsequently isolated from a mouse brain cDNA and these CDC25-like cDNAs seem to be expressed in a tissue specific manner (Cen *et al.*, 1992). Antibodies raised against a peptide encoded by all cDNAs detected proteins of 75 and 95 kDa in NIH3T3 cells. In addition, two putative mammalian GNRPs for p21ras have been cloned by homology to SOS from a mouse eye cDNA library and these seem to be expressed ubiquitously (Bowtell *et al.*, 1992). However, evidence that their gene products can specifically enhance exchange of guanine nucleotides on p21ras has not yet been provided. Finally, smg p21 GDS, originally cloned as GNRP for two ras-related small GTP-binding proteins, smg p21 A and -B (or rap1A and rap1B), can also stimulate exchange on p21K-ras, but not p21Ha-ras or p21N-ras (Kaibuchi *et al.*, 1991; Mizuno *et al.*, 1991). Thus, it appears that a class of mammalian GNRPs for p21ras exists with a tissue specific distribution.

Remarkably, in addition to the homology with CDC25 in the C-terminal region of the proteins encoded by the mammalian CDC25 homologs, a 241 amino acid domain near the N-terminus shares homology with the human breakpoint cluster region (bcr) protein (Hariharan and Adams, 1987), the vav oncogene product (Bustelo *et al.*, 1992; Katzav *et al.*, 1989; Margolis *et al.*, 1992) and the dbl oncogene product (Ron *et al.*, 1988) (see Fig.2).

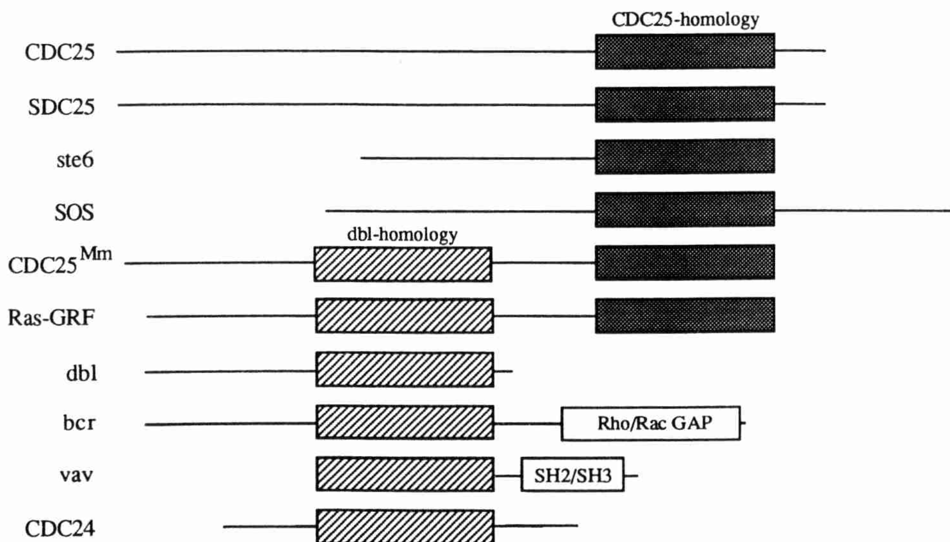

Fig.2. Homologous regions in guanine nucleotide release proteins (GNRPs). (Putative) GNRPs for ras and ras-like proteins from *S. cerevisiae* (CDC25, SDC25, CDC24), *S. pombe* (ste6), Drosophila (SOS) and mammalians (Ras-GRF, $CDC25^{Mm}$, dbl, bcr and vav) and their homologous domains. Regions of CDC25-homology are represented by grey boxes, regions of dbl-homology are indicated by hatched boxes. In addition, the SH2/SH3 domains in vav and the region of bcr that is involved in stimulation the GTPase activity of Rho- and Rac-like proteins are indicated.

These latter proteins are assumed to regulate ras-like small GTP-binding proteins. Bcr encodes a GAP protein for p21rac (Diekmann *et al.*, 1991), which is involved in growth factor-induced membrane ruffling (Ridley *et al.*, 1992). The region of bcr that is important for this activity is distinct from the region of homology shared with dbl and the ras-GNRPs. The dbl oncogene appears to be the mammalian homolog of CDC24 from *Saccharomyces cerevisiae* and has been shown to encode a GNRP for CDC42Hs (Hart *et al.*, 1991), which is closely related to the rac and rho proteins (Munemitsu *et al.*, 1990). Therefore, it is possible that the GNRPs for p21ras are active on different members of the ras superfamily, allowing crosstalk between different signaling pathways. Such crosstalk is also possible at the level of the GAP proteins, since p120GAP has been shown to associate with a protein of 190 kDa (Ellis *et al.*, 1990), which has a region of similarity with bcr (Settleman *et al.*, 1992b). The 190 kDa protein has been shown to have GAP-like activity for the rho family of small GTP-binding proteins (Settleman *et al.*, 1992a). Therefore, the activation state of a number of small GTP-binding proteins may be tightly linked through bifunctionality, or direct interaction, of their regulatory proteins. Such a fine-tuning of small GTP-binding proteins has been observed in the case of rac and rho, where growth-factor induced activation of p21rac seems to drive activation of p21rho (Downward, 1992; Ridley and Hall, 1992; Ridley *et al.*, 1992).

1.3. Ras-mediated effects.

The transforming potential of oncogenic forms of p21ras and the similarity to other small GTP-binding proteins, implies that ras proteins function in signal transduction pathways that control cell proliferation. This hypothesis was initially tested by microinjection of recombinant ras proteins to analyze the direct effects of p21ras on different cell types. Such an approach has the advantage that long term and indirect effects of transformation that occur after introduction of oncogenic p21ras by DNA transfection can be disregarded. In NIH3T3 cells microinjection of p21ras causes morphological transformation and stimulation of DNA synthesis in the absence of growth factors (Feramisco *et al.*, 1984; Stacey and Kung, 1984). Also, a large variety of other effects have been reported, such as; membrane ruffling (Bar-Sagi and Feramisco, 1986), induction of c-fos expression (Stacey *et al.*, 1987), maturation and stimulation of diacylglycerol production in *Xenopus laevis* oocytes (Birchmeier *et al.*, 1985; Lacal *et al.*, 1987b) and differentiation of PC12 cells (Bar-Sagi and Feramisco, 1985; Noda *et al.*, 1985). Since microinjection is rather laborious and only a limited number of cells can be studied, scrape-loading of p21ras into quiescent cells was used to study the role of p21ras in the activation of cellular kinases. Scrape-loading of active p21ras in Swiss 3T3 cells leads to the activation of protein kinase C (PKC) (Morris *et al.*, 1989), induction of c-myc expression (Lloyd *et al.*, 1989) and DNA synthesis (Morris *et al.*, 1989). In addition, a rapid activation of extracellular signal-regulated kinases (erks) was observed after scrape-loading of oncogenic p21ras (Leevers and Marshall, 1992). These findings indicated that active p21ras can indeed mimic the action of growth factors, suggesting that p21ras functions as mediator of their message.

To analyze which cellular stimuli actually require the function of p21ras for efficient signal

transduction, a monoclonal antibody (Y13-259) that can neutralize the function of p21ras, was used in microinjection experiments. Microinjection of Y13-259 has shown that the function of p21ras is required for growth factor-induced cell proliferation (Mulcahy *et al.*, 1985), as well as transformation by a number of viral oncogenes (Smith *et al.*, 1986). In *Xenopus laevis* oocytes, ras proteins are required for insulin-induced maturation (Korn *et al.*, 1987) and in PC12 cells NGF-induced differentiation is blocked by microinjection of Y13-259 (Hagag *et al.*, 1986). An alternative approach to elucidate the involvement of p21ras in certain signaling routes was made possible by the isolation of a mutant of p21ras with the ability to inhibit cellular p21ras function *in vivo* (Feig and Cooper, 1988a). This dominant inhibitory mutant, p21ras(Asn17), has a reduced affinity for GTP, and is therefore locked in the inactive state. Its inhibitory potential is thought to be due to a very efficient binding of the GNRP for p21ras, thereby blocking the activation of normal p21ras (Farnsworth and Feig, 1991). Recently, direct proof that this mutant inhibits the activation of p21ras by different extracellular stimuli has been obtained (Medema *et al.*, 1993). Expression of p21ras(Asn17) inhibits proliferation of normal and v-src transformed NIH3T3 cells (Feig and Cooper, 1988a). Also, p21ras(Asn17) has been used to show a requirement for p21ras in NGF-induced differentiation of PC12 cells (Szeberényi *et al.*, 1990), growth factor-induced stimulation of DNA synthesis (Cai *et al.*, 1990) and growth factor-induced fos-expression in NIH 3T3 cells (Cai *et al.*, 1990; Medema *et al.*, 1991b). Furthermore, induction of IL-2 expression in response to protein kinase C and T cell receptor stimulation in T lymphoblasts is inhibited in cells expressing p21ras(Asn17) (Rayter *et al.*, 1992). Recently, the activation of extracellular signal-regulated kinases (erks), raf-1 kinase and a ribosomal S6 kinase (pp90rsk) by a variety of growth factors was shown to require the function of p21ras by making use of p21ras(Asn17) (de Vries-Smits *et al.*, 1992; Thomas *et al.*, 1992; Wood *et al.*, 1992). Another dominant inhibitory mutant of p21ras, p21ras(Leu61Ser 186), which apparently interferes with binding of the effector molecule to active p21ras at the cell membrane, inhibits insulin-induced maturation of *Xenopus laevis* oocytes, and insulin-induced gene expression (Gibbs *et al.*, 1989; Medema *et al.*, 1991b). Altogether, these observations place p21ras at a crucial point in signaling pathways that regulate proliferation, as well as differentiation. Ras proteins apparently exert their effects through activation of cellular kinases and induction of expression of certain early response genes. The most straightforward cascade of events would be activation of p21ras in response to these extracellular stimuli, in turn causing activation of cellular kinases. However, based on the experiments described above it is also possible that p21ras activation is not a direct consequence of these extracellular stimuli but that manifestation of their effects requires the function of p21ras in a separate pathway.

2. p21ras in receptor tyrosine kinase signaling.

2.1. Activation of p21ras.

Although a requirement for p21ras activity in RTK signaling had been proposed as early as 1986 on the basis of experiments with neutralizing antibodies against p21ras (Smith *et al.*,

1986), no direct evidence for activation of p21ras by these RTKs was available for some time. The first reports that external signals control the activation state of p21ras came from different groups, one group used stimulation of the antigen receptor of T lymphocytes to induce a rapid and dramatic activation of p21ras (Downward *et al.*, 1990a), whereas others showed that PDGF and serum can cause a small but consistent increase in the level of p21ras.GTP (Gibbs *et al.*, 1990; Satoh *et al.*, 1990b). Since then a variety of extracellular stimuli have been shown to cause an activation of p21ras. Epidermal growth factor (EGF) (Osterop *et al.*, 1993; Satoh *et al.*, 1990a), insulin (Burgering *et al.*, 1991), thrombin and lysophosphatidic acid (LPA) (van Corven *et al.*, 1993), were shown to drive p21ras into its active conformation in fibroblasts. Also, in cells carrying activated oncogenes with tyrosine kinase activity, such as v-src, v-abl or erbB2/neu, the levels of p21ras-GTP are increased (Gibbs *et al.*, 1990; Satoh *et al.*, 1990a). In PC12 cells NGF and EGF induce an accumulation of the GTP-bound conformation of p21ras (Qiu and Green, 1991). This can also be achieved by stimulation of PC12 cells with fibroblasts growth factor (FGF), insulin, insulin-like growth factor (IGF)-1 and interleukin (IL)-6, but only in the presence of sodium vanadate (Nakafuku *et al.*, 1992). In addition, many cytokines including IL-2, IL-3, IL-5 (Duronio *et al.*, 1992), granulocyte/macrophage colony stimulating factor (GM-CSF) (Satoh *et al.*, 1991), erythropoietin (Torti *et al.*, 1992) and steel factor (Duronio *et al.*, 1992) activate p21ras in haematopoietic cells. Also, stimulation of epithelial cells with transforming growth factor β (TGFβ) results in activation of p21ras (Mulder and Morris, 1992). Most recently, a relatively slow, but dramatic activation of p21ras was observed in response to UV-irradiation (Devary *et al.*, 1992). Activation of p21ras by a specific extracellular stimulus appears to be independent of the cell type, since transfection of the IL-2 receptor into a murine myeloid progenitor cell line confers IL-2 responsiveness to these cells with respect to p21ras activation (Izquierdo *et al.*, 1991). Similarly, EGF-stimulation of a myeloid cell line containing a stably transfected EGF receptor results in activation of p21ras (Satoh *et al.*, 1991). It is clear that activation of p21ras can occur in response to a large variety of extracellular stimuli, which indicates that they share at least some common intracellular pathways. This is somewhat surprising since the growth regulatory receptors through which these different factors signal and the respective cellular responses they elicit are rather diverse.

2.2. Mechanism of activation.

Activation of p21ras in response to triggering of a growth regulatory receptor could occur through two basically different mechanisms. First, in analogy with receptor coupled G proteins, agonist binding could stimulate GTP/GDP exchange. Conversely, growth factors could cause an inactivation of GAP activity. In T lymphocytes activation of p21ras by the TCR and CD2 antigens correlates well with a decrease in GAP activity measured in cell extracts (Downward *et al.*, 1990a; Graves *et al.*, 1991). Also, erythropoietin-treatment of human erythroleukemia cells causes an inhibition of *in vitro* GAP activity, although the timing of this inhibition does not exactly coincide with the time course of p21ras-activation (Torti *et al.*, 1992). Remarkably, exchange of nucleotide onto p21ras

in permeabilized T lymphocytes is very rapid, both in quiescent and stimulated cells, suggesting a constitutively active GNRP is present in these cells (Downward *et al.*, 1990a). In contrast, in fibroblasts no evidence for an inactivation of GAP activity in response to different growth factors has been obtained, suggesting a different mechanism of activation. Indeed, activation of nucleotide exchange is (at least partially) responsible for insulin- and EGF-induced activation of p21ras (Medema *et al.*, 1993). Also, NGF-induced activation of p21ras correlates with an increase in membrane-associated exchange activity, although this activity may not be ras-specific and coincides with a matching increase in GAP-activity (Li *et al.*, 1992a). Indirect evidence has also suggested that activation of p21ras in response to serum or PDGF is mediated by enhanced nucleotide exchange rather than by inactivation of GAPs (Zhang *et al.*, 1992). Therefore, the intracellular pathway that eventually leads to activation of p21ras seems to be dependent on the type of receptor that is causing the effect.

Diversity in the mechanism by which activation of p21ras can occur is also illustrated by the fact that PKC can regulate p21ras in T lymphocytes, whereas a non-PKC-dependent pathway has been proposed to cause activation of p21ras in response to insulin (Medema *et al.*, 1991a), PDGF (Molloy *et al.*, 1992), IL-2 (Izquierdo *et al.*, 1991) and NGF (Nakafuku *et al.*, 1992). Moreover, PKC activation by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in fibroblasts does not result in activation of p21ras (Medema *et al.*, 1991a). The TCR-induced activation of p21ras is mediated only partially by PKC, and an alternative pathway independent of PKC coexists in T cells (Izquierdo *et al.*, 1992). This PKC-independent pathway also causes a reduction in GAP activity and is sensitive to tyrosine kinase inhibitors.

Tyrosine phosphorylation seems to be an important event for p21ras activation by all growth regulatory factors studied so far. For example, tyrosine kinase inhibitors can block activation of p21ras by NGF, EGF (Qiu and Green, 1991), steel factor, IL-3 (Duronio *et al.*, 1992), erythropoietin (Torti *et al.*, 1992), phorbol esters, IL-6 (Nakafuku *et al.*, 1992) and LPA (van Corven *et al.*, 1993). Activation of p21ras by insulin, EGF and LPA is inhibited by phenylarsine oxide, a putative tyrosine phosphatase inhibitor (Medema *et al.*, 1991a and R.H.M. and J.L.B. unpublished observations). Tyrosine kinase activity seems also to be one of the initial events in TCR signaling. Although the T cell receptor (TCR) itself has no tyrosine kinase activity, a large body of evidence indicates that it is functionally coupled to non-receptor tyrosine kinases in T cells (reviewed in Klausner and Samelson, 1991) (see Fig.3). Among the candidates are p56lck and p59fyn, two tyrosine kinases of the src-family, and ZAP-70, a tyrosine kinase of 70 kDa that associates to the ζ-subunit of the TCR in response to receptor activation (Chan *et al.*, 1992). As already mentioned, the PKC-independent pathway that causes p21ras activation in response to TCR-activation is inhibited by a tyrosine kinase inhibitor (Izquierdo *et al.*, 1992). Therefore, a tyrosine phosphorylation/dephosphorylation cascade could very well precede activation of p21ras.

Clearly, signaling via p21ras is a common pathway for many growth regulatory factors. However, variations exist in the mechanism of ras activation, as well as the persistence and extent of activation. For instance, some growth

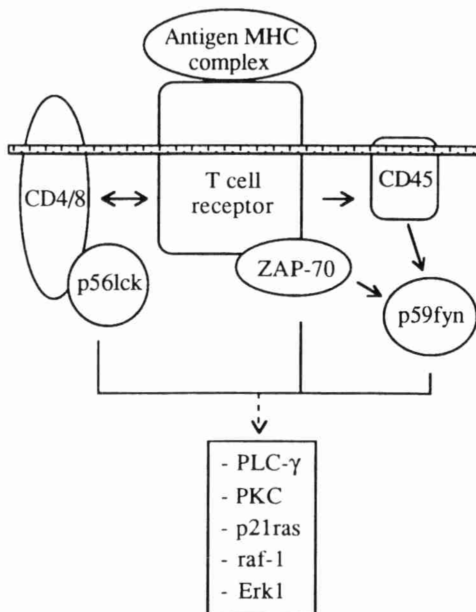

Fig.3. T cell receptor signaling via non-receptor tyrosine kinases. The T cell receptor complex, composed of a αβ heterodimer, associated with a number of CD3 polypeptides, namely: a ζζ homodimer (ζ-module) and a δε and γε heterodimer (ε-module) (see Abraham *et al.*, 1992 for a review), is activated upon binding of the antigen-major histocompatibility complex. This binding results in activation of non-receptor tyrosine kinases, p56lck (which is associated to the CD4 and CD8 molecules), p59fyn and ZAP-70 (which associates to the ζ-subunits). Efficient coupling of TCR-activation to p56lck and p59fyn activation is dependent on dephosphorylation of p56lck and p59fyn by the transmembrane tyrosine phosphatase CD45. Activation of this signaling complex results in activation of PLC-γ, PKC, p21ras (via inhibition of GAP-activity) raf-1 kinase and erk1.

regulatory factors seem to exert their effect on p21ras through inactivation of GAP activity (Downward *et al.*, 1990a; Torti *et al.*, 1992), whereas others can activate exchange factors (Li *et al.*, 1992a; Medema *et al.*, 1993), but it is very well possible that a combination of both is required for the full effect. Also, the increase in p21ras-GTP can be very transient, as is the case for thrombin-induced ras activation in CCL39 cells (van Corven *et al.*, 1993), but on the other hand, insulin-induced ras activation can persist for more than one hour (Osterop *et al.*, 1993). Finally, activation of p21ras in response to T cell activation results in 50% of total p21ras complexed to GTP (Downward *et al.*, 1990a), but PDGF-stimulation of NIH 3T3 cells causes only 15% of total p21ras to bind GTP (Gibbs *et al.*, 1990). Thus, the mechanism of the activation of p21ras and the ultimate effect on p21ras-GTP levels seem to be dependent on the stimulus and the cell type under investigation.

2.3. Receptor tyrosine kinases.

Some of the early events that occur upon RTK activation have been studied extensively. Receptors with tyrosine kinase activity possess a glycosylated extracellular ligand binding domain, a single hydrophobic transmembrane region and a cytoplasmic domain that contains a tyrosine kinase catalytic domain. The insulin-like growth factor (IGF)- and insulin-receptor tyrosine kinases are somewhat exceptional since they pre-exist as a disulfide stabilized receptor dimer of two αβ chains. Ligand binding to its cognate RTK triggers a large number of cellular responses, eventually leading to a stimulation of cell proliferation. Depending on the cell type and the type of receptor, these effects include; stimulation of tyrosine kinase activity, stimulation of serine/threonine kinase activity, elevation of intracellular Ca^{2+} concentration, activation of phospholipase C-γ (PLC-γ), activation of phosphatidylinositol-3'-kinase (PI3K), increased phosphatidylinositol turnover, stimulation of glucose and amino acid transport and stimulation of Na/H antiport activity (Yarden and Ullrich, 1988) (see Fig.4). The tyrosine kinase domain of all RTKs and non-receptor tyrosine kinases con-

tains a consensus sequence, GlyXGlyXXGlyX (15-20)Lys, that functions as part of the binding site for ATP, which is the phosphate-donor for the kinase reaction (Hanks *et al.*, 1988). Replacement of the lysine residue in this consensus in PDGF-, EGF- and insulin receptors completely abolishes their kinase activity (Chen *et al.*, 1987; Chou *et al.*, 1987; Ebina *et al.*, 1987; Escobedo *et al.*, 1988; McClain *et al.*, 1987). Although ligand binding characteristics are normal, these kinase minus mutants are not mitogenic when transfected into cells (Chen *et al.*, 1987; Chou *et al.*, 1987; Honegger *et al.*, 1987; Williams, 1989). In addition, many ligand-induced effects appear to be dependent on the tyrosine kinase activity of the receptor (Escobedo *et al.*, 1988; Glenney *et al.*, 1988; Honegger *et al.*, 1987; Moolenaar *et al.*, 1988; Russell *et al.*, 1987; Westermark *et al.*, 1990). This indicates that the activation of tyrosine kinase activity is the first event in the signaling cascades initiated by RTKs. Activation of these cascades will eventually lead to proliferation, an event that seems to require the function of p21ras. Other receptors that lack intrinsic tyrosine kinase activity, such as the T cell receptor, are thought to be linked to non-receptor tyrosine kinases. The events that occur upon activation of members of the non-receptor tyrosine kinases, like for instance c-src, show many similarities to those observed in response to activated RTKs (Cantley *et al.*, 1991). However, here we will limit ourselves to a discussion of signaling pathways initiated by RTKs.

The tyrosine kinase activity of RTKs is stimulated by ligand binding to the extracellular domain, which induces dimerization (except for the IGF- and insulin-receptors). The adjacent cytosolic domains will then cross-phosphorylate each other on tyrosine residues, which causes a conformational change that presumably enhances kinase activity (Schlessinger, 1988). In the case of the IGF- and insulin-receptor, ligand binding is thought to cause a conformational change in the extracellular domain and, consequently, a conformational change in the cytosolic domain could result in kinase activation (Ullrich and Schlessinger, 1990). Upon agonist binding, RTKs will autophosphorylate on specific tyrosine residues and phosphorylate a panel of cellular substrates, that control distinct signaling pathways, including activation of p21ras.

2.4. RTK signaling complex.

Signaling proteins that physically associate to various activated RTKs include; PLC-γ (Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989; Rottapel *et al.*, 1991), p120GAP (Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990; Margolis *et al.*, 1990a; Pronk *et al.*, 1992; Reedijk *et al.*, 1990), PI3K (Bjorge and Kudlow, 1987; Coughlin *et al.*, 1989; Kazlauskas and Cooper, 1989; Reedijk *et al.*, 1990; Rottapel *et al.*, 1991; Varticovski *et al.*, 1989), pp60src (Kypta *et al.*, 1990) and raf-1 kinase (App *et al.*, 1991; Morrison *et al.*, 1989; Morrison *et al.*, 1988) (see Fig.4). In case of the insulin receptor, PI3K associates to the docking protein IRS-1 (Sun *et al.*, 1991). Therefore, ligand binding may result in the formation of a signaling complex, consisting of the active RTK and a number of associated proteins, which triggers the activation of cellular signal transduction molecules. The fact that these RTKs seem to require the function of p21ras for stimulation of mitogenicity, raises the question which components of this signaling complex are involved in activation of p21ras.

The association of p120GAP to various acti-

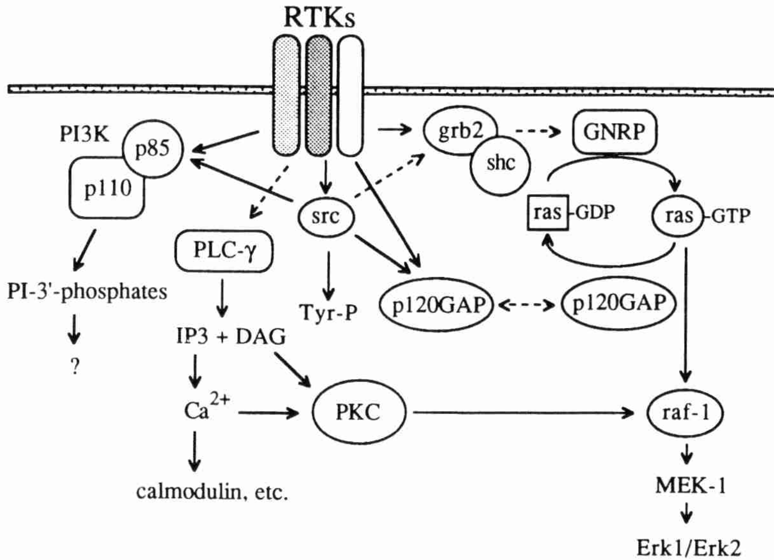

Fig.4. RTK-signaling pathways. Signaling pathways that are activated upon agonist binding to its cognate RTK. 1) PI3K associates to and becomes phosphorylated by the activated RTKs and catalyzes phosphorylation of phosphatidylinositols at the 3' position of the inositol rings. 2) Phospholipase C-γ, associates to and is phosphorylated by the active RTKs and produces DAG and IP3, which in turn will cause activation of PKC and a release of Ca^{2+} from intracellular stores. 3) c-src is activated in response to various growth factors, and will result in increased tyrosine phosphorylation of a number of proteins. 4) p120GAP associates to a number of activated RTKs and is also involved in regulation of p21ras, although a clear linkage between both phenomena is unclear at this moment. 5) Grb2 and shc associate to activated RTKs and are most likely involved in the pathway towards p21ras, presumably via the GNRP for p21ras. Activation of p21ras results in activation of raf-1, which in turn activates MEK-1 and erk1 and erk2.

vated RTKs and its subsequent tyrosine phosphorylation provides the most obvious possible link between RTK signaling and p21ras signal transduction pathways (Ellis *et al.*, 1990; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990; Molloy *et al.*, 1989; Pronk *et al.*, 1992; Reedijk *et al.*, 1990). However, growth factors that drive p21ras into the active conformation and simultaneously stimulate tyrosine phosphorylation of p120GAP have no obvious effect on the overall GAP-activity as measured in an *in vitro* assay (Gideon Bollag and Frank McCormick unpublished results, Boudewijn Burgering unpublished observations). Moreover, for insulin-induced activation of p21ras in NIH 3T3-L1 fibroblasts, p120GAP phosphorylation is not required (Porras *et al.*, 1992) and PDGF receptor mutants that will no longer bind and phosphorylate p120GAP on tyrosine can still signal through p21ras (Burgering *et al.*, 1993b). In response to various activated RTKs, p120GAP tightly associates to two tyrosine-phosphorylated proteins of 62 kDa and 190 kDa (Ellis *et al.*, 1990). Although a reduction in the *in vitro* GAP-activity towards p21ras has been observed upon association with the 190 kDa protein (Moran *et al.*, 1991) and the activated EGF-receptor (Serth *et al.*, 1992), no correlation has been established between these associations and activation state of p21ras. Thus, even if these modifications of p120GAP would affect its activity towards p21ras, they are not the only determinants of the

activation state of p21ras. These findings do not necessarily mean that regulation of p120GAP can not contribute to the activation of p21ras, but indicate that mechanisms, different from tyrosine phosphorylation and receptor association must exist. For example, a reduction in GAP-activity could be caused by activation of an inhibitor protein (or lipid) and this inhibition could be lost upon lysis of the cells and would therefore remain undetected in an *in vitro* assay. Tyrosine phosphorylation of p120GAP could of course have important consequences for its function as effector molecule (as will be discussed later). Whatever the effect of RTKs on the enzymatic activity of p120GAP, some RTKs have been shown to stimulate nucleotide exchange on p21ras, which indicates that other pathways exist.

Raf-1 kinase is a serine/threonine kinase that is phosphorylated on serine residues in response to many growth factors, concomitant with an increase in kinase activity (Morrison *et al.*, 1988). The raf-1 kinase has been proposed to participate in the RTK signaling complex, since receptor-association has been observed with the PDGF- and EGF-receptors (App *et al.*, 1991; Morrison *et al.*, 1989). However, receptor-association to the PDGF-receptor was not observed by others (Baccarini *et al.*, 1990) and no data about the requirement of this association for mitogenesis have been obtained, nor has the site of association for raf-1 kinase been mapped. Also, unlike the other RTK-substrates discussed here, raf-1 kinase does not contain an SH2 domain (discussed in detail below). Therefore, a definite conclusion about the significance of the association of raf-1 kinase to the PDGF- and EGF-receptor signaling complexes awaits further study. As for a possible role for raf-1 kinase as intermediate between the RTKs and p21ras, data have been obtained that place raf-1 kinase downstream of p21ras in RTK signaling (as will be discussed below).

PLC-γ converts phosphatidylinositol(PI)-4,5-biphosphate (PIP_2) into diacylglycerol (DAG) and inositol-triphosphate (IP_3), both representing second messengers (Berridge, 1987; Nishizuka, 1984). DAG is the natural activator of PKC, which in turn may phosphorylate a variety of proteins on serine and threonine and IP_3 is released into the cytosol where it binds to a specific intracellular receptor, leading to a release of Ca^{2+} from intracellular stores. Ca^{2+} can act as a cofactor for many enzymes, including PKC and calmodulin (Berridge, 1987). PLC-γ_1 and -γ_2 couple PDGF stimulation to PI turnover, whereas the β-isoforms appear to mediate the classical G protein coupled PI response (Margolis *et al.*, 1990b; Sultzman *et al.*, 1991). PLC-γ seems to require tyrosine phosphorylation for its activity *in vivo* (Kim *et al.*, 1991). The activity of PLC-γ is negatively regulated by profilin, which binds the major substrate of PLC-γ, PIP_2, with high affinity (Goldschmidt-Clermont *et al.*, 1990). Phosphorylation of PLC-γ is necessary to hydrolyze PIP_2 that is bound to profilin (Goldschmidt-Clermont *et al.*, 1991). Hydrolysis of PIP_2 is assumed to relieve the inhibition of PIP_2 on the function of profilin as a cofactor in actin polymerization and cytoskeletal reorganization (Goldschmidt-Clermont and Janmey, 1991). This indicates that PLC-γ might control growth factor-induced actin rearrangement, although activation of PLC-γ per se is not sufficient for this effect (Severinsson *et al.*, 1990). Also, activation of PLC-γ by different RTKs does not seem to mediate the effects on DNA synthesis. For instance, a mutant of the

PDGF receptor that lacks the kinase-insert domain (ΔKi), can induce PI turnover, but is no longer mitogenic (Escobedo and Williams, 1988). Furthermore, insulin- and CSF-1-receptors can stimulate mitogenesis although no effect on PLC-γ activity has been observed (Downing *et al.*, 1989) and mutants of the FGF receptor that do not stimulate PI turnover are still mitogenic (Mohammadi *et al.*, 1992; Peters *et al.*, 1992). These findings make it unlikely that PLC-γ could in some way mediate activation of p21ras, since this seems to be required for the mitogenic effect of RTKs.

PI3K catalyzes the phosphorylation of PI at the 3' position of the inositol ring (Whitman *et al.*, 1988). This leads to formation of phosphatidylinositides that are resistant to the action of known PLCs (Lips *et al.*, 1989; Serunian *et al.*, 1989). PI3K was first identified as associating protein of v-src and v-ros (Macara *et al.*, 1984; Sugimoto *et al.*, 1984). This association was subsequently observed to polyoma middle T, where a strong correlation between PI3K association and transforming potential could be demonstrated (Whitman *et al.*, 1985). The activity of PI3K can be stimulated by a number of mitogenic growth factors, such as PDGF (Auger *et al.*, 1989), CSF-1 (Reedijk *et al.*, 1990), EGF (Bjorge and Kudlow, 1987) and insulin (Endemann *et al.*, 1990; Ruderman *et al.*, 1990). The presence of PI3K activity complexed to pp60src, middle T and the PDGF receptor has been correlated with a 85 kDa phosphoprotein (Courtneidge and Heber, 1987; Kaplan *et al.*, 1987) and partial purification of PI3K has shown that the active fraction consists of a tightly associated heterodimer of the 85 kDa protein and a 110 kDa protein (Carpenter *et al.*, 1990). Both subunits of PI3K were recently cloned and the 85 kDa protein appears to be a regulatory subunit of the enzyme (Escobedo *et al.*, 1991b; Otsu *et al.*, 1991; Skolnik *et al.*, 1991), whereas the 110 kDa subunit carries the catalytic activity (Hiles *et al.*, 1992). Apparently, tyrosine phosphorylation is not required for activation of PI3K, since binding to IRS-1 alone results in activation (Backer *et al.*, 1992). Conflicting results have been obtained with mutations within the PDGF receptor that abrogate PI3K association. In some cases a loss of mitogenicity was observed (Coughlin *et al.*, 1989; Escobedo and Williams, 1988; Fantl *et al.*, 1992), whereas other groups report loss of PI3K association without an effect on the mitogenic response (Heideran *et al.*, 1991; Kazlauskas *et al.*, 1992; Yu *et al.*, 1991). Therefore, no straightforward conclusions as to the role of PI3K in RTK-stimulated mitogenesis can be drawn from these experiments. However, PI3K has also been found associated to oncogenic ras proteins, which suggested a direct involvement of PI3K in p21ras signaling (Sjölander *et al.*, 1991). Nevertheless, the fact that the ΔKi PDGF receptor mutant can no longer activate PI3K can still signal through p21ras towards erk1 and erk2 makes it unlikely that PI3K functions upstream or downstream of p21ras (Burgering *et al.*, 1993b).

2.5. SH2 domains and tyrosine kinase signaling.

The association of substrates of RTKs to the activated receptors occurs between specific autophosphorylation sites in the RTKs and conserved domains in the substrates. These domains are known as src homology 2 (SH2) domains and were originally identified in members of the src family of non-receptor tyrosine kinases, such as v-src and v-fps (Sadowski *et al.*, 1986). SH2 do-

mains have since then been identified in a large variety of proteins, such as p120GAP, PLC-γ, the 85 kD PI3K-subunit, PTP1C (a phosphotyrosine phosphatase (Shen *et al.*, 1991) and a number of novel transforming genes, including v-crk, nck, grb2, shc and vav (which will be discussed below) (see Fig.5). An SH2 domain consists of about 100 amino acids and mutations within this domain often reduce the transforming potential of v-src (Koch *et al.*, 1991). This finding suggested that these SH2 domains may direct the interaction of src with several cellular proteins that are necessary for transformation. This interaction was shown to be mediated by binding of src-SH2 domains to phosphorylated tyrosine residues of target proteins (Anderson *et al.*, 1990a). In addition, the SH2 domains can negatively regulate the tyrosine kinase activity of c-src, through interaction with phosphorylated Tyr-527 (Cantley *et al.*, 1991). This is supported by the finding that certain mutations in the SH2 domains can enhance the catalytic and transforming potential of c-src (Hirai and Varmus, 1990; Seidel-Dugan *et al.*, 1992). Most proteins with SH2 domains also contain one or more copies of another conserved src homology domain of about 50 amino acids, the SH3 domain (Koch *et al.*, 1991). Mutational analysis of SH3 domains suggests a role for this domain in regulation of the tyrosine kinase activity of c-src and c-abl (Franz *et al.*, 1989; Hirai and Varmus, 1990; Jackson and Baltimore, 1989; Kato *et al.*, 1986; Seidel-Dugan *et al.*, 1992). SH3 domains have also been identified in several proteins without SH2 domains that associate with the cytoskeleton or cellular membranes (Chenevert *et al.*, 1992; Drubin *et al.*, 1990; Koch *et al.*, 1991; Rodaway *et al.*, 1989), which indicates that this region may be involved in the targeting of proteins to specific subcellular locations.

A first indication that SH2 domains are involved in binding of phosphorylated tyrosine residues, came from the analysis of the p47gag-crk oncoprotein. This protein activates tyrosine kinase activity, although it has no apparent enzymatic activity by itself (Mayer *et al.*, 1988). p47gag-crk associates with a large number of tyrosine phosphorylated proteins, including pp60-v-src (Matsuda *et al.*, 1990; Mayer and Hanafusa, 1990). The region of p47gag-crk that is important for association with tyrosine phosphorylated proteins appeared to be its SH2 domain. This finding was followed by the observation that the isolated SH2 domains of different RTK-substrates could bind to the activated RTKs (Anderson *et al.*, 1990a; Hu *et al.*, 1992; Mayer *et al.*, 1991; McGlade *et al.*, 1992; Mohammadi *et al.*, 1991; Moran *et al.*, 1990; Reedijk *et al.*, 1992). This suggests that the autophosphorylation of the RTKs triggered by ligand binding results in a panel of phosphotyrosine residues, that function as recognition sites for different substrates. The insulin receptor is somewhat exceptional in this respect, since it seems to require a separate SH2-docking protein to bind certain SH2-containing proteins, such as PI3K (Lavan *et al.*, 1992; Sun *et al.*, 1991). This function is fulfilled by the most prominent substrate of the insulin receptor, insulin receptor substrate-1 (IRS-1), a protein of approximately 185 kDa, which contains several potential tyrosine phosphorylation sites that could serve as recognition sites for SH2 domains (Sun *et al.*, 1991).

The amino acids adjacent to the phosphorylated tyrosine appear to determine the affinity of different SH2-containing proteins for that specific site (Cantley *et al.*, 1991). Thus, binding specificity of a given SH2-containing protein is

dictated by the amino acid sequence surrounding each phosphorylated tyrosine. This is exemplified by the fact that small phosphopeptides (as small as 5 amino acids), identical to the proposed binding sites, can specifically inhibit binding of SH2-containing proteins that normally bind at this position (Escobedo *et al.*, 1991a; Fantl *et al.*, 1992; Kashishian *et al.*, 1992). With these peptides and by making use of mutants of the PDGF-receptor that lack specific tyrosine residues, it has been demonstrated that the PDGF-receptor has different binding sites for PLC-γ, p120GAP, and PI3K (Fantl *et al.*, 1992; Kashishian *et al.*, 1992; Kazlauskas *et al.*, 1992). This suggests that a given receptor can mediate its signal through a receptor-specific panel of SH2-containing proteins for which it contains high-affinity binding sites. However, the fact that each substrate will bind to the PDGF-receptor at a different position does not necessarily mean that one receptor molecule can bind all of these substrates at the same time.

The identification of SH2 domains as specific binding sites for tyrosine phosphorylated proteins sheds more light on the possible mechanism of signaling from activated RTKs to p21ras. Recently, candidate proteins containing SH2/SH3 domains that could link activated RTKs to signaling pathways involving ras proteins have been identified. First, in *C. elegans*, the sem-5 gene encodes a small protein with two SH3 domains flanking a SH2 domain and this protein plays an important role in vulval development (Clark *et al.*, 1992). Genetic evidence places sem-5 between the lin-23 gene and the let-60 gene, also involved in correct vulva formation. The lin-23 gene shows similarity to the EGF-receptor and let-60 is homologous to mammalian ras genes. This suggests that in *C. elegans* sem-5 is required for signaling from the RTK to p21ras. Interestingly, grb2, the human homolog of the sem-5 gene has been identified and cloned by virtue of its association to the EGF receptor (Lowenstein *et al.*, 1992). Microinjection of grb2 protein together with normal p21ras, at concentrations where neither protein alone had any effect, results in an induction of DNA synthesis (Lowenstein *et al.*, 1992). This strongly suggests that the function of grb2 and that of p21ras is somehow linked, although they are not necessarily involved in the same pathway. Each could function in a separate pathway, which act synergistically in the induction of DNA synthesis. Second, a gene was cloned by screening cDNA libraries with DNA probes of SH2 domains (Pelicci *et al.*, 1992). The resulting cDNA, which encodes a protein with a single SH2 domain and a region homologous to the α1 chain of collagen, was named shc. The shc protein associates to the activated EGF-receptor (McGlade *et al.*, 1992; Pelicci *et al.*, 1992). In addition, this protein is phosphorylated on tyrosine upon activation of the EGF-receptor and in v-src or v-fps transformed cells (McGlade *et al.*, 1992; Pelicci *et al.*, 1992; Pronk *et al.*, 1993). Overexpression of the shc gene transforms NIH 3T3 cells, which indicates the importance of the gene product in regulation of cellular proliferation. A link between shc and p21ras signaling was recently provided by the finding that the shc protein will bind to the grb2 protein upon RTK activation and overexpression of shc induces ras-dependent neurite outgrowth in PC12 cells (Rozakis-Adcock *et al.*, 1992). Third, the nck protein, which consists of three SH3 domains and one SH2 domain (Lehman *et al.*, 1990), is phosphorylated on serine and tyrosine in response to stimulation with EGF, PDGF and NGF, in A431

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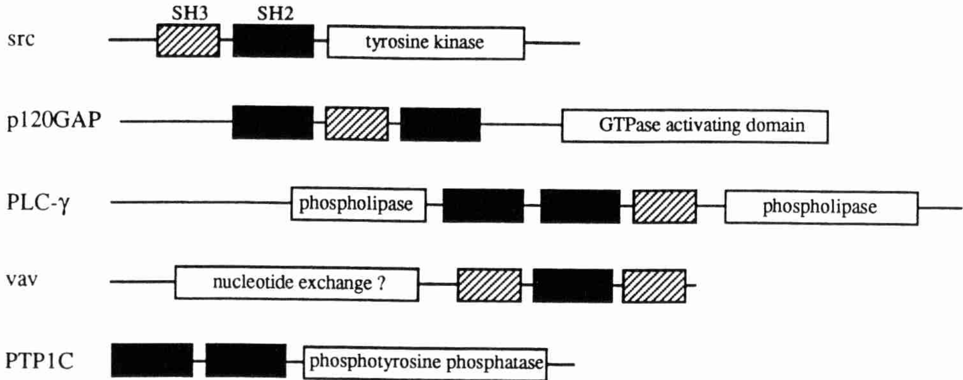

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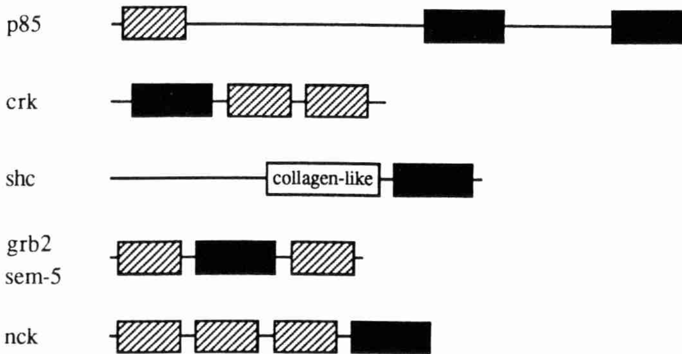

Fig.5. Proteins containing SH2/SH3 domains. SH2 domains are depicted as black boxes (approx. 100 amino acids) and the hatched boxes represent the SH3 domains (approx. 50 amino acids). The SH2/SH3-containing proteins can be grouped into two groups, ones that have a known enzymatic activity and ones that presumably function as adaptor proteins for other proteins.

cells, NIH 3T3 cells and PC12 cells, respectively (Li *et al.*, 1992b; Meisenhelder and Hunter, 1992; Park and Rhee, 1992). In addition, increased phosphorylation of nck is also observed in src-transformed NIH 3T3 cells (Meisenhelder and Hunter, 1992) and in T lymphocytes upon activation of the T cell receptor (Park and Rhee, 1992). Nck binds to the activated EGF- and PDGF-receptors and this association is mediated by the SH2 domain of nck (Li *et al.*, 1992b; Park and Rhee, 1992). At present no data have been obtained that place nck in one signaling pathway with p21ras, but overexpression of nck in NIH 3T3 cells and 3Y1 rat fibroblasts causes oncogenic transformation (Chou *et al.*, 1992; Li *et al.*, 1992b), suggesting that nck plays an important role in the mitogenic signaling of RTKs. Taken together, this suggests that a cascade of

SH2/phosphotyrosine interactions might link the RTKs to the eventual activation of p21ras. However, one should bear in mind that some of the expreiments rely on overexpression of the SH2-containing proteins. High levels of the SH2-containing proteins could result in interaction with proteins for which they have a relatively low affinity. Under physiological conditions these interactions might not occur.

Similarly, other regulatory proteins of small G-proteins which contain SH2/SH3 domains or interact with SH2/SH3-containing proteins have been identified. For example, 3BP-1, a protein that associates with the SH3-domain of c-abl, shows similarity to the bcr protein and n-chimaerin, suggesting it might have GAP activity for the rho-family of small GTP-binding proteins (Cicchetti *et al.*, 1992). Also, the vav protein, which contains an SH2 domain flanked by two SH3 domains (Margolis *et al.*, 1992) has a region of homology to the dbl oncogene, a possible exchange factor for rho- or rac-like GTP-binding proteins (Hart *et al.*, 1991). Moreover, BUD5, a putative GTP-GDP exchange factor cloned from *S. cerevisiae* interacts with BEM1 which contains two SH3 domains (Chant *et al.*, 1991; Chenevert *et al.*, 1992; Powers *et al.*, 1991). Therefore the SH2/SH3 domains could perform important functions in regulation of small GTP-binding proteins.

2.6. Lipids as activators of p21ras.

The activation state of p21ras can be controlled by lipids. For instance, activation of PKC, in response to phorbol esters, causes activation of p21ras in T lymphocytes (Downward *et al.*, 1990a), indicating that diacylglycerol, the physiological activator of PKC, is involved in the regulation of p21ras. However, such activation of p21ras by PKC has only been observed in T cells, which shows that the RTKs, most of which will activate PKC, do not signal to p21ras via PKC. Also, the phospholipid LPA can induce a transient activation of p21ras in rat-1 fibroblasts (van Corven *et al.*, 1993). The effect of LPA, however, is probably mediated by a protein tyrosine kinase (van Corven *et al.*, 1993).

In addition to these more or less indirect effects of lipids on the activation state of p21ras, a more direct role for lipids in the regulation of p21ras by GAPs has been proposed. Initially, it was found that the mitogenicity of certain lipids is virtually completely inhibited by microinjection of the neutralizing anti-ras antibody Y13-259 (Yu *et al.*, 1988), suggesting that their mitogenic signals are mediated exclusively by p21ras. Subsequent studies have shown that the ability of the purified GAPs to stimulate the GTPase activity of p21ras *in vitro* is inhibited by several lipids (Tsai *et al.*, 1989). Interestingly, the metabolism of the lipids with inhibitory potential, such as phosphatidic acid, arachidonic acid and PI monophosphate, changes upon mitogenic stimulation. Lipids with the ability to inhibit GAP-activity *in vitro* are produced within 1-5 min of serum stimulation of NIH 3T3 cells (Yu *et al.*, 1990). These findings have led to the proposal that certain growth factors may regulate the activation state of p21ras by regulating lipid metabolism, resulting in the inhibition of GAP-activity. The inhibition appears to be a consequence of a direct interaction of p120GAP with the mitogenically active lipids and this interaction is dependent on divalent ions (Tsai *et al.*, 1991). However, the concentration-dependence of the inhibition suggests that formation of micelles could be the prerequisite of this effect, especially since the lipids with low critical micel-

lar concentration (CMC) values are the most potent inhibitors (Serth *et al.*, 1991). Nevertheless, the local concentration of certain lipids at the cellular membrane could reach the values required for inhibition and certain lipids can inhibit the activity of both p120GAP and neurofibromin at concentrations well below their CMC (Golubic *et al.*, 1991). Mitogenically active phospholipids may also regulate p21ras via activation of a putative GTPase inhibitory protein. Such a protein, which binds phospholipids was partially purified from mouse brain (Tsai *et al.*, 1990), although no further evidence for the identity and physiological importance of the protein has been provided.

Neurofibromin and p120GAP are affected differently by certain lipids. For instance, neurofibromin is very sensitive to PIP_2, arachidonic acid and phosphatidic acid, whereas p120GAP is much less sensitive to these compounds (Bollag and McCormick, 1991). These lipids do not prevent the interaction of p120GAP or neurofibromin with p21ras, suggesting that lipids could stabilize the interaction of p21ras-GTP with a specific GAP. This could have important implications for the interaction of p21ras with its downstream target, as will be discussed later. However, regulation of the activation state of p21ras requires more than production of mitogenically responsive lipids, since serum, which efficiently stimulates formation of these lipids, is a poor activator of p21ras. Of course it remains to be determined if inhibition of lipid production can block activation of p21ras. It could very well be that these lipids potentiate the effect of a growth factor that has an effect on nucleotide exchange on p21ras, a possibility that requires further study.

3. Downstream signaling of p21ras.

3.1. p120GAP as effector.

A number of experimental observations have indicated that p120GAP, in addition to negatively regulating p21ras, might play a role in the downstream signaling from p21ras as well (reviewed in McCormick, 1989). Initial data showed that p120GAP interacts with p21ras at a site denoted as the effector region. Antibodies directed against the effector region of p21ras can block the interaction with p120GAP (Rey *et al.*, 1989) and most mutations in the effector region that reduce the transforming potential of ras-oncoproteins also impair the interaction with p120GAP (Adari *et al.*, 1988; Cales *et al.*, 1988). Moreover, mutants of p21ras which have a high affinity for p120GAP, but lack the consensus membrane localization site, inhibit signaling from active p21ras (Gibbs *et al.*, 1989; Medema *et al.*, 1991b; Michaeli *et al.*, 1989). Also, K-rev (rap1A), a member of the ras-family that can revert ras-transformed cells, has been suggested to do so through competition for p120GAP, on the basis of its identical effector region (Frech *et al.*, 1990; Kitayama *et al.*, 1989). However, all these observations could just as well plead for an effector different from p120GAP, that has a binding site on p21ras that (partially) overlaps the binding site for p120GAP.

Further evidence for an effector function for p120GAP came from experiments with isolated atrial cell membranes, where p120GAP can cause an inhibition of potassium channel opening induced by the activated muscarinic receptor (Yatani *et al.*, 1990). This effect requires interaction of p120GAP with p21ras, since addition of Y13-259 blocked the p120GAP-induced inhibition (Yatani *et al.*, 1990). In *Xenopus laevis*

oocytes a similar situation has been observed for the activation of the maturation promoting factor (Dominguez *et al.*, 1991). Here, an induction of H1-kinase activity was measured upon micro-injection of p120GAP or oncogenic p21ras and this effect was blocked by Y13-259 or a neutralizing anti-p120GAP antibody (Dominguez *et al.*, 1991). In addition, antibodies against phosphocholine(PC)-specific PLC (PC-PLC) also blocked the effect of oncogenic p21ras and p120GAP, suggesting that PC-PLC is somehow activated by the active p21ras/p120GAP-complex and mediates oocyte maturation (as will be discussed below). However, in contrast to microinjection of oncogenic p21ras, microinjection of p120GAP did not stimulate germinal vesicle breakdown (GVBD), which is a good parameter of oocyte maturation. This was explained by the fact that only a minor fraction of endogenous p21ras (required for interaction with p120GAP) will be in the active conformation in resting oocytes (Dominguez *et al.*, 1991). Nevertheless, insulin-induced maturation of oocytes was inhibited by microinjection of p120GAP in the same study. Since the insulin-induced maturation is mediated by p21ras, one would expect that this effect would be enhanced, rather than inhibited by p120GAP. Thus, interaction of p21ras and p120GAP alone appears to be insufficient for induction of oocyte maturation.

In the isolated atrial cell membranes it was shown that p120GAP deletion mutants encoding only the SH2/SH3 domains can establish inhibition of potassium channel opening independently of p21ras (Martin *et al.*, 1992). This suggests that the effector function of p120GAP resides in the SH2/SH3 domains, and that interaction of p120GAP with p21ras causes a conformational change in the GAP protein which enables the SH2/SH3 domains to interact with their downstream target. The same deletion mutants of p120GAP can induce expression from the fos-promoter in a transient expression system (Medema *et al.*, 1992). However, in this case the induction is dependent on ras-activity, since co-expression of p21ras(Asn17) blocks the effect. Therefore, it is unlikely that p120GAP alone is the target of p21ras in this particular signaling pathway, but other signals that stem from p21ras are required to induce various effects (see Fig.1). This notion is supported by the finding that p21ras(Glu38), has a normal affinity for p120GAP (Krengel *et al.*, 1990), although the transforming ability of normal p21ras is destroyed by this mutation (Cales *et al.*, 1988), indicating that interaction between p21ras and p120GAP per se is not sufficient for transformation. Deletion mutants of p120GAP that encode only the catalytic domain involved in interaction with p21ras (Marshall *et al.*, 1989), can inhibit induction of gene-expression by activated forms of p21ras, an effect that can be reverted by coexpression of full length p120GAP (Schweighoffer *et al.*, 1992). This suggests that the catalytic domain of p120GAP competes with full length p120GAP for binding to p21ras, but that the catalytic domain of p120GAP alone cannot perform the effector function, in agreement with the finding that this function is localized in the SH2/SH3 domains.

The targets of other G-proteins have been shown to contain GAP activity. For instance, in the control of protein synthesis, the ribosomes act both as effector and GAP for the bacterial elongation factor EF-Tu (Bourne *et al.*, 1990a). Interaction of the photoreceptor heterotrimeric G protein, transducin, with its effector, cGMP phosphodiesterase, accelerates GTP hydrolysis

on transducin (Arshavsky and Bownds, 1992). Similarly, phospholipase C-β1, which is an effector for the heterotrimeric $G_{q/11}$ protein, has GTPase stimulatory activity towards $G_{q/11}$ (Berstein *et al.*, 1992). These observations point to the possibility that a general theme exists, in which the effector molecule for GTP-binding proteins could control their upstream regulators (Bourne and Stryer, 1992). This raises the question whether neurofibromin, the other GTPase activating protein for p21ras, can also function as an effector. In that way multiple signals could stem from p21ras by selection of different effector proteins, a decision that could be controlled by mitogenic lipids (Bollag and McCormick, 1991). It should be noted however, that deletion of neurofibromin results in increased levels of the GTP-bound form of p21ras, which is thought to be causing the transformed phenotype of the Schwannoma cell lines (Basu *et al.*, 1992; DeClue *et al.*, 1992). If so, then the effector function of neurofibromin, if such exists, seems dispensable for transformation by p21ras. Introduction of oncogenic p21ras in normal Schwann cells causes a block in cell proliferation (Ridley *et al.*, 1988), indicating that active p21ras in the presence of neurofibromin leads to growth arrest in these cells. This has led to the proposal that neurofibromin could be an effector for p21ras, which mediates growth-inhibitory effects (Bollag and McCormick, 1992) (see also Fig.1).

Proteins that bind to p120GAP are potential targets of the p21ras-p120GAP effector-complex. Firstly, two p120GAP-associated proteins, p62 and p190, that were cloned recently could participate in p21ras signaling (see Fig.1). The p62 protein has significant homology to a putative hnRNP protein and binds RNA, suggesting a role in RNA processing, but the relevance of this with respect to p21ras signal transduction is still obscure (Wong *et al.*, 1992). The p190 protein can function as a GAP for p21rho, and interaction of p120GAP and p190 may allow coupling of signaling pathways involving p21ras and rho (Settleman *et al.*, 1992a). In addition, the protein appears to be identical to the transcriptional repressor of the glucocorticoid receptor, which raises the possibility that p21ras can signal directly to the nucleus through the p120GAP-p190 complex (Settleman *et al.*, 1992b).

3.2. Kinases downstream of p21ras.

Raf-1 kinase is a 70-75 kDa phosphoprotein with intrinsic kinase activity towards serine and threonine residues (Rapp, 1991). Several lines of evidence suggested that p21ras and raf-1 kinase function in the same signaling pathway. Firstly, expression of oncogenic forms of p21ras leads to enhanced phosphorylation of raf-1 kinase in NIH 3T3 and PC12 cells (Morrison *et al.*, 1988; Wood *et al.*, 1992). Secondly, a dominant negative mutant of raf-1 kinase or antisense raf-1 RNA can suppress transformation of NIH 3T3 and normal rat kidney (NRK) cells by oncogenic forms of p21ras (Kizaka-Kondoh *et al.*, 1992; Kolch *et al.*, 1991). Thirdly, transformation by v-raf does not require the function of p21ras (Feig and Cooper, 1988a; Smith *et al.*, 1986). In addition, both raf-1 kinase and p21ras appear to be essential components of a signaling pathway shared by EGF and PDGF (Kizaka-Kondoh *et al.*, 1992). This notion is supported by the fact that raf-1 kinase activity is stimulated in response to many different ligands, a situation that is remarkably similar to activation of p21ras (App *et al.*, 1991; Baccarini *et al.*, 1990; Blackshear *et al.*, 1990; Carroll *et al.*, 1990;

Kovacina *et al.*, 1990; Morrison *et al.*, 1988; Siegel *et al.*, 1990; Turner *et al.*, 1991). In addition, activation of raf-1 kinase by different growth factors is blocked in the presence of p21ras(Asn17) (Troppmair *et al.*, 1992; Wood *et al.*, 1992), demonstrating that activation of raf-1 kinase by RTKs is mediated by p21ras. Such a mechanism is somewhat contradictory to the direct activation of raf-1 kinase, assumed to occur upon stimulation of the PDGF-receptors (Morrison *et al.*, 1989). However, PDGF-stimulation of tyrosine phosphorylation of raf-1 kinase was not observed by others (Baccarini *et al.*, 1990) and other growth factors, such as EGF, insulin and CSF-1, elicit only serine phosphorylation of raf-1 kinase (App *et al.*, 1991; Baccarini *et al.*, 1990; Blackshear *et al.*, 1990; Kovacina *et al.*, 1990). Thus, activation of raf-1 kinase in general appears to be a more indirect effect of RTK-activation.

In addition to raf-1 kinase, the activation erks, or mitogen activated protein (MAP) kinases, (erk1 or p44mapk and erk2 or p42mapk) by a number of growth factors requires the activity of p21ras (de Vries-Smits *et al.*, 1992; Thomas *et al.*, 1992; Wood *et al.*, 1992). In some ras-transformed cells these kinases are constitutively activated, and oncogenic p21ras can cause activation of erk1 and erk2 when introduced into quiescent cells (Leevers and Marshall, 1992). Erks are serine/threonine kinases, and activation requires phosphorylation of erk1 and erk2 at a threonine and a tyrosine residue separated by one amino acid (Anderson *et al.*, 1990b; Boulton *et al.*, 1991; Payne *et al.*, 1991). The activation of a serine/threonine kinase by phosphorylation at tyrosine residues was initially thought to provide the link between RTK-activation and the observed increase in serine/threonine phosphorylation of cellular proteins (Anderson *et al.*, 1990b). However, the kinase responsible for activation of erk1 and erk2 (MAP kinase kinase or mapkk) has characteristics of a kinase that is activated by serine/threonine phosphorylation (Alessandrini *et al.*, 1992; Crews and Erikson, 1992; Gómez and Cohen, 1991; Kosako *et al.*, 1992; L'Allemain *et al.*, 1992; Matsuda *et al.*, 1992; Nakielny *et al.*, 1992a; Nakielny *et al.*, 1992b). Mapkk is a "dual kinase" since it can phosphorylate erks both on tyrosine and threonine residues (Nakielny *et al.*, 1992b; Posada and Cooper, 1992; Rossomando *et al.*, 1992). Recently, the mapkk was cloned from a murine pre-B cell cDNA library (and named MEK) (Crews *et al.*, 1992). It should be noted that activation of erks can also occur in a ras-independent manner. For example, TPA-induced activation of erks in rat-1 cells is not dependent on the activity of p21ras (de Vries-Smits *et al.*, 1992). Moreover, activation of erk2 by EGF in rat-1 cells is only partly inhibited by expression of p21ras(Asn17) and full inhibition is accomplished when the calcium chelator EGTA is added in combination with expression of p21ras(Asn17) (Burgering *et al.*, 1993a). This suggests that EGF stimulates both a ras-dependent, as well as a Ca^{2+}-dependent route, each of which can mediate erk2-activation.

The findings that both activation of raf-1 kinase and of erk1/erk2 require the function of p21ras suggested a relation between these protein kinases. Initially, two groups reported that erks could phosphorylate raf-1 kinase *in vitro* (Anderson *et al.*, 1991; Lee *et al.*, 1992), suggesting that raf-1 kinase is downstream of erk. However, raf-1 kinase which is phosphorylated by erk1/erk2 *in vitro* is only slightly activated. More recently, different groups have shown that

immunoprecipitated raf-1 kinase can reactivate dephosphorylated mapkk (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992) and that erks are constitutively activated in raf-transformed cells (Dent *et al.*, 1992; Howe *et al.*, 1992). This suggests that raf-1 kinase is the mapkk kinase or activates this latter kinase through another kinase present in the preparations used for *in vitro* phosphorylation. This places raf-1 kinase upstream of erk1 and erk2 and downstream of p21ras. Thus, an important signal transduction pathway is emerging in which p21ras controls raf-1 kinase, which via mapkk can activate erk1 and erk2 (see Fig.6). The fact that erk1/erk2 can also phosphorylate raf-1 kinase suggests that some feedback mechanism may exist by which erks regulate raf-1 kinase activity. Nevertheless, some data which are apparently in conflict with this straightforward pathway have been obtained. For example, mutants of the EGF receptor that lack kinase activity can no longer activate raf-1 kinase (Baccarini *et al.*, 1991), whereas activation of erk1 and erk2 is still observed (although the response is sharply reduced) (Campos-González and Glenney, 1992), suggesting that other (redundant) routes may exist. However, a small residual kinase activity of the mutant EGF-receptor could also explain this result. In addition, optimal activation of raf-1 kinase in insect cells requires co-expression of oncogenic p21ras and pp60src, suggesting that the mechanism of activation is more complex (Williams *et al.*, 1992). Also, v-raf expression in PC12 cells fails to activate erk2 (Wood *et al.*, 1992), although raf-immunoprecipitates from PC12 cells are able to reactivate dephosphorylated mapkk (Howe *et al.*, 1992). The reason for this difference is unclear, but suggests that for the activation of erk2 *in vivo* the presence of active raf-1 kinase is not sufficient.

Activation of erk1 and erk2 has important consequences for transcriptional and translational activity in the cell. Erk2 can activate other kinases, such as p90rsk (S6 kinase II) (Erikson, 1991; Sturgill *et al.*, 1988) and a newly identified kinase, termed MAP kinase activated protein kinase-2 (MAPKAP kinase-2) of which the substrates are still unknown (Stokoe *et al.*, 1992) (see Fig.6). Also, the relevant substrates of

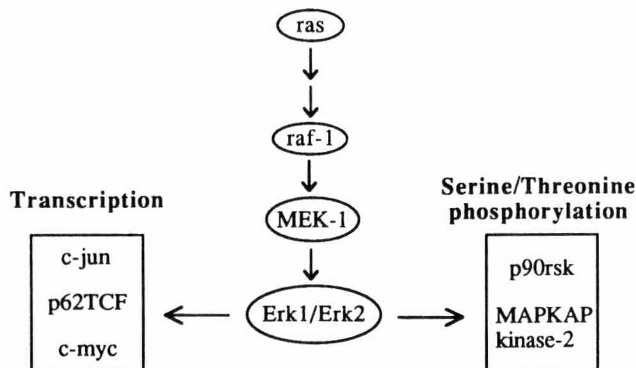

Fig.6. A kinase cascade that is initiated by p21ras. Activation of p21ras will result in activation of raf-1, which can in turn activate the mapkk (MEK-1). MEK-1 can activate erk1 and erk2 and these latter kinases are thought to be involved in activation of a variety of transcription factors and other serine/threonine kinases. Therefore, this pathway could mediate many ras-induced effects, such as an altered gene expression.

p90rsk are still elusive, since phosphorylation of S6 by p90rsk *in vitro* only results in a partial reactivation (Ahn *et al.*, 1990) and another kinase, p70 S6 kinase, is held responsible for the activation of S6 *in vivo* (Ahn *et al.*, 1990; Ballou *et al.*, 1991; Chung *et al.*, 1992). This latter kinase is not activated by erk1 or erk2 and is part of a distinct signaling pathway (Ballou *et al.*, 1991; Chung *et al.*, 1992), for which the involvement of p21ras has not yet been addressed. In addition, purified erks can directly phosphorylate a number of transcription factors *in vitro*, such as; c-myc (Alvarez *et al.*, 1991; Seth *et al.*, 1991), c-jun (Alvarez *et al.*, 1991; Pulverer *et al.*, 1991) and p62TCF (Gille *et al.*, 1992) (see Fig.6). Thus, activation of erks could be responsible for the induction of gene expression that is observed in response to different growth factors. However, one should bear in mind that phosphorylation of these transcription factors is observed with purified kinases in an *in vitro* kinase assay. Therefore, kinases that copurify with erk1/erk2 or associate to them might be responsible for the observed phosphorylations.

Several possibilities exist by which p21ras could cause activation of raf-1 kinase. The raf-1 protein has its kinase domain in the carboxy-terminal half of the protein, whereas the amino-terminal region of the protein negatively regulates kinase activity (Li *et al.*, 1991). The amino-terminal domain of raf-1 kinase has been implicated to function like the regulatory lipid-binding domain of PKC and activation of raf-1 kinase might take place by an allosteric regulatory mechanism (Stanton *et al.*, 1989). On the other hand, activation of the kinase activity of raf-1 kinase correlates with increased phosphorylation of raf-1 kinase, suggesting that activation could also be a consequence of phosphorylation. Therefore, p21ras should either activate the allosteric activator of raf-1 kinase or activate (directly or indirectly) the raf-1 kinase kinase. The fact that phorbol esters can cause activation of raf-1 kinase in all cells, suggests that PKC is a common intermediate (Li *et al.*, 1991), which would argue against allosteric regulation of raf-1 kinase by p21ras. Nevertheless, TPA-sensitive PKC is not involved in ras-dependent activation of erk1 and erk2 by PDGF or insulin in fibroblasts. Possibly p120GAP could affect the activity of a kinase through binding of a tyrosine phosphorylated residue by means of its SH2 domains. A positive regulatory function for the SH2 domains of p120GAP in p21ras signaling has indeed been demonstrated (Martin *et al.*, 1992; Medema *et al.*, 1992; Schweighoffer *et al.*, 1992). Whether these domains can cause activation of a protein kinase remains to be shown.

3.3. Phospholipases.

RTK-stimulation frequently results in generation of DAG, which is a potent activator of PKC (Nishizuka, 1988). The initial formation of DAG is a consequence of a rapid and transient hydrolysis of PI by PI-specific PLC, which also produces IP3, causing a transient Ca^{2+} release from intracellular stores (Berridge, 1987). This phospholipase C-mediated degradation of inositol phospholipids is an important step in the signal transduction cascades triggered by different growth factors (Berridge, 1987; Williams, 1989). This transient DAG production is often followed by a more sustained elevation of DAG, which is thought to be a consequence of phosphatidylcholine (PC) hydrolysis (Exton, 1990). Also, cells transformed with ras-oncogenes display increased steady-state levels of DAG (Fleischman *et al.*, 1986; Wolfman and Macara,

1987) and microinjection of oncogenic p21ras in *Xenopus laevis* oocytes causes a rapid production of DAG (Lacal *et al.*, 1987a). The increased level of DAG is most likely responsible for the sustained activation of PKC observed in ras-transformed cells (Diaz-Laviada *et al.*, 1990). Sustained activation of PKC is a prerequisite for long-term physiological responses, such as proliferation and differentiation (Asaoka *et al.*, 1991; Berry *et al.*, 1990; William *et al.*, 1990) and cells that stably overexpress PKC show disturbed growth control and enhanced tumorigenicity (Cuadrado *et al.*, 1990; Housey *et al.*, 1988; Krauss *et al.*, 1989; Megidish and Mazurek, 1989; Persons *et al.*, 1988; Watanabe *et al.*, 1992). This suggests that formation of DAG may be an important event in ras-transformation, although the mechanism by which p21ras can stimulate DAG formation is not fully understood.

The elevated levels of DAG in ras-transformed cells could be a consequence of an induction of PI-specific PLC (PI-PLC), since microinjection of neutralizing anti-PI-PLC antibodies can block ras-induced DNA synthesis (Smith *et al.*, 1990). However, ras-transformed cells do not contain elevated levels of inositol phosphates (Lacal *et al.*, 1987d), nor do they show enhanced basal PI-specific PLC activity (Seuwen *et al.*, 1988) and scrape-loading of p21ras into Swiss 3T3 cells causes a rapid activation of PKC in the absence of any measurable effect on PI turnover (Morris *et al.*, 1989). Also, microinjection of oncogenic p21ras in oocytes does cause a dramatic increase in DAG, but only minor differences in the levels of other products of PI turnover are observed (Lacal *et al.*, 1987a). Therefore, PI-PLC-catalyzed hydrolysis of PI is an unlikely source for DAG in ras-transformed cells.

An alternative source for DAG is hydrolysis of PC. Indeed, analysis of the molecular species of 1,2-diacylglycerides produced upon EGF- and PDGF-treatment demonstrates that DAG formation is primarily due to PC hydrolysis and hydrolysis of other phospholipids contributes only to a very minor extent (Pessin *et al.*, 1990). In addition, ras-transformed cells exhibit increased levels of phosphocholine (PCho), the other product of PC-PLC-catalyzed hydrolysis of PC (Lacal, 1990). A similar elevation of PCho has been observed in PDGF-stimulated fibroblasts (Besterman *et al.*, 1986; Pessin *et al.*, 1990). However, it should be noted that the increased levels of PCho in ras-transformed cells, as well as in response to various mitogens, may be caused in part by an elevated choline-kinase activity (Besterman *et al.*, 1986; Lacal, 1990; Macara, 1989; Warden and Friedkin, 1985). Also, the increase in PCho precedes the observed increase in DAG in the oocytes microinjected with active p21ras, suggesting that PC is not the only source of DAG (Lacal *et al.*, 1987b). Nevertheless, sufficient data indicate that part of the DAG produced in ras-transformed cells is a result of PC-PLC-catalyzed hydrolysis of PC.

Indeed, PC-PLC plays an important role in ras- and growth factor-induced mitogenesis. For instance, addition of PC-PLC from *Bacillus cereus* to the medium of Swiss 3T3 fibroblasts stimulates DNA synthesis (Larrodera *et al.*, 1990) and can induce gene-expression (Diaz-Meco *et al.*, 1991). Remarkably, maximal induction of DNA synthesis by addition of PC-PLC to the medium is detected ~9 hr before the maximal stimulation triggered by PDGF. This is the same time lag required for PDGF to stimulate formation of PCho, suggesting that activation of PC-

PLC is a relatively late event (Larrodera *et al.*, 1990). Similarly, microinjection of PC-PLC in *Xenopus laevis* oocytes induces maturation and neutralizing antibodies against PC-PLC can block maturation induced by insulin or microinjected p21ras (Garcia de Herreros *et al.*, 1991). Again, induction of maturation by PC-PLC is faster than insulin-induced maturation and the time lag correlates with the time required to stimulate PCho production. The time-course of induction of oocyte maturation by microinjection of p21ras closely resembles that generated by microinjection of PC-PLC, indicating that activation of p21ras and PC-PLC are both late events. Interestingly, expression of PC-PLC can overcome inhibition of proliferation of NIH 3T3 cells by a dominant inhibitory p21ras mutant (p21rasAsn17) (Cai *et al.*, 1992), suggesting that PC hydrolysis is the target of p21ras which mediates mitogenic signaling. In addition, stimulation of PC-PLC-catalyzed hydrolysis of PC induced by EGF or serum is inhibited by expression of p21ras(Asn17), indicating that p21ras mediates growth factor-induced activation of PC-PLC (Cai *et al.*, 1992). Altogether, these data suggest the existence of a mitogenic signaling pathway, in which p21ras would, as a late but crucial event in mitogenesis, activate PC-PLC.

Some questions remain, however. Firstly, the timing of this mitogenic signaling pathway. For PDGF-stimulated mitogenesis, activation of PC-PLC seems to occur around eight hours after addition of PDGF (Larrodera *et al.*, 1990). Also, serum-stimulated DNA synthesis is sensitive to inhibition by the anti-ras neutralizing antibody Y13-259 up to around eight hours after addition of serum (Mulcahy *et al.*, 1985). This suggests that activation of PC-PLC by p21ras occurs at a very late stage in mitogenic signaling. However, we know that p21ras is activated rapidly in response to different growth factors and from experiments with *Xenopus laevis* oocytes, it is suggested that PC-PLC is activated rapidly upon microinjection of oncogenic p21ras (Lacal *et al.*, 1987a). Why then would activation of PC-PLC occur as a late event in mitogenic signaling by these growth factors? One could argue that the levels of p21ras-GTP after growth factor stimulation are not comparable with those as a result of microinjection, but this would lead to the conclusion that activation of PC-PLC can not occur in response to physiological levels of p21ras-GTP. To add to this confusion, PDGF-stimulation was initially reported to give rise to a very rapid release in PCho (Besterman *et al.*, 1986; Pessin *et al.*, 1990), but these early time points have not been studied in later studies where a delayed response was observed. Because addition of PC-PLC seems sufficient to overcome inhibition of p21ras, this would suggest that activation of PC-PLC is the only crucial event in mitogenic signaling downstream of p21ras. If so, then one would come to the remarkable conclusion that all early events occuring within minutes of growth factor stimulation (such as activation of p21ras, erks, induction of fos-expression, etc.) are only required for the eventual induction PC-PLC, which occurs eight hours later. It should be noted that discrepancy exists as to the kinetics of PCho-release upon microinjection of oncogenic p21ras in *Xenopus laevis*. In one particular study, PCho-release is detected 2 hours after microinjection of oncogenic p21ras (Diaz-Meco *et al.*, 1992), whereas others already detect increased levels of PCho at 30 minutes after microinjection (Garcia de Herreros *et al.*, 1991; Lacal *et al.*, 1987a). Therefore, activation p21ras could be very closely linked to activation of PC-

PLC, but these events might just as well be separated by more than an hour in mitogenic signaling.

Secondly, confusion exists as to the signals produced by PC-PLC. At first sight the production of DAG would provide an adequate second messenger, causing activation of PKC. However, PC-PLC-induced DNA synthesis in Swiss 3T3 fibroblasts (Larrodera *et al.*, 1990), as well as induction of stromelysin expression (Diaz-Meco *et al.*, 1991) is independent of phorbol ester-sensitive PKC. In contrast, maturation of *Xenopus laevis* oocytes by PC-PLC requires the function of PKC-ζ (Dominguez *et al.*, 1992). It could be that this isoform is also responsible for induction of DNA synthesis in Swiss 3T3 fibroblasts, since it is insensitive to TPA-downregulation (Ono *et al.*, 1989). In addition, activation of PC hydrolysis by oncogenic p21ras in Swiss 3T3 cells seems to require a phorbol ester PKC (Price *et al.*, 1989). This would place PKC both upstream and downstream of PC-PLC in Swiss 3T3 cells, the PKC-isoform upstream being sensitive to phorbol esters, the PKC-isoform downstream being insensitive to phorbol esters. Other metabolites that are produced as a result of increased levels of DAG, such as arachidonic acid, phosphatidic acid (PA) and LPA could also function as "second" messengers. Indeed, PA and LPA are very good mitogens when added to A431 and rat-1 cells (Moolenaar *et al.*, 1986; van Corven *et al.*, 1989) and mitogenic signaling by EGF in Balb/c 3T3 cells requires arachidonic acid metabolism, although sole addition of arachidonic acid metabolites is not sufficient for mitogenesis (Handler *et al.*, 1990). Taken together, a function for PC-PLC somewhere downstream of p21ras in mitogenic signal transduction is a serious possibility, although many questions are still unanswered concerning the timing of this effect and the character of the signal that is generated.

3.4. Role of protein kinase C.

As already mentioned, PKC appears to play an important role in transformation of cells by oncogenic forms of p21ras. However, the exact position and role of PKC in signal transduction by p21ras is a matter of debate. Most of this controversy stems from the fact that no experimental approaches are available at present that can inhibit or distinguish all of the different isoforms of PKC. At present ten different isoforms of PKC have been identified, with distinct enzymological characteristics and tissue specific distribution. The four classical isoforms, or cPKC-isoforms (α, β1, β2 and γ) are activated by Ca^{2+} and DAG (Nishizuka, 1988), whereas the isoforms of the nPKC (novel PKC) subspecies (δ, ε, η(L) and θ) are activated by DAG in the absence of Ca^{2+} (Koide *et al.*, 1992; Ogita *et al.*, 1992). All these isoforms respond well to phorbol esters (Koide *et al.*, 1992; Nishizuka, 1988; Ogita *et al.*, 1992). The two isoforms of the most recently discovered PKC subspecies, the atypical PKC isoforms (aPKC) (ζ and λ) apparently do not respond to DAG or Ca^{2+}, nor to phorbol esters (Ono *et al.*, 1989; Ways *et al.*, 1992). This indicates that retention of PKC-activity is possible after prolonged treatment with phorbol esters, an approach that is widely used to investigate the involvement of PKC in signaling pathways. Indeed, examples exists that not all PKC-activity is downregulated after prolonged treatment with phorbol esters (Cooper *et al.*, 1989; Isakov *et al.*, 1990; Ways *et al.*, 1992). Moreover, even isoforms that are sensitive to phorbol esters can be retained (Borner *et al.*,

1992; Strulovici *et al.*, 1991). Therefore, conclusions based on depletion of PKC-activity by prolonged exposure to phorbol esters need to be taken with caution. Even more frustrating is the fact that some isoforms of PKC, which are downregulated in one specific cell type, might not behave as such in another cellular background. For instance, PKC-ζ is not downregulated by phorbol esters in a variety of cell lines (Ways *et al.*, 1992), but can be downregulated in R6 rat embryo fibroblasts (Borner *et al.*, 1992). Specific phosphorylation of PKC-substrates, such as the 80 kDa MARCKS protein, is often used as measure for the state of total PKC-activity in the cell. Since this MARCKS protein itself was recently found to be downregulated by prolonged treatment with TPA this measurement may not be a good read-out for total PKC-activity (Brooks *et al.*, 1991). The use of PKC-inhibitors, such as staurosporine, sphingosine and H7 would be more appropriate, but the specificity of these compounds is also questionable. More specific inhibition can be accomplished by making use of the pseudosubstrates of PKC-isoforms, but these compounds do not easily enter the cell, so that permeabilization or transfection of expression plasmids is required. It seems that with the current knowledge of PKC, it would be more appropriate to draw conclusions about the involvement of specific isoforms and not to address PKC as one enzymatic entity.

Nevertheless, some conclusions can be drawn from the vast amount of data that is available on the role of PKC in p21ras signaling. For instance, TPA-induced DNA synthesis in NIH 3T3 cells can be inhibited by microinjection of Y13-259 or expression of p21ras(Asn17) (Cai *et al.*, 1990; Yu *et al.*, 1988). Also, overexpression of p120GAP can block c-fos expression, activation of erk2 and DNA synthesis induced by TPA in Swiss 3T3 cells (Nori *et al.*, 1991), suggesting that p21ras mediates TPA-induced PKC signal transduction. In PC12 cells, TPA-induced activation of raf-1 kinase, erks and p90rsk is blocked by expression of p21ras(Asn17) (Thomas *et al.*, 1992; Wood *et al.*, 1992). In contrast, TPA-induced activation of erk2 in COS-1 cells (Howe *et al.*, 1992) or rat-1 cells (de Vries-Smits *et al.*, 1992) and induction of fos-expression by TPA is also not blocked by expression of p21ras(Asn17) (Cai *et al.*, 1990). It is possible that these conflicting results are a consequence of the difference in cell type. But alternatively, as mentioned previously, inhibition through interference with the function of p21ras does not necessarily point to a common pathway, but could just as well plead for cooperating routes.

Direct proof that PKC can function as upstream activator of p21ras comes from T cells, where a rapid and profound increase in the level of p21ras-GTP could be observed after TPA treatment (Downward *et al.*, 1990a). Also, in PC12 cells the combination of TPA and the tyrosine phosphatase inhibitor vanadate can cause activation of p21ras, through the TPA-sensitive PKC (Satoh *et al.*, 1991). However, functional PKC does not seem to be required for activation of p21ras in T cells, since pseudosubstrates of PKC could not prevent activation of p21ras by stimulation of the T cell receptor in permeabilized cells (Izquierdo *et al.*, 1992), demonstrating that the TCR signaling pathway does not necessarily signal via PKC. Also, in fibroblasts, no evidence has been found that activation of PKC can drive p21ras in the active conformation. Indeed, phorbol ester-sensitive isoforms of PKC are not required for activation of p21ras by dif-

ferent growth factors in fibroblasts (Medema *et al.*, 1991a; Satoh *et al.*, 1991), which shows that activation of p21ras by (phorbol-ester sensitive) PKC is limited to certain cell types.

PKC also plays an important role downstream of p21ras. For instance, scrape-loading of p21ras into Swiss 3T3 cells rapidly activates PKC (Morris *et al.*, 1989). Moreover, microinjection or scrape-loading of oncogenic p21ras fails to induce DNA synthesis in cells where PKC has been downregulated by prolonged treatment with phorbol esters (Lacal *et al.*, 1987c; Morris *et al.*, 1989). In ras-transformed cells, a sustained activation of PKC is observed, without a concomitant down-regulation of the enzyme (Diaz-Laviada *et al.*, 1990). Furthermore, PKC-ζ functions downstream of p21ras in insulin-induced oocyte maturation (Dominguez *et al.*, 1992). As mentioned previously, different isoforms of PKC mediate signaling to and from PC-PLC in ras-signaling (Diaz-Laviada *et al.*, 1990; Price *et al.*, 1989). These data suggest that PKC could be required at multiple stages during p21ras signal transduction. Most likely, separate isoforms are responsible for signal transduction at each stage. However, a reliable determination of the position of PKC-isoforms in this pathway will await the development of good reagents that can specifically inhibit each of the different isoforms.

3.5. Transcription factors.

In ras-transformed cells many genes are differentially expressed, but initially it was unclear whether the altered expression of these genes was a direct consequence of the presence of active p21ras, or rather an indirect consequence of transformation. Therefore, investigators analyzed the expression of certain genes in cells where oncogenic p21ras could be conditionally expressed or introduced by microinjection. Using these methods, a number of genes were shown to be up-regulated upon appearance of oncogenic p21ras, such as the stromelysin gene (Matrisian *et al.*, 1986), the collagenase gene (Schönthal, 1988), the c-fos gene (Schönthal, 1988; Stacey *et al.*, 1987), VL30-driven constructs (Owen *et al.*, 1990) and the TGF-β gene (Owen and Ostrowski, 1990). Transcriptional activation of these genes by oncogenic p21ras is mediated by a number of binding sites, namely: the TRE (TPA responsive element) (Gutman and Wasylyk, 1990; Imler *et al.*, 1988), the PEA3 binding site (Wasylyk *et al.*, 1989) or the RRE (ras responsive element) (Owen and Ostrowski, 1990). This indicated that p21ras can specifically regulate the activity of the transcription factors that bind to these sites. The AP-1 transcription factor (which binds to the TRE) is composed of heterodimers of c-jun and c-fos family members, which are themselves oncoproteins (reviewed in Vogt and Bos, 1990), the products of the ets-1 and ets-2 oncogenes can activate transcription by binding to the PEA3 binding site (Wasylyk *et al.*, 1990) and the RRE binds AP-1, ets-1 and a protein of 120 kDa functionally related to known ets-coded proteins (Langer *et al.*, 1992; Owen and Ostrowski, 1990; Wasylyk *et al.*, 1990). The ets-1 protein cooperates with c-jun and c-fos for activation of transcription from the polyoma enhancer (Gutman and Wasylyk, 1990). The AP-1 binding site in the collagenase promoter is sufficient to confer ras-responsiveness, but when the PEA3 binding site is present this response is enhanced (Imler *et al.*, 1988). This indicates that p21ras can activate transcription through c-jun or c-fos and that ets can enhance this effect. Several mechanisms by which p21ras can stimu-

late AP-1 dependent transcription have evolved. First, the c-jun protein is posttranslationally modified upon the appearance of active p21ras, resulting in a stimulation of its transactivating potential (Binétruy *et al.*, 1991; Pulverer *et al.*, 1991; Smeal *et al.*, 1991; Smeal *et al.*, 1992). In addition, the expression of c-fos is transiently induced by active p21ras, resulting in increased AP-1 binding activity (Schönthal, 1988; Stacey *et al.*, 1987). The transient nature of this induction is a consequence of repression of fos-promoter activity by elevated levels of c-fos (Schönthal, 1988). Finally, this initial stimulation of AP-1 activity gives rise to a more sustained increase in c-jun expression mediated by a TRE in the c-jun promoter (Angel *et al.*, 1988).

Activation of the c-jun protein is associated with phosphorylation of its activation domain, as well as dephosphorylation of at least one of three inhibitory phosphorylation sites next to its DNA-binding domain (Boyle *et al.*, 1991). In unstimulated cells, c-jun is phosphorylated on the inhibitory sites (Boyle *et al.*, 1991). Initially, glycogen synthase kinase 3 (GSK3) was held responsible for this phosphorylation (Woodgett, 1991), but recently evidence has been obtained that casein kinase II (CKII) phosphorylates c-jun at these sites (Lin *et al.*, 1992). This finding suggests that CKII negatively regulates expression of genes that are transactivated by c-jun. This is in apparent contradiction with the finding that several growth factors that stimulate expression of AP-1-inducible genes, enhance CKII activity (Ackermann *et al.*, 1990; Klarlund and Czech, 1988; Sommercorn *et al.*, 1987). Also, CKII can stimulate c-fos expression through activation/phosphorylation of the serum response factor (SRF), suggesting CKII has a positive effect on gene expression (Gauthier-Rouvière *et al.*, 1991; Manak *et al.*, 1990). At present, no data are available concerning the effect of p21ras on the activity of CKII.

Phosphorylation of the sites in the activation domain of c-jun can be established by erk2 *in vitro*, suggesting that erk2 is the connection between p21ras and c-jun (Pulverer *et al.*, 1991). However, a c-jun protein kinase has been isolated by association to recombinant jun protein, but does not appear to be erk1 or erk2 (Adler *et al.*, 1992). Jun-activity is required for ras-transformation, indicating that c-jun is a mediator of p21ras action (Lloyd *et al.*, 1991). Moreover, ras-induced expression from promoters containing AP-1 and PEA3 binding sites requires raf-1 kinase, suggesting that this route is likely to involve erk2 (Bruder *et al.*, 1992). Other possible candidates for phosphorylation by erk2 are p62TCF and myc (Gille *et al.*, 1992; Seth *et al.*, 1991), but not much is known about the involvement of p21ras in the regulation of the transcriptional activity of these factors.

4. p21ras in development.

4.1. Proliferation versus differentiation.

The identification of ras genes as transforming oncogenes made researchers initially focus on the positive role of p21ras in the proliferative signals of various growth factors. Clearly, as has been discussed above, p21ras activation plays a decisive role in the onset of proliferation in fibroblasts. However, not long after the discovery of p21ras as an oncoprotein, it became apparent that ras proteins also play a crucial role in differentiation. For instance, microinjection of oncogenic forms of p21ras into PC12 cells causes an induction of neurite outgrowth and a

block of cellular proliferation (Bar-Sagi and Feramisco, 1985; Noda *et al.*, 1985). Expression of oncogenic p21ras leads to proliferation arrest in Schwann cells (Ridley *et al.*, 1988) and causes differentiation in F9 embryonal carcinoma cells (Yamaguchi-Iwai *et al.*, 1990). Also, introduction of v-H-ras in cultured human medullary thyroid carcinomas (MTC), induces differentiation, a process that actually opposes the normal tumor progression of these carcinomas (Nakagawa *et al.*, 1987). Again, introduction of oncogenic p21ras causes a reduced cellular proliferation. Furthermore, differentiation of 3T3-L1 into adipocytes can be induced by expression of oncogenic p21ras, a process that normally requires insulin or IGF-1 (Benito *et al.*, 1991). In contrast, oncogenic p21ras seems to have a negative effect on myogenic differentiation (Olson *et al.*, 1987) and Ca^{2+}-induced differentiation of keratinocytes (Weissman and Aaronson, 1983). Thus, it seems that the cellular context determines whether p21ras controls differentiation or proliferation.

This explanation was challenged, however, by the finding that both NGF and EGF can cause an activation of endogenous p21ras in PC12 cells (Qiu and Green, 1991). Remarkably, only NGF induces differentiation of PC12 cells, whereas EGF will induce proliferation. Using neutralizing antibodies and dominant inhibitory mutants, it was shown that induction of differentiation depends on the activity of p21ras, whereas normal cellular proliferation is not affected (Hagag *et al.*, 1986; Szeberényi *et al.*, 1990). Moreover, microinjection of oncogenic p21ras alone is sufficient for neuronal differentiation (Bar-Sagi and Feramisco, 1985; Noda *et al.*, 1985). This raises an intriguing question; why does EGF not induce differentiation of PC12 cells? Of course one could argue that microinjection of oncogenic p21ras overrules the requirement of synergizing pathways that are normally activated by NGF, but not by EGF, which function independently of p21ras. Also, EGF could activate additional routes which will cause the cell to proliferate. Alternatively, quantitative differences in the activation of p21ras could exist that can explain the difference in response. As a matter of fact, the activation of p21ras by EGF is transient, whereas the activation by NGF is a more lasting response (Qiu and Green, 1992). This might seem a minor difference at first sight, but one that may have major consequences for the downstream signaling from p21ras. It turns out that translocation of erk2 to the nucleus only occurs in response to NGF. EGF causes activation of erk2, but no erk2 can be detected in the nucleus after stimulation with EGF (Traverse *et al.*, 1992), possibly a consequence of the transient nature of the erk2 activation. Active erk2 in the nucleus may phosphorylate various transcription factors that can not serve as substrates of cytoplasmic erk2. These transcription factors could then regulate transcription of genes involved in differentiation of PC12 cells. However, it remains to be established whether this translocation is the decisive factor for the cell to differentiate.

Although these data suggest that in PC12 cells the time course of p21ras-activation is crucial for it to function in induction of proliferation or differentiation, such criteria might not apply to other cells types. For instance, insulin stimulation of A14 cells (NIH 3T3 cells overexpressing the human insulin receptor) results in a durable activation of p21ras, concomitant with an increased DNA synthesis (Osterop *et al.*, 1993). In contrast, EGF-induced activation of p21ras in rat-1 cells is transient, but also induces a marked

stimulation of DNA synthesis. Moreover, the hypothesis that translocation of erk2 is required for it to activate transcription factors remains to be tested. Clearly, the different effects of EGF and NGF in PC12 cells plus the fact that they utilize common pathways make these cells an ideal model system to study the events required to trigger either differentiation or proliferation.

4.2. Development.

So far, we have mainly discussed the involvement of p21ras in signal transduction in mammalian cells. Nevertheless, elegant studies concerning the role of p21ras in signal transduction have been performed in a number of different organisms, in particular *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Caenorhabditis elegans* and *Drosophila melanogaster* . These studies show a remarkable conservation of the signaling pathways in which p21ras is involved, but alos show that certain conceptual differences exist. It is not within the scope of this review to give a complete description of the developmental processes that are controlled by p21ras in the various organisms, excellent reviews have appeared on those issues (Broach, 1991; Greenwald and Rubin, 1992; Rubin, 1989; Rubin, 1991; Sternberg and Horvitz, 1991). Here we will limit ourselves to an overview of the more recent findings and discuss their relevance with respect to the function of p21ras in mammalian cells.

In budding yeast (*S. cerevisiae*) two genes, RAS1 and RAS2, have been identified that are structurally and functionally homologous to mammalian ras genes (Broek *et al.*, 1985; DeFeo-Jones *et al.*, 1983; Powers *et al.*, 1984). However, despite the functional conservation, in budding yeast RAS proteins function (exclusively) as upstream activators of adenylate cyclase (CYR1) (Kataoka *et al.*, 1985; Toda *et al.*, 1985), an enzyme that is not directly regulated by p21ras in mammalian cells (Beckner *et al.*, 1985). This RAS/cAMP system in budding yeast controls initiation of the cell cycle (reviewed in Broach, 1991). When nutrient levels are sufficiently high cells enter the mitotic cycle. On the other hand, starvation causes an inactivation of this pathway and consequently cells will enter G_0 and sporulate. Therefore, the activation state of RAS1 and RAS2 is in some way linked to the nutrient status. Several regulatory proteins of RAS in budding yeast have been cloned, such as two different GNRPs; CDC25 and SDC25 (Broek *et al.*, 1987; Camonis *et al.*, 1986; Créchet *et al.*, 1990; Jones *et al.*, 1991) and two different GAPs: IRA1 and IRA2 (Tanaka *et al.*, 1989; Tanaka *et al.*, 1990). These proteins show significant homology with their respective mammalian counterparts and in fact one mammalian GNRP has been cloned by functional complementation of a CDC25-minus strain (Martegani *et al.*, 1992). Interestingly, both IRA1 and IRA2 are not believed to function as effectors for RAS proteins, but solely down-modulate RAS activity, since loss of function of both IRA1 and IRA2 suppresses lethality CDC25 null mutations (Tanaka *et al.*, 1989). Finally, SRV2 (or CAP) seems to be required for efficient coupling of RAS to adenylate cyclase (Fedor-Chaiken *et al.*, 1990; Field *et al.*, 1990), which raises the question whether similar proteins exist in mammalian cells. Despite the clear linkage of RAS and adenylate cyclase in budding yeast, it is unclear how the nutrient status can control the RAS/cAMP pathway. Recently, it was reported that in response to glucose the CDC25 protein dissociates from RAS upon phosphoryla-

tion by cAMP dependent protein kinase (Gross *et al.*, 1992a). This indicates that activation of the cAMP/RAS pathway results in a reduced interaction of RAS with its upstream activator CDC25, which could serve as a feedback mechanism. It has been proposed that allosteric (in)activation of regulatory proteins of RAS by certain metabolites of glucose could trigger the activation of RAS (Broach, 1991). If so, this provides us with interesting options for regulation of p21ras in mammalian cells. This situation would be somewhat similar to regulation of p21ras by lipid metabolites discussed previously.

The fission yeast (*S. pombe*) contains a single ras gene, ras1 (Fukui and Kaziro, 1985). This ras gene is not required for growth control, like in budding yeast, but functions in the sexual differentiation pathways (Fukui *et al.*, 1986; Nadin-Davis *et al.*, 1986). Loss of function of ras1 results in a loss of pheromone response and sterile yeast strains. In fission yeast no effector for ras1 has been identified and the ste6 gene is the only regulatory gene for ras1 identified thus far. The ste6 gene encodes a CDC25 homolog and is therefore believed to be the GNRP for ras1 (Fukui *et al.*, 1986; Hughes *et al.*, 1990; Nadin-Davis *et al.*, 1986). However, activation of ras1 alone does not seem to be sufficient for entry into meiosis, indicating that other pathways must be activated which synergize with ras1 (Nielsen *et al.*, 1992).

Recently, kinase cascades showing homology to the erk-kinase cascades in mammalian cells have been partially elucidated in both budding and fission yeast (see Fig.7). In budding yeast, FUS3 and KSS1 encode erk-homologs. Analogous to erks, FUS3 is phosphorylated on both tyrosine and threonine residues (separated by one amino acid) upon pheromone treatment (Gartner *et al.*, 1992). Genetic evidence places the protein kinases STE7 and STE11 upstream of FUS3 and KSS1, in a pathway in which activation of STE11 would precede activation of STE7 (Cairns *et al.*, 1992; Stevenson *et al.*, 1992). Similarly, in fission yeast, the protein kinase spk1 represents an erk-homolog (Toda *et al.*, 1991), whereas byr1 (Nadin-Davis and Nasim, 1988; Nadin-Davis and Nasim, 1990) and byr2 (Wang *et al.*, 1991) represent protein kinases that are both believed to function downstream of ras1. Sequence comparison shows that byr1 and STE7 resemble mapkk (Crews *et al.*, 1992; Howe *et al.*, 1992), suggesting the signal transduction pathway has been conserved. However, the mammalian raf-1 kinase, which has been proposed to be the kinase that activates mapkk (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992), shows no apparent homology to STE11 or byr2 (Howe *et al.*, 1992). These kinases have been proposed to play a role upstream of STE7 and byr1, respectively, but are not necessarily immediately upstream of STE7 and byr-1 and could just as well represent the yeast raf-kinase kinases. Altogether, the signal transduction pathway of fission yeast, more than that in budding yeast, shows a remarkable resemblance to the pathway that has recently become apparent in mammals. That is, p21ras activates a kinase cascade that includes mapkk (STE11, byr2), which phosphorylates and activates erk (STE7, byr1). Further genetic analysis of this pathway in yeast will provide us with novel intermediates and targets of this pathway. Since the pathway is apparently so well conserved, homologs in mammals are likely to exist, although the RAS/cAMP system in *S. cerevisiae* has clearly demonstrated that such extrapolations do not always hold.

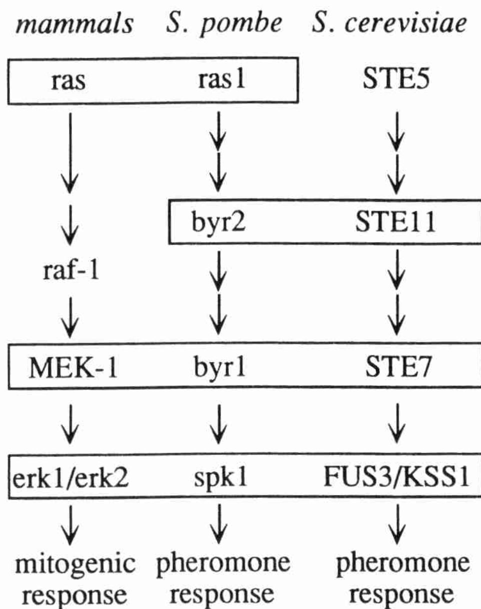

Fig.7. Conservation of a kinase cascade in yeast and mammals. Known intermediates of the kinase cascade initiated by p21ras in mammalian cells, eventually leading to activation of erk1 and erk2 are depicted for *S. cerevisiae*, *S. pombe* and *mammals*. Intermediates that show homology throughout these species are boxed. At places where two arrows are present between two intermediates, one or more proteins could be required for transduction of the signal.

C. elegans is the only multicellular organism for which a complete cell lineage map is available. The induction of vulva formation in this nematode is believed to be initiated by an inductive signal produced by a gonadal anchor cell that triggers the vulva precursor cells to divide and produce the vulva. Correct development of the vulva depends on the strength of the inductive signal. The inductive signal produced by the anchor cells will override an inhibitory signal and cause the cells close to the anchor cell to have the vulval fate. Interest for this organism from the ras-field boomed when a gene was identified in *C. elegans* (let-60), that was shown to encode a protein homologous to mammalian p21ras (Han and Sternberg, 1990) (see Fig.8). This gene was originally isolated as a gene involved in vulva induction (Ferguson *et al.*, 1987). Dominant mutations in let-60, which are similar to the oncogenic mutations in mammalian ras-genes, lead to formation of the multivulva phenotype, whereas loss-of-function mutations invariably result in a vulvaless phenotype (Beitel *et al.*, 1990). By genetics many other genes involved in vulva induction have been identified (Ferguson *et al.*, 1987). Let-23, the first to be characterized, encodes a RTK with similarities to the EGF-receptor (Aroian *et al.*, 1990). More recently, a protein containing SH2/SH3 domains was cloned from *C. elegans* (sem-5), involved in vulva induction (Clark *et al.*, 1992). Genetic evidence places this protein downstream of let-23 and upstream of let-60, suggesting that SH2/SH3 domains perform important functions in this pathway. An homologous protein from humans was tested for its function in p21ras signaling, based on the genetic linkage in nematodes. Remarkably, the human homolog, grb2, could cause DNA synthesis in cells when coinjected with normal p21ras (see above). This clearly exemplifies that the biological approach benefits from the groundwork layed by the genetic approach to p21ras signaling.

In *Drosophila melanogaster*, ras proteins regulate cell fate in the developing eye (see Fig.8). The signaling pathway is somewhat reminiscent of that in *C. elegans*. Development of the R7 photoreceptor cell requires the interaction of the sevenless receptor (*sev*) with its ligand bride-of-sevenless (*boss*), that is expressed on the surface of an adjacent R8 cell (Krämer *et al.*, 1991). The sevenless receptor encodes a transmembrane receptor with tyrosine kinase activity (Hafen *et al.*, 1987). Several mutant flies

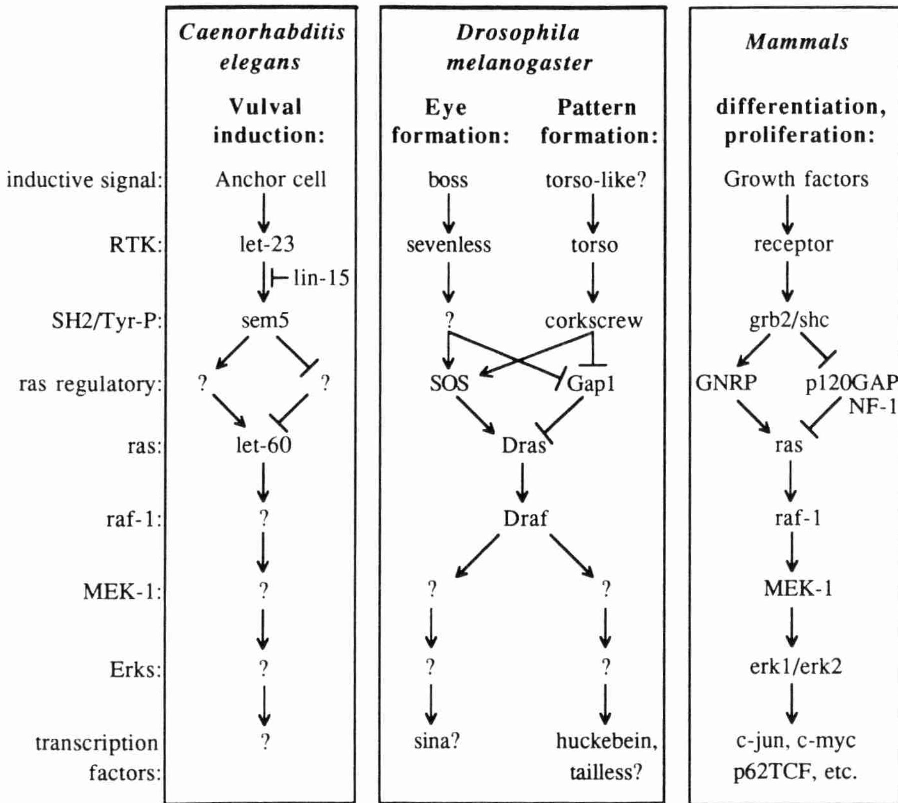

Fig.8. Ras-signal transduction pathways in *C. elegans*, *D. melanogaster* and *mammals*. RTK-signaling via p21ras is initiated by an inductive signal, resulting in activation of the RTK. The next step most likely involves a network of tyrosine phosphorylations/dephosphorylations and SH2-mediated interactions, of which a few components have become clear just recently. This network must somehow result in activation of p21ras by means of activation of a GNRP or inactivation of a GAP, the level indicated as ras regulatory proteins. Ras-activation then can activate the raf-1 kinase, although many intermediates could precede this particular activation. Raf-1 kinase initiates a kinase cascade, which will eventually result in the transduction of the signal to the nucleus, by phosphorylation of a number of transcription factors. The simple schemes presented here are partially based on speculations and serve to indicate the conservation in this particular signaling pathway, but it is likely that many other intermediates are critical to this pathway.

were subsequently isolated with aberrant R7 development. Analysis of these mutants learned that the *Drosophila* ras1 gene was involved in this signal transduction pathway in control of R7 cell fate (Fortini *et al.*, 1992; Simon *et al.*, 1991). Moreover, a CDC25 homolog, son-of-sevenless (SOS) was identified as another gene involved in this pathway (Bonfini *et al.*, 1992; Fortini *et al.*, 1992; Simon *et al.*, 1991). Therefore, it seems acceptable that interaction of *boss* with the *sevenless* receptor causes activation of *ras1*. The activation of *ras1*, like in mammalian cells, requires the action of an guanine nucleotide release factor, a function that is most likely fulfilled by the *sos* protein. Also, a gene encoding a *Drosophila* homolog of the mammalian GAPs has been identified, called Gap1 (Gaul *et al.*, 1992). Deletion of the Gap1 gene leads to formation of supernumerary R7 cells, similar to what is seen in flies expressing

ras1(Val12). This suggests that, at least for R7 induction, the *Gap1* protein does not function as effector for *ras1*. Most interestingly, a *Drosophila* homolog of human rap1A (*roughened*), has been identified that apparently antagonizes the function of *ras1* (Hariharan *et al.*, 1991). This is very similar to the finding that overexpression of rap1A can revert ras-transformed cells (Kitayama *et al.*, 1989). The gain of function mutations of *roughened* affect a phenylalanine at position 157, that is conserved in rap1A and H-ras, but no functional role has been ascribed to this amino acid. It will be of great interest to see whether a similar mutation in rap1A could also potentiate its role as suppressor of ras-transformation. Finally, the *Drosophila* homolog of raf-1 kinase, *lethal*(1)*polehole* or *Draf* (Mark *et al.*, 1987), plays a crucial role in the R7 pathway (Dickson *et al.*, 1992). Therefore, also in *Drosophila* the p21ras signaling pathway seems to be similar to what has been proposed in mammalian cells.

In addition to the function in eye development, functional *Draf* is required for head and tail formation in the developing *Drosophila* embryo (Ambrosio *et al.*, 1989; St Johnston and Nüsslein-Volhard, 1992). This developmental process requires the protein products of torso and corkscrew (csw) (Casanova and Struhl, 1989; Klingler *et al.*, 1988; Perkins *et al.*, 1992; Sprenger *et al.*, 1989), as well as two transcription factors, *tailless* and *huckebein* (reviewed in St Johnston and Nüsslein-Volhard, 1992) (see Fig.8). Torso appears to encode a RTK and corkscrew encodes a putative tyrosine phosphatase with two SH2 domains, similar to mammalian PTP1C (Shen *et al.*, 1991). The involvement of *Draf* in this pathway suggests a function for *ras1* as well, based on the proposed signaling pathway in mammalian cells. Indeed, it seems that also *ras1* is required for *torso* signaling (cited in Roberts, 1992). Therefore, like in mammalian cells, both p21ras and raf-1 kinase are important intermediates in signaling by different RTKs in *Drosophila* and SH2 containing proteins perform important functions. The finding that a tyrosine phosphatase with SH2 domains plays a role in a RTK signaling route that also requires p21ras opens up new perspectives. The importance of phosphatases in p21ras signaling in mammalian cells has indeed been implicated (Medema *et al.*, 1991a) and therefore it will be of great interest to identify the mammalian homolog of corkscrew. This protein might prove to be an important mediator of RTK signaling towards p21ras.

Clearly, the conserved function of ras genes in different organisms enables us to take a genetic approach to tackle dilemmas such as the identification of the effector of p21ras, intermediates required for activation by RTKs, etc. Genetic and biochemical approaches to signal transduction have a mutual benefit from the progress booked in the separate fields and it seems that future research will depend on a combination of both.

5. Concluding remarks.

The last few years have provided us with many new insights in the role of p21ras in signal transduction, although important topics, such as the effector molecule of p21ras, mechanism of p21ras activation and the role of PKC in the p21ras pathway remain to be solved. Most importantly, the ras protein seems to couple RTKs to kinase cascades, involving raf-1 kinase and erks, making signal transduction towards the

nucleus possible. Clearly, SH2-containing proteins will play an important adaptor function in this signaling pathway, possibly by altering the enzymatic activity of certain proteins by an allosteric activation. These functions, as well as many components of the p21ras signaling pathway are apparently conserved through evolution. The available model systems in genetically well-defined organisms will certainly speed up the identification of regulatory proteins, as well as downstream targets of p21ras. As usual, some questions have been answered, but many more have been created. Most importantly, a general scheme for signal transduction from RTKs via p21ras has evolved, which will direct future research to fill in many of the missing links. Ultimately, combined research efforts will elude the versatile role of p21ras in many key processes in development, i.e. cellular proliferation and differentiation. That our understanding of disease processes like cancer will benefit from this knowledge needs no explanation.

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CHAPTER 2

Insulin stimulation of gene expression mediated by p21ras activation.

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Insulin stimulation of gene expression mediated by p21ras activation

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In fibroblasts, insulin is a weak mitogen and does not induce expression of c-*fos*, c-*jun* or *p33*. However, increasing the expression levels of either normal p21Hras or the insulin receptor, but not mutant p21Hras, enables insulin to induce the expression of these genes. In cells expressing elevated levels of insulin receptor, this process involves a rapid increase in p21rasGTP levels (from 20% to 70% GTP as a percentage of total guanine nucleotides). No increase in p21rasGTP levels was observed after PDGF and EGF stimulation of cells expressing high levels of the cognate receptor, stressing the specificity of the insulin-induced increase. We conclude that in fibroblasts, p21ras is an intermediate of the insulin signal transduction pathway involved in the regulation of gene expression and mitogenicity.

Key words: GDP–GTP exchange/GTPase/insulin/phosphatidylinositol-3-kinase/signal transduction

Introduction

Proteins encoded by members of the p21ras gene family bind guanine nucleotides and have low intrinsic GTPase activity (Barbacid, 1987). p21ras GTPase activity is strongly increased by a cytosolic protein of 120 kd called GTPase activating protein (GAP) (Trahey and McCormick, 1987). Specific point mutations at positions 12, 13 or 61 of p21ras result in proteins that have lost their intrinsic GTPase activity (Gibbs *et al.*, 1984; Sweet *et al.*, 1984) as well as regulation by GAP (Trahey and McCormick, 1987). These mutant p21ras proteins are capable of transforming immortalized cells *in vitro*, indicating that the GTP form of p21ras is a positive signal in cell proliferation (Gibbs *et al.*, 1987; Hoshino *et al.*, 1988; Satoh *et al.*, 1988; Trahey and McCormick, 1987). Moreover, microinjection of p21rasGTP but not p21rasGDP induces the expression of c-*fos*, showing that also in the regulation of gene expression p21rasGTP is the active state (Stacey *et al.*, 1987). By analogy to the heterotrimeric G proteins, it has been postulated that GTP binding to p21ras is under the control of growth factors. Thus, a specific growth factor may constitute or induce a messenger upstream of p21ras and drive p21ras into an active, signal generating state (Hall, 1990). Recently, Downward *et al.* (1990a) strengthened this concept, by showing that upon stimulation of T cells with phytohaemagglutinin, the GTP form of p21ras accumulates rapidly.

In most cell types the signal that activates p21ras is still unknown, although several candidates have been put forward (see also Hall, 1990). Korn *et al.* (1987) showed that p21ras mediates insulin-induced maturation of *Xenopus* oocytes and we recently obtained evidence that in rat fibroblasts, p21ras might mediate insulin- or insulin-like growth factor I-induced processes as well (Burgering *et al.*, 1989). For the neuronal PC12 cells it has been suggested that p21ras might be involved in nerve growth factor-induced neurite outgrowth (Bar-Sagi and Feramisco, 1985; Hagag *et al.*, 1986; Noda *et al.*, 1985). Furthermore, using interfering p21ras mutants (Asn17), a role for p21ras has been suggested in EGF and TPA signal transduction (Cai *et al.*, 1990). Also, a close linkage between p21ras and PDGF-stimulated responses has been implicated (Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990; Molley *et al.*, 1989). The fact that microinjection of antibodies against p21ras can block the action of a variety of growth factors, suggests a more general role for p21ras in growth control, and that, for instance, p21ras might be a connecting point for several growth factor mediated signalling pathways (Mulcahy *et al.*, 1985; Yu *et al.*, 1988).

We have studied whether p21ras can mediate signals induced by stimulation of cells with insulin. For that purpose we investigated the effects of expression of mutant p21Hras or overexpression of normal p21Hras and of insulin receptor on insulin-induced gene expression. Furthermore, we have measured the effect of insulin treatment on the activation state of p21ras. Our results show that insulin stimulation results in a rapid conversion of p21rasGDP into p21rasGTP, which provides evidence that the activation of p21ras may be part of the insulin signal transduction pathway leading to the induction of gene expression.

Results

Overexpression of insulin receptor and overexpression of normal p21Hras enhance insulin-induced gene expression

We have previously proposed a role for normal p21ras in the mitogenic effect of insulin (Burgering *et al.*, 1989). To extend our initial observations, we analysed the effect of overexpression of normal p21Hras and mutant p21Hras on insulin-induced expression of c-*fos*, c-*jun* and *p33* [*p33* is an insulin-inducible gene isolated from rat hepatocytes (Messina *et al.*, 1985)]. The different cells lines were serum starved for 24 h, insulin or serum was added for various lengths of time, and RNA was isolated and probed for the expression of c-*fos*, c-*jun* and *p33* mRNA. A clear induction by insulin of c-*fos*, c-*jun* and *p33* gene expression was

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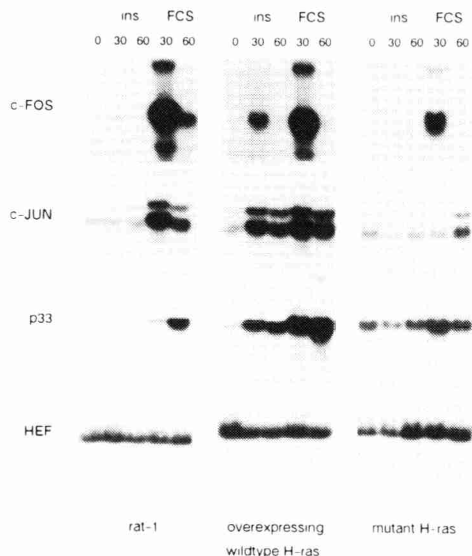


Fig. 1. Induction of c-*fos*, c-*jun* and *p33* mRNAs after growth factor treatment of rat-1 parental cells and p21Hras-transformed cell lines. Subconfluent cultures of different cell lines were serum-arrested for 24 h and stimulated with insulin (ins, 10 μg/ml) or fetal calf serum (FCS, 10%). Total RNA was isolated at the indicated time points (minutes) and probed for the expression of the genes indicated. Hybridizations with human elongation factor 1 (HEF) cDNA were performed to indicate equal amounts of RNA. Control inductions with the solution in which insulin is dissolved (1% BSA, 4 mM HCl) did not show any increase in the expression of the genes analysed (not shown). Results shown were obtained with the H13 cell line, overexpressing normal p21ras (Downward *et al.*, 1988) and RR3, a mutant p21ras expressing cell line. Similar results were obtained with two other p21Hras overexpressing cell lines, H9 (Downward *et al.*, 1988) and HE$^+$ (Burgering *et al.*, 1989) and with two other mutant p21Hras expressing cells, RR2 and RR7.

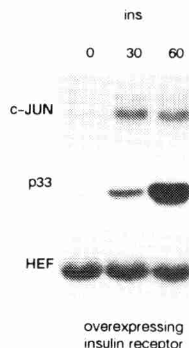


Fig. 2. Induction of c-*jun* and *p33* expression after insulin stimulation of insulin receptor overexpressing cells. Subconfluent cultures of A14 cells, overexpressing the insulin receptor, were serum-arrested for 24 h and stimulated with insulin (ins, 10 μg/ml). Total RNA was isolated at the indicated time points (minutes) and probed for the expression of the genes indicated. Hybridizations with human elongation factor 1 (HEF) cDNA were performed to indicate equal amounts of RNA.

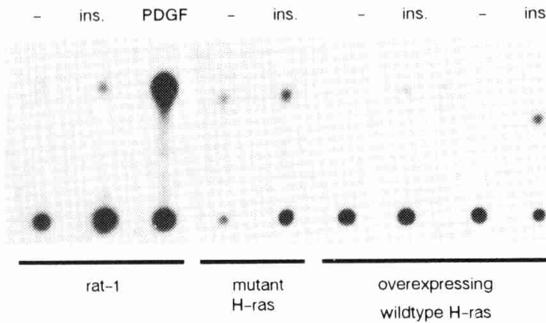
observed in cells overexpressing normal p21Hras (Figure 1). In contrast, we observed only a small, hardly detectable, insulin-induced increase in the expression of these genes in the untransformed, parental rat-1 cells or in the mutant p21Hras-transformed cells. As in the normal p21Hras overexpressing cell lines, insulin also stimulated expression of c-*jun* and *p33* in the insulin receptor-overexpressing A14 cell line (Figure 2). These results confirm those obtained by us (Burgering *et al.*, 1989) and others (Stumpo and Blackshear, 1986) for insulin induced c-*fos* expression. Taken together these results show that both increased expression of insulin receptor as well as increased expression of p21Hras result in enhanced insulin signalling, as reflected by the ability of insulin to stimulate gene expression.

Overexpression of p21Hras does not enhance all cellular responses to insulin

In cell lines expressing high levels of insulin receptor, several other processes are induced rapidly by insulin, including an increase in tyrosine phosphate-containing phosphatidylinositol-3-kinase (PI-3-K) activity (Endemann *et al.*, 1990; Ruderman *et al.*, 1990). We have investigated whether overexpression of p21Hras potentiates this insulin response in a way similar to the induction of gene expression. For this purpose we measured insulin-induced PI-3-K activity in anti-phosphotyrosine immunoprecipitates. The various cell lines were serum starved for 24 h and stimulated with insulin. Cells were lysed 10 min after stimulation and phosphotyrosine-containing proteins were immunoprecipitated with a polyclonal antibody. PI-3-K activity was measured in the immunoprecipitate collected on protein A–Sepharose beads. As shown in Figure 3, PI-3-K activity was strongly induced in the cells overexpressing the insulin receptor, but not in the cell lines overexpressing normal p21Hras or in any of the other cell lines. In the cell lines overexpressing normal p21Hras and mutant p21Hras, PI-3-K is not defective, since in these cells PDGF can induce PI-3-K activity normally (B.M.Th.Burgering, A.M.M.de Vries Smits, F.McCormick, J.L.Bos, manuscript in preparation). These results show that overexpression of p21Hras does not augment all cellular responses to insulin and that overexpression of p21Hras specifically contributes to the response pathway of insulin involved in the regulation of gene expression.

Insulin stimulates increase of p21rasGTP levels

The simplest model deduced from the experiments described above would assume that p21ras is an intermediate in insulin signalling. To demonstrate this more directly, we have analysed the effect of growth factor treatment, in particular insulin, on the relative levels of GTP bound to p21ras. To this end, cells were labelled *in vivo* with [^{32}P]orthophosphate for 3 h and after growth factor stimulation for an additional 5 min, cells were lysed and p21ras was collected by immunoprecipitation with the monoclonal antibody Y13-259. Bound nucleotides were eluted and separated by thin layer chromatography. Using this protocol, we observed in untransformed cells a low amount of GTP bound to p21ras as a proportion of total nucleotides (20% GTP; Figure 4A), compared with the level of GTP bound



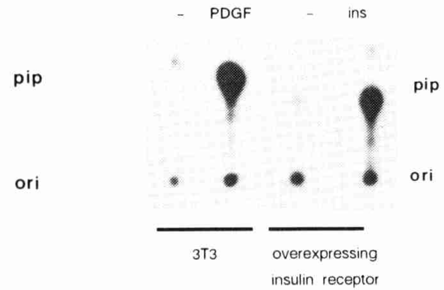


Fig. 3. Activation of PI-3-K after growth factor treatment. Cells were serum-arrested for 24 h and stimulated with insulin (ins. 10 μg/ml) or PDGF (40 ng/ml) for 15 min. Cells were lysed and 400 μg of protein was precipitated with polyclonal anti-phosphotyrosine serum. The precipitates bound to protein A-Sepharose beads were assayed for PI-3-K activity using phosphatidylinositol as substrate. Lipids were separated by thin layer chromatography and detected by autoradiography. −, no growth factor added. Cell lines used are (**A**) rat-1, RR2 (mutant p21Hras), H9 and H13 (overexpressing normal p21Hras); (**B**) NIH 3T3 and A14 (overexpressing insulin receptor). The position of the different phosphatidylinositols (PI, PIP and PIP_2) were determined by running standards and staining with iodine vapour.

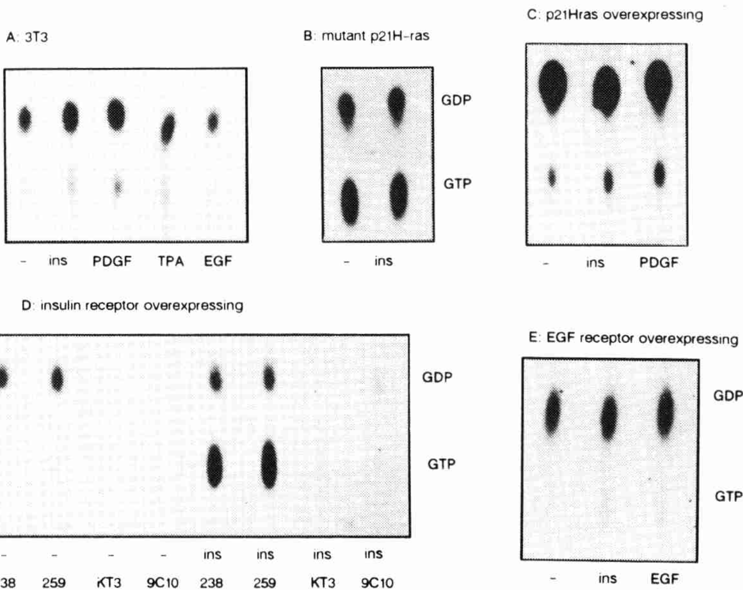


Fig. 4. GTP/GDP bound to p21ras. Autoradiographs after chromatographic separation of GTP and GDP eluted from p21ras. Cells were labelled with [^{3}P]orthophosphate for 3 h and stimulated with growth factors for 5 min. Cells were lysed and p21ras was collected by immunoprecipitation with the ras-specific monoclonal Y13-259 or, when indicated, with the p21ras-specific monoclonal Y13-238 or the control monoclonals KT3 and 9C10. GDP/GTP was eluted and separated by thin layer chromatography. **A**. NIH/3T3 cells, unstimulated (−) and stimulated with insulin (ins, 10 μg/ml), PDGF (20 ng/ml), TPA (100 ng/ml) and EFG (20 ng/ml). **B**. RR3 cells expressing mutant Hras, unstimulated (−) and stimulated with insulin. **C**. H13 cells, overexpressing normal p21Hras, unstimulated (−) and stimulated with insulin or PDGF. **D**. A14 cells, overexpressing the insulin receptor, unstimulated (−) and stimulated with insulin. **E**. HER14 cells, overexpressing the EGF receptor, unstimulated (−) or stimulated with insulin or EGF.

to p21ras in mutant p21Hras-expressing cells (70% GTP; Figure 4B). Treatment of normal fibroblasts—NIH 3T3 cells (Figure 4A) and rat-1 cells (not shown)—with various growth factors did not result in a detectable shift in the GTP/GDP balance on p21ras. Next, we analysed the effect of insulin stimulation on the cells expressing increased levels of

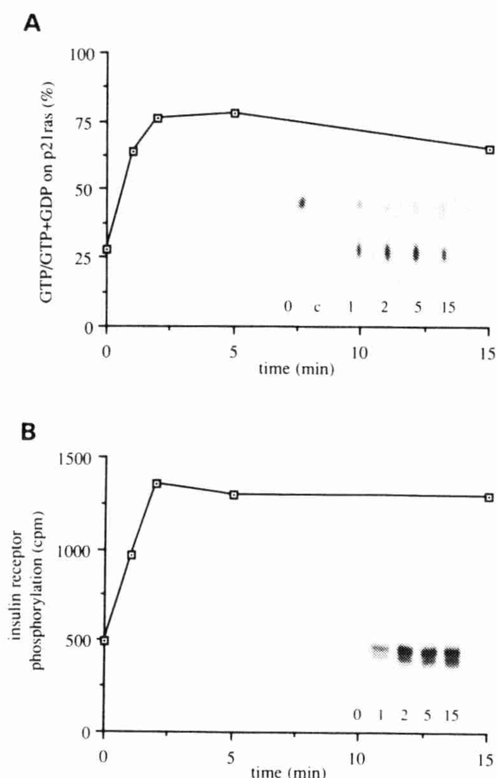


Fig. 5. Time course of insulin-induced increase in GTP bound to p21ras and increase in insulin receptor phosphorylation. **A**. Graphic presentation of the p21rasGTP/GDP ratio in A14 cells after stimulation with insulin for the indicated times. The insert shows the autoradiograph of the thin layer separation of GTP and GDP bound to p21ras (for details see legend to Figure 4) c: control unstimulated with antibody KT3. **B**. Graphic presentation of the phosphorylation of the β-chain of the insulin receptor. *In vivo* phosphorylated insulin receptor was immunoprecipitated and the β-chain was separated by SDS−polyacrylamide gel electrophoresis. After autoradiography (see insert), the phosphorylated bands corresponding to the insulin receptor β-chain were cut out and the ^{32}P content was quantified by liquid scintillation counting.

p21Hras. Insulin did not significantly stimulate a shift in the balance of GTP/GDP bound to p21ras in the normal p21Hras overexpressing cells, although in some experiments a small increase was observed (from 3 to 5% GTP; Figure 4C). This insulin-induced increase was within the same order as that observed after stimulation of p21Hras overexpressing cell lines with PGDF (Figure 4C) or EGF (data not shown: see also Satoh *et al.*, 1990a,b). However, insulin stimulation of cells expressing increased levels of insulin receptor resulted in drastic accumulation of GTP on p21ras (20% to 70% GTP; Figure 4D). The insulin-induced activation of p21ras in the insulin receptor overexpressing cells was analysed using the Triton X114 phase-split method (Bordier, 1981; Hancock *et al.*, 1990). In this method palmitoylated/farnesylated p21ras is partitioned in the detergent phase, whereas the non-modified p21ras is retained in the aqueous phase. It demonstrates that the activation of p21ras occurred within the fraction of processed p21ras. To substantiate the specificity of the observed activation of p21ras, we performed several control experiments. First, precipitation with another anti-ras monoclonal antibody (Y13-238) yielded similar results, whereas precipitation with two unrelated antibodies [KT3 (anti-SV40 LT) and 9C10 (anti-adenovirus E1B)] were negative, underscoring that the GTP/GDP binding was to p21ras. Secondly, we analysed the time course of the insulin-induced increase of GTP bound to p21ras (Figure 5A). After 1 min, the accumulation of GTP on p21ras was clearly visible and almost maximal, indicating that the activation of p21ras is a very rapid process. The increased GTP/GDP ratio on p21ras remained elevated for at least 15 min. Thirdly, after precipitation of p21ras, the ^{32}P-labelled lysates used for the time course experiment were re-used to immunoprecipitate the insulin receptor with a polyclonal anti-insulin receptor serum. After SDS−PAGE and autoradiography, the phosphorylated bands corresponding to the insulin receptor β-chain were cut out and ^{32}P content was quantified by liquid scintillation counting. The kinetics of insulin-stimulated phosphorylation of the insulin receptor (Figure 5B) were rapid and almost identical to the kinetics of insulin stimulated accumulation of GTP on p21ras (Figure 5A), indicating a tight link between receptor activation and p21ras activation. Finally, we analysed whether EGF can stimulate the increase of GTP on p21ras in cells overexpressing the EGF receptor [HER14 cells, $\sim 3 \times 10^5$ EGF receptors (Honegger *et al.*, 1987)]. As shown in Figure 4E, EGF stimulation of HER14 cells did not result in a significant increase in the levels of GTP bound to p21ras.

Discussion

Insulin induced gene expression

In this paper we have studied the effects of insulin on gene expression, PI-3-K activity and the GTP/GDP balance on p21ras in cell lines expressing different levels of insulin receptor, normal p21Hras and mutant p21Hras. The data presented provide evidence that insulin-induced activation of p21ras is an intermediate in the route leading from insulin receptor activation to the induction of gene expression.

The premise that normal p21ras proteins are mediators of growth factor-induced signals to downstream targets implies that overexpression of normal p21Hras should result in a qualitative and/or quantitative change in growth factor response. In this respect there is no conceptual difference between the effect of overexpression of a growth factor receptor or a key intermediate in the signal transduction pathway. Indeed, we do not observe a difference between the effect of overexpression of the insulin receptor and the effect of overexpression of p21Hras in insulin-induced gene expression. Both in cells overexpressing the insulin receptor and in cells overexpressing normal p21Hras, but not in the parental cells, insulin can induce the expression of genes like c-*fos*, c-*jun* or *p33*. This therefore indicates the p21ras may be part of the insulin signalling pathway to induce gene expression. The observation that in mutant p21Hras expressing cells insulin did not induce gene expression is consistent with this conclusion. In these cells, p21Hras is

postulated to be active constitutively and is likely to be insensitive to the upstream regulatory mechanisms controlling normal p21Hras function. The elevated level of p33 expression might be due to the constitutive activation of the downstream part of the p21ras pathway. The expression of *c-fos* and *c-jun* is not elevated in mutant p21Hras transformed cells, presumably because these genes are expressed only transiently after growth factor stimulation.

Insulin induced activation of p21ras

The direct link between p21ras and insulin-induced receptor activation was shown in cells expressing increased amounts of the insulin receptor. In these cells the level of GTP bound to p21ras increases dramatically within 1 min after insulin stimulation (Figure 4). Furthermore, the time course of insulin-induced phosphorylation of the insulin receptor β-chain, a measure for insulin receptor activation, and of insulin-induced activation of p21ras is identical (Figure 5). We do not observe insulin-induced stimulation of p21ras in normal fibroblasts, presumably due to the low number of insulin receptors (2×10^3 receptors/cell compared with 3×10^5 receptors/cell in the insulin receptor overexpressing cells). This does not imply that insulin-induced activation of p21ras does not occur in these cells. A small shift in the GTP/GDP balance might be brought about after insulin stimulation, yet remain undetected. In this respect it is interesting to note that other growth factors tested, including serum, do not cause a significant shift of the GTP/GDP balance on p21ras in NIH 3T3 cells and rat-1 cells (Figure 1A; J.Downward, personal communication), while blocking p21ras, by micro-injection of Y13-259 in these cells, inhibits mitogenic signalling of these growth factors (Mulcahy *et al.*, 1985; Yu *et al.*, 1988). Similarly, in cells overexpressing normal p21Hras, hardly any increase in the level of GTP bound on p21ras after insulin stimulation was observed. It should be noted, however, that in these cells, which overexpress p21Hras 100-fold, an increase of 0.5% GTP bound to p21ras represents an absolute increase of GTP bound to p21ras that is similar to the increase observed after insulin stimulation of the cells expressing increased levels of insulin receptor but normal levels of p21ras. Such an increase, although observed by Satoh *et al.* (1990a,b) in p21Hras overexpressing cells after PDGF and EGF stimulation, would be undetectable in our assay.

An effect on the nucleotide balance of p21ras similar to that found in the cells expressing increased levels of the insulin receptor with insulin, has been shown for T cells, where stimulation of the T cell receptor or treatment with the phorbol-ester TPA results in a rapid and strong shift in the GTP/GDP balance on p21ras (Downward *et al.*, 1990a).

p21ras is part of the insulin signal transduction pathway

From our results we conclude that insulin-induced activation of p21ras is an intermediate step in the insulin signal transduction pathway leading to the activation of gene expression. However, one could argue that increased expression of the insulin receptor results in an improper linkage to other pathways and, thus, is permissive in the activation of p21ras. Although this possibility cannot be formally excluded, it would imply that overexpression of p21Hras is also permissive for stimulation of gene expression by insulin. We therefore consider a role for p21ras as an intermediate in the insulin signal transduction more likely than a mechanism involving some kind of interchangeable permissiveness.

Insulin has many effects on the cell and a variety of pathways has been proposed (Rosen, 1987). Our results do not imply that p21ras mediates all these insulin-induced processes. Indeed, the analysis of insulin-induced PI-3-K activity suggests that this process is not mediated by p21ras. Apparently, p21ras mediates only part of the insulin-induced signal transduction pathway. This notion is further strengthened by observations that insulin-induced hexose uptake and 1,2- and 1,3-diacylgylcerol increase are also unaffected by overexpression of p21Hras (unpublished observations). The involvement of p21ras in only a specific branch of the insulin signal transduction pathway may also explain the observation that under certain experimental conditions insulin can synergize with p21ras elicited signals (Morris *et al.*, 1989), maybe through the increase in glucose and/or metabolite uptake.

p21ras: mediator of multiple signal transduction pathways?

The fact that p21ras is not involved in all insulin-induced cellular responses, but only in a part that is common to many different growth factors, i.e. the induction of gene expression, suggests that p21ras may also be involved in the signal transduction pathway of these growth factors. Indeed, activation of p21ras appears to be obligatory for the induction of mitogenesis by several growth factors and the induction of *c-fos* by serum (Cai *et al.*, 1990; Mulcahy *et al.*, 1985; Yu *et al.*, 1988). However, whether it is the relative or absolute level of p21rasGTP that is required to achieve stimulation of the downstream part of the '*ras*-pathway', is still unknown. The situation is further complicated by the possibility that partial activation of p21ras may synergize with other intracellular routes activated by the same growth factor. Our results show that p21ras is more sensitive to activation by insulin than by other growth factors. For instance, normal fibroblasts express an amount of PDGF-B receptors within the same order of magnitude as insulin receptors on the A14 insulin receptor overexpressing cell line (W.H.Moolenaar, personal communication). PDGF, however, although a strong mitogen, does not activate p21ras significantly. Also after EGF stimulation of NIH 3T3 cells expressing increased levels of the EGF receptor, no increase in GTP bound to p21ras was observed. These observations do not rule out the involvement of p21ras in PDGF and EGF signal transduction, but they indicate that insulin increases p21rasGTP levels more effectively than do PDGF and EGF. Clearly, the involvement of p21ras activation in insulin-induced mitogenesis and gene expression differs from its involvement in PDGF and EGF signalling. In fibroblasts p21ras may be the main mediator in insulin-induced mitogenesis and gene induction, whereas for other growth factors activation of p21ras, although necessary, may synergize with other activated pathways.

Activation of p21ras may be a main crossroad in growth factor-induced signal transduction and it will be of interest to study the relative contributions of the various growth factors in the activation of p21ras and the different mechanisms employed. In this respect insulin-induced activation of p21ras may prove to be an important paradigm for

study of the mechanism by which p21rasGTP/GDP cycling is regulated. At present we do not know what precise mechanism is employed by insulin to increase the level of GTP on p21ras. In analogy to the results obtained with T lymphocytes, inactivation of GAP activity via protein kinase C (Downward *et al.*, 1990a), is a possibility we are currently investigating. On the other hand, activation of an exchange factor remains a possibility as well. The existence of multiple GAP activities, such as the recently identified *NF1* gene product (Ballester *et al.*, 1990; Martin *et al.*, 1990; Xu *et al.*, 1990) and different exchange activities [membrane-bound (West *et al.*, 1990) and cytoplasmic (Downward *et al.*, 1990b; Wolfman and Macara, 1900)] indicates the complexity of p21ras regulation.

Materials and methods

Materials and cells

Tissue culture media and sera were from Gibco Laboratories. Insulin, protease inhibitors, phosphatidylinositol and lipid standards were from Sigma Chemical Co. Platelet-derived growth factor (PDGF) BB homodimer was from Amersham. Polyclonal anti-PY was prepared as described (Pang *et al.*, 1985), polyclonal anti-insulin receptor has been described (Maassen *et al.*, 1987). The overexpressing H-*ras* cell lines (H9 and H13) have been described (Downward *et al.*, 1988) and were kindly provided by J.de Gunzburg (Paris). Mutant p21Hras cell lines (RR2, RR3, and RR7) were made by transfecting a human mutant H-*ras* gene (pEJ 6.6, G12V) and kindly provided by R.Offringa (Leiden). An insulin receptor-overexpressing cell line (A14) was made by transfection of NIH 3T3 cells with a plasmid expressing a full length human insulin receptor cDNA under control of the SV40 early promoter, in combination with a dihydrofolate reductase gene for amplification and a neomycin resistance gene for selection. The cells contain 3×10^5 high affinity insulin receptors ($K_D \leq 10^{-9}$ M) per cell (J.A.Maassen, manuscript in preparation). HER14 cells overexpressing 3×10^5 EGF receptors per cell (Honegger *et al.*, 1987) were kindly obtained from J.Schlessinger. Cells were cultured routinely in DMEM supplemented with 8% fetal calf serum (FCS). For serum starvation subconfluent cultures were cultured in DMEM plus 0.5% FCS and 10 μg/ml transferrin. After 24 h the cells were stimulated with the indicated growth factor.

RNA analysis

RNA was isolated by LiCl–urea lysis and prepared for Northern blotting as described (Schrier *et al.*, 1983). The probes used, HEF and c-*fos*, have been described previously (Burgering *et al.*, 1989). Mouse *c-jun* cDNA was kindly provided by R.Bernards (Boston, MA) and the *p33* cDNA (Messina *et al.*, 1985) was kindly provided by D.K.Granner (Nashville, TN).

Determination of GTP/GDP ratio

The determination of the GTP/GDP ratio of p21ras was essentially as described (Downward *et al.*, 1990a). Cells were serum arrested for 18 h and subsequently labelled for 3 h with 400 μCi [^{32}P]orthophosphate per 9 cm dish in phosphate-free/serum-free medium (Gibco). Cells were stimulated with insulin (10 μg/ml) for the times indicated. Cells were put on ice and rapidly washed with ice-cold Tris-buffered saline and lysed in 50 mM HEPES buffer, pH 7.4, 1% Triton X-114, 100 mM NaCl, 5 mM $MgCl_2$, 1 mg/ml BSA, 10 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor. 100 μM GTP, 100 μM GDP, 1 mM ATP and 1 mM sodium phosphate pH 7.4 was included to prevent post-lysis labelling of p21ras. Nuclei were removed by centrifugation and the Triton X-114 and aqueous phases were split at 37°C for 2 min followed by a brief spin (Bordier, 1981). The detergent phase was deluted 10-fold with lysis buffer without Triton X-114. The lysate was precleared for 5 min with protein G–Sepharose beads and further incubated for 40 min with anti-p21ras monoclonal Y13-259 or Y13-238 (Oncogene Science) or with a control monoclonal KT3 (anti-SV40 large T) or 9C10, a rat antibody directed against adenovirus E1B protein (kindly provided by A.Zantema), all bound to protein G–Sepharose. Immunoprecipitates were collected and washed eight times with 50 mM HEPES buffer, pH 7.4, 500 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 0.005% SDS. GTP/GDP was eluted in 2 mM EDTA, 2 mM DTT, 0.2% SDS, 0.5 mM GDP, 0.5 mM GTP at 68°C for 20 min and separated on PEI–cellulose plates (Merck) developed in 1.2 M ammonium formate, 0.8 M HCl. Plates were autoradiographed and the GTP/GDP ratio was determined by scintillation counting.

Phosphatidylinositol-3-kinase assay

Cells were washed twice with cold PBS containing 1 mM sodium vanadate and lysed by scraping in 1 ml of lysis buffer containing 2 mM Tris–Cl (pH 7.8), 137 mM NaCl, 1% NP40, 10% glycerol, 2 mM sodium EDTA, 1 mM sodium vanadate, 1 mM phenylmethylsulphonyl fluoride, 0.15 U/ml aprotinin and 20 μM leupeptin. After 5 min on ice the nuclei were removed by an Eppendorf spin for 10 min at 4°C. Supernatant was transferred to a clean tube and protein content was measured by the Bradford method (BioRad). 400 μg of protein was incubated with polyclonal anti-phosphotyrosine serum for at least 3 h at 4°C. Protein A–Sepharose beads were used to collect the antigen–antibody complexes. The complexes were washed twice with lysis buffer in the presence of 1M LiCl, twice with lysis buffer and once with 10 mM Tris–Cl (pH 7.5), 1 mM sodium vanadate. PI-3-K activity was determined essentially as described by Kaplan *et al.* (1990). In brief, anti-phosphotyrosine immunoprecipitates collected on protein A–Sepharose were incubated in 30 mM HEPES (pH 7.5), 200 μM adenosine and 0.2 mg/ml sonicated phosphatidylinositol for 15 min at 25°C in a total volume of 50 μl. Adenosine was included to inhibit any contaminating PI-4-K activity (Whitman *et al.*, 1987). The reaction was started by adding 30 mM $MgCl_2$, 40 μM ATP and 10 μCi [γ-^{32}P]ATP (final concentrations) and incubation continued for another 25 min at 25°C. The reaction was terminated by the addition of 100 μl 1 M HCl and quickly mixing. Lipids were extracted by addition of 200 μl chloroform–methanol (1:1). The organic phase was washed once more with methanol–1 M HCl (1:1). An aliquot of the organic phase of 50 μl was applied to a silica gel G plate and developed in chloroform–methanol–4 M NH_4OH (45:35:10).

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CHAPTER 3

Two dominant inhibitory mutants of p21ras
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Two Dominant Inhibitory Mutants of p21^{ras} Interfere with Insulin-Induced Gene Expression

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Insulin induces a rapid activation of p21^{ras} in NIH 3T3 and Chinese hamster ovary cells that overexpress the insulin receptor. Previously, we suggested that p21^{ras} may mediate insulin-induced gene expression. To test such a function of p21^{ras} more directly, we studied the effect of different dominant inhibitory mutants of p21^{ras} on the induction of gene expression in response to insulin. We transfected a collagenase promoter-chloramphenicol acetyltransferase (CAT) gene or a *fos* promoter-luciferase gene into NIH 3T3 cells that overexpressed the insulin receptor. The activities of both promoters were strongly induced after treatment with insulin. This induction could be suppressed by cotransfection of two inhibitory mutant *ras* genes, H-*ras*(Asn-17) or H-*ras*(Leu-61,Ser-186). In particular, insulin-induced activation of the *fos* promoter was inhibited completely by H-*ras*(Asn-17). These results show that p21^{ras} functions as an intermediate in the insulin signal transduction route leading to the induction of gene expression.

The mammalian *ras* genes encode small GTP-binding proteins of approximately 21 kDa which are associated with the inner side of the plasma membrane and which alternate between an inactive GDP-bound conformation and an active GTP-bound conformation (for a review, see reference 2). p21^{ras} proteins are most likely involved in the transduction of signals from various external stimuli to intracellular targets (for a review, see reference 14). This hypothesis is based on a variety of observations. For instance, microinjection of a neutralizing anti-*ras* antibody in NIH 3T3 cells blocks the mitogenic effects of a number of growth factors (17) and the induction of c-*fos* expression by serum (25). Expression of a dominant inhibitory mutant H-*ras* gene which interferes with normal p21^{ras} function inhibits the induction of DNA synthesis by serum and several growth factors in NIH 3T3 cells, as well as the induction of c-*fos* expression by epidermal growth factor (EGF) and other growth factors (4). In addition, nerve growth factor-induced differentiation of PC12 cells is inhibited by either microinjection of neutralizing p21^{ras} antibodies (13) or transfection of a dominant inhibitory *ras* mutant (28). More direct proof for a role of p21^{ras} in growth factor signal transduction came from the observations that several growth factors can rapidly activate p21^{ras}, as measured by an increase in its GTP-bound conformation (3, 7, 10, 22, 23). The precise mechanism of p21^{ras} activation and the direct effector(s) of p21^{ras} in mammalian cells are still elusive, although several proteins involved in these processes have been identified (for a review, see reference 14).

We have recently found that stimulation with insulin can activate p21^{ras} very rapidly in NIH 3T3 and Chinese hamster ovary cells that overexpress the insulin receptor, indicating that p21^{ras} may be involved in insulin-induced signal transduction (3, 19). One of the effects of insulin in these cells is the induction of the expression of a variety of early response genes, such as c-*fos* and c-*jun*. Since overexpression of normal p21^{ras} also enables insulin to induce the expression of these genes, we proposed that p21^{ras} mediates insulin-induced gene expression (3). In addition, in a transient expression assay, activated p21^{ras} by itself can increase the expression of a number of genes, including c-*fos* and the collagenase gene (21, 24), and thus seems to be sufficient to increase gene expression. Therefore, in the case of insulin signal transduction, p21^{ras} activation might be the trigger for induction of gene expression. In this paper, we describe the use of two dominant inhibitory mutants of p21^{ras} to obtain direct proof for a possible role of p21^{ras} in insulin-induced gene expression.

MATERIALS AND METHODS

Recombinant plasmids. Rous sarcoma virus (RSV) promoter-driven mutant p21^{ras}-encoding plasmids were constructed by insertion of the various coding regions into the polylinker of pRSV.H20 (18). The following coding regions were inserted: the 1.2-kb *Bam*HI fragments from pZIP*ras*(Leu-61) and pZIP*ras*(Leu-61,Ser-186) (21), the 4.0-kb *Eco*RI-*Cla*I fragment from pSVET24 (Val-12) (5), and a 4.8-kb *Bam*HI fragment from pLTR H-*ras*(N) (15), which was mutated at codon 17 by site-directed mutagenesis (Asn-17). pRSV*neo* and pRSVc-*jun* have been described previously (18). Col-CAT contains part of the human collagenase promoter (−517 to +63) and has been described previously (1). The *Hin*dIII-*Bgl*II fragment from Fos-CAT (24), which contains part of the human c-*fos* promoter (−711 to +42), was cloned into *Hin*dIII-*Bgl*II-digested pSLA3 (30) to obtain Fos-luciferase.

Cell culture and transfection. Cells were cultured in Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% fetal calf serum (GIBCO) and 0.05% L-glutamine. A14 cells were obtained by transfection of a full-length human insulin receptor under the control of the simian virus 40 early promoter into NIH 3T3 cells (3). Transient transfections on NIH 3T3 and A14 cells were carried out by using the calcium phosphate method (29). After a 5-h treatment with the DNA-calcium phosphate precipitate, the cells were given a 1.5-min glycerol shock. HeLa cells were transfected by using the DEAE-dextran method (12). The amount of DNA in each precipitate was normalized to 12 μg with

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RSV-*neo*. After transfection, cells were cultured for 48 h in Dulbecco's modified Eagle medium containing 0.5% fetal calf serum. Induction with insulin or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was carried out by the addition of insulin (10 μg/ml) or TPA (100 ng/ml) 24 h prior to harvesting of the cells. Protein extracts were prepared by repeated freeze-thawing, and protein concentrations were measured with a Bradford protein assay (Bio-Rad). CAT activity and luciferase activity were assayed as described elsewhere (1, 6).

RESULTS

Biological activity of two dominant inhibitory p21$^{H\text{-}ras}$ mutants. In this study, we have used two dominant inhibitory mutant *ras* genes in a transient transfection assay. The first, *ras*(Leu-61,Ser-186), was originally selected in *Saccharomyces cerevisiae* as an interfering mutant (16) and blocks *ras*-induced maturation in oocytes (11). The mutant p21^{ras} has a serine at position 186 replacing a cysteine. This alteration prevents proper membrane attachment and consequently renders the protein nonfunctional (32). Furthermore, the mutant protein has a change at position 61, where leucine is substituted for glutamine, resulting in a loss of GTPase activity and an affinity for the GTPase-activating protein that is 50-fold higher than that of wild-type p21^{ras} (11, 31). This mutant protein has been proposed to inhibit the function of p21^{ras} by competition for its cellular target (11, 16). The second dominant inhibitory *ras* mutant, *ras*(Asn-17), inhibits *ras*-induced differentiation of PC12 cells and proliferation of NIH 3T3 cells (9, 28). In addition, in transient expression assays this mutant prevents the activation of the c-*fos* promoter by several growth factors (4). The mutant protein has a single amino acid substitution at position 17, where serine is changed to asparagine, causing a reduced affinity for GTP without affecting affinity for GDP (9). This results in a mutant protein that is mostly GDP bound, which might interfere with normal p21^{ras} activation.

To measure the effect of these dominant inhibitory mutants on p21^{ras}-mediated gene induction, we have used the collagenase promoter in a transient expression assay. The collagenase promoter was reported to be induced by oncogenic p21^{ras}, mediated through the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element (24).

A collagenase promoter-chloramphenicol acetyltransferase construct (Col-CAT) was transfected into HeLa cells in combination with a number of mutant *ras* genes, all under control of the RSV long terminal repeat promoter. Cotransfection of Col-CAT with RSV-*ras*(Val-12) or RSV-*ras*(Leu-61) resulted in a 10- to 80-fold induction of CAT expression in HeLa cells (Fig. 1), confirming previous reports (24). Cotransfection of Col-CAT with either one of the two dominant inhibitory mutant *ras* genes, *ras*(Leu-61,Ser-186) or *ras*(Asn-17), did not increase Col-CAT expression (Fig. 1 and 2). Expression of Col-CAT induced by transiently expressed oncogenic p21^{ras}, however, was completely blocked by cotransfection of RSV-*ras*(Leu-61,Ser-186) (Fig. 1A). This result indicates that this mutant protein efficiently inhibits *ras*-induced gene expression in this transient expression assay.

As a control for the specificity of *ras*(Leu-61,Ser-186), we investigated whether *ras*(Leu-61,Ser-186) interferes with the induction of collagenase expression by TPA and c-*jun*. As shown in Fig. 1A and 1B, both stimulation with TPA or cotransfection with RSV-c-*jun* lead to an induction of CAT expression, and neither one of these inductions is sensitive

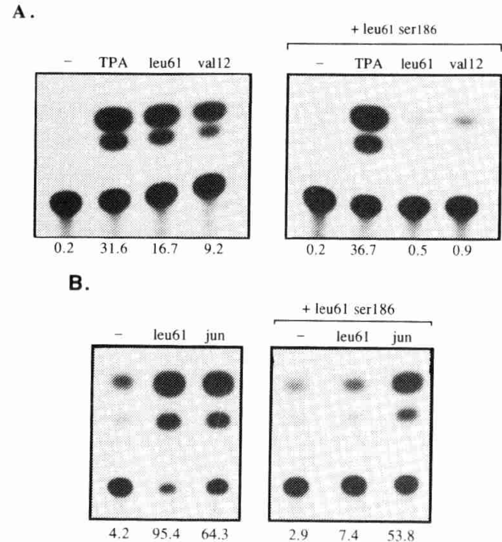

FIG. 1. Biological activity of *ras*(Leu-61,Ser-186). HeLa cells were transfected with 2 μg of Col-CAT in combination with RSV-*neo* (left panels) or 8 μg of RSV-*ras*(Leu-61,Ser-186) (right panels) per 9-cm dish. (A) Basal expression of the Col-CAT construct (–), TPA induction performed by adding 100 ng of TPA per ml at 24 h prior to cell lysis (TPA), cotransfection with 2 μg of RSV-*ras*(Leu-61) (leu61), and cotransfection with 2 μg of RSV-*ras*(Val-12) (val12). (B) Col-CAT expression alone (–), cotransfected with 2 μg of RSV-*ras*(Leu-61) (leu61), or cotransfected with 4 μg of RSV-c-*jun* (jun). The amount of DNA in each precipitate was normalized by addition of RSV-*neo*. After 48 h, protein extracts were prepared and tested for CAT activity. Numbers at the bottom indicate the percentage of conversion of chloramphenicol into acetylated chloramphenicol.

to coexpression of *ras*(Leu-61,Ser-186). This indicates that *ras*(Leu61,Ser-186) interferes specifically with the activation of the collagenase promoter by activated (mutant) p21^{ras}. Therefore, induction of gene expression through a pathway

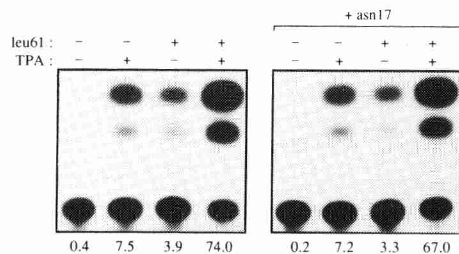

FIG. 2. Biological activity of *ras*(Asn-17). HeLa cells were transfected with 2 μg of Col-CAT in combination with RSV-*neo* (left panel) or 8 μg of RSV-*ras*(Asn-17) (right panel) per 9-cm dish. RSV-*ras*(Leu-61) (2 μg) was cotransfected where indicated above the lanes, and TPA (100 ng/ml) was added to the cells 24 h after transfection where indicated. CAT activity was determined as described for Fig. 1. Numbers at the bottom indicate the percentage of chloramphenicol acetylation in each lane.

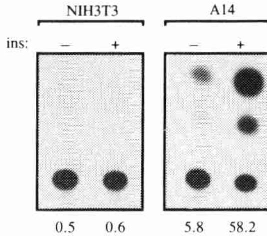

FIG. 3. Insulin induction of Col-CAT expression. NIH 3T3 (left panel) and A14 (right panel) cells were transfected with 2 μg of Col-CAT plus 8 μg of RSV-*neo*. Cells were treated with insulin (ins) (10 μg/ml) 24 h after transfection where indicated. CAT activity was determined 48 h after transfection. Numbers at the bottom indicate the percentage of chloramphenicol acetylation in each lane.

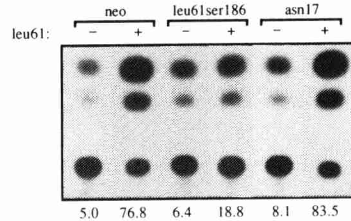

FIG. 4. Inhibition of *ras*-induced Col-CAT expression in A14 cells. A14 cells were transfected with 2 μg of Col-CAT, with or without 2 μg of RSV-*ras*(Leu-61) as indicated above the lanes, to test basal (−) and *ras*(Leu-61)-induced (+) Col-CAT expression. Eight micrograms of RSV-*neo* (neo), RSV-*ras*(Leu-61,Ser-186) (leu61ser186), or RSV-*ras*(Asn-17) (asn17) were added to the DNA precipitates as indicated. After 48 h, cells were lysed and CAT activity was determined. Numbers at the bottom indicate the percentage of chloramphenicol acetylation in each lane.

in which the activity of p21^ras is required is expected to be blocked in the presence of this inhibitory mutant protein. p21^ras(Asn-17) does not inhibit p21^ras(Leu61)-induced collagenase expression, which is consistent with the hypothesis that this inhibitory mutant interferes with upstream elements of the p21^ras signal transduction route (9). Also, TPA induction of collagenase expression remained unaltered in the presence of *ras*(Asn-17), demonstrating that p21^ras(Asn-17) does not interfere aspecifically with the induction of gene expression.

Inhibition of Col-CAT expression induced by activated p21^ras was also observed in NIH 3T3 cells after cotransfection of pRSV-*ras*(Leu-61,Ser-186) (data not shown).

Insulin-induced Col-CAT expression. It had been shown previously that insulin stimulates the expression of a number of endogenous genes in fibroblasts that overexpress the insulin receptor (3, 26, 27). In these cells, p21^ras is rapidly activated upon stimulation with insulin (3). To study the effect of the dominant inhibitory mutant *ras* genes on insulin-induced gene expression, we transfected the Col-CAT construct in A14 cells (NIH 3T3 cells overexpressing the human insulin receptor). As shown in Fig. 3, a strong induction of CAT expression was observed after stimulation with insulin. Apparently insulin can, like cotransfection of activated p21^ras, induce collagenase promoter activity in a transient expression assay. In contrast to HeLa and NIH 3T3 cells, we observed some basal expression of Col-CAT in A14 cells. This may be due to basal insulin receptor activity in the A14 cells caused by the high levels of expression. Indeed, the percentage of p21^ras bound to GTP is slightly elevated in A14 cells compared with in NIH 3T3 cells (~7 versus ~15%; see reference 3). In the parental NIH 3T3 cells, insulin had no effect on the expression of Col-CAT; this is similar to the lack of induction of endogenous c-*fos*, c-*jun*, and p33 mRNA expression in response to insulin in these cells (3).

Inhibition of insulin-induced gene expression. To investigate the role of p21^ras in insulin-induced gene expression, we compared the effects of the dominant inhibitory mutants on *ras*- (Fig. 4) and insulin-induced (Fig. 5) Col-CAT expression in A14 cells. As shown in Fig. 4, a 15-fold induction of CAT expression was observed in A14 cells after cotransfection of Col-CAT with *ras*(Leu-61). This *ras*-induced expression of Col-CAT was inhibited (about fourfold) by cotransfection of *ras*(Leu-61,Ser-186), whereas cotransfection of *ras*(Asn-17) had no effect. These results are in agreement with the results obtained with the HeLa cells. Figure 5 shows the effects of the dominant inhibitory mutants on insulin-induced Col-CAT expression in A14 cells. Cotransfection of the Col-CAT construct together with RSV-*ras*(Leu-61,Ser-186) resulted in a 4.5-fold reduction of the insulin-induced Col-CAT expression, whereas the basal level of Col-CAT expression was lowered by a factor of 1.6 (Fig. 5A). This is very similar to what we found for the inhibition of Col-CAT expression induced by cotransfection of activated p21^ras in the A14 cells (compare Fig. 4 and Fig. 5A). Cotransfection with RSV-

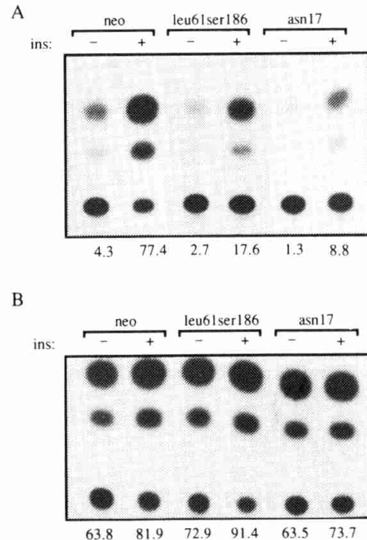

FIG. 5. Inhibition of insulin-induced Col-CAT expression. A14 cells were transfected with 2 μg of Col-CAT (A) or 2 μg of RSV-CAT (B) per 9-cm dish. We cotransfected the cells with 8 μg of RSV-*neo* (neo), RSV-*ras*(Leu-61,Ser-186) (leu61ser186), or RSV-*ras*(Asn-17) (asn17) as indicated. Cells were stimulated with insulin (ins) (10 μg/ml) 24 h after transfection. At 48 h after transfection, protein extractions were performed and CAT activity was determined. Numbers at the bottom indicate the percentage chloramphenicol acetylation in each lane.

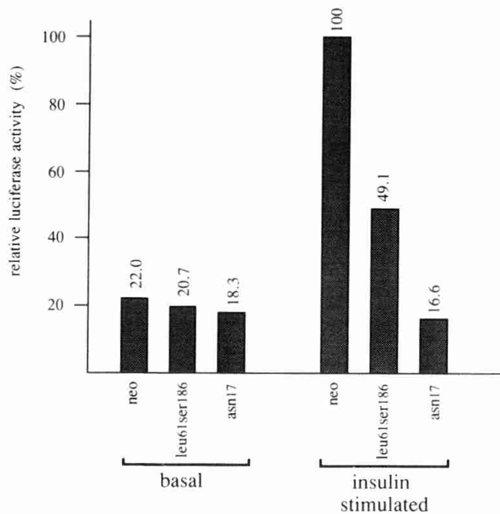

FIG. 6. Inhibition of insulin-induced Fos-luciferase expression. A14 cells were transfected with 2 μg of Fos-luciferase together with 8 μg of RSV-*neo* (neo), RSV-*ras*(Leu-61,Ser-186) (leu61ser186), or RSV-*ras*(Asn-17) (asn17). Cells were left unstimulated (basal) or stimulated with insulin (10 μg/ml) 24 h after transfection. Cells were lysed 48 h after transfection, and protein extracts were prepared for determination of luciferase activity. Luciferase activity is expressed as the percentage of the activity in insulin-stimulated A14 cells transfected with RSV-*neo* and Fos-luciferase.

ras(Asn-17) resulted in a 8.8-fold reduction of insulin-stimulated CAT expression, while this mutant protein had no effect on the induction by activated p21^{ras} (compare Fig. 4 and Fig. 5A). With RSV-*ras*(Asn-17), the basal level of expression was 3.3-fold lower. Thus, the function of normal p21^{ras}, but not that of activated p21^{ras}, is inhibited by coexpression of *ras*(Asn-17). This is in agreement with the proposal that *ras*(Asn-17) inhibits by interference with the activation of normal p21^{ras} (9). Cotransfection of either one of the dominant inhibitory mutant *ras* genes with RSV-CAT had no effect on CAT expression (Fig. 5B), indicating that these mutants do not influence the transfection efficiency.

Effect of dominant interfering mutants of p21^{ras} on insulin-induced c-*fos* promoter activity in A14 cells. The expression of endogenous c-*fos* is induced by insulin treatment of A14 cells (3). In addition, constructs containing the human c-*fos* promoter are both *ras* and insulin inducible in a transient assay (21, 27). Therefore, we also transfected cells with a reporter gene carrying the human c-*fos* promoter linked to the luciferase gene (Fos-luciferase). Insulin stimulation of A14 cells transfected with Fos-luciferase resulted in a five-fold induction of luciferase expression (Fig. 6), consistent with the finding that c-*fos* mRNA levels increase in these cells after stimulation with insulin. Cotransfection with RSV-*ras*(Leu61,Ser-186) resulted in a twofold inhibition, whereas cotransfection of RSV-*ras*(Asn-17) completely abolished insulin-induced Fos-luciferase expression.

DISCUSSION

We previously suggested that the activation of p21^{ras} may mediate insulin-induced gene expression (3). In this paper we obtained further evidence for this hypothesis by showing that insulin-induced activation of the collagenase promoter and the *fos* promoter can be inhibited by either of two dominant inhibitory *ras* proteins. The mutant proteins used in this study, p21^{ras}(Leu-61,Ser-186) and p21^{ras}(Asn-17), interfere at different levels in the p21^{ras} pathway. p21^{ras}(Leu-61,Ser-186) presumably interferes with the effector function of p21^{ras}, and p21^{ras}(Asn-17) presumably interferes with the activation of p21^{ras}. These different specificities strongly support the argument that the observed inhibition of insulin-induced gene expression is due to interference with p21^{ras} function and not with a function other than that of p21^{ras}. Therefore, these results imply that p21^{ras} is directly involved in the insulin signaling pathway. Furthermore, our previous finding that insulin rapidly activates p21^{ras} in the A14 cells excludes the possibility that p21^{ras} functions in an alternative route which cooperates with the insulin signaling pathway. Taken together, we conclude that p21^{ras} is an operative component of the insulin signaling pathway; it receives and transmits a signal upon stimulation with insulin, leading to the induction of gene expression.

In the A14 cells, the induction of Col-CAT expression by insulin is only partly suppressed by either one of the inhibitory mutants. Thus, it might be that insulin activates the collagenase promoter via a *ras*-independent route as well. Alternatively, the transiently expressed inhibitory mutant proteins might not completely block the signal mediated by endogenous p21^{ras}. The induction of *fos* promoter activity, however, is completely inhibited by coexpression of p21^{ras}(Asn-17), indicating that in this transient expression system only a p21^{ras}-dependent route is responsible for this effect. In contrast, p21^{ras}(Leu-61,Ser-186) consistently shows a partial inhibition of insulin-induced Fos-luciferase expression. Apparently, p21^{ras}(Leu-61,Ser-186) inhibits the function of endogenous p21^{ras} less efficiently than does p21^{ras}(Asn-17), in agreement with the recent observation that a mutation interfering with downstream coupling of p21^{ras} has little effect on the function of normal p21^{ras} (8).

The observation that p21^{ras} mediates insulin-induced activation of the collagenase promoter and *fos* promoter in transient expression systems strongly suggests that p21^{ras} mediates insulin-induced expression of the endogenous genes as well. In addition, insulin can elicit a number of other effects in the cell, such as increased glucose and amino acid uptake, increased lipid turnover, and induction of tyrosine and serine phosphorylation of a large number of substrates (20). The role of p21^{ras} activation in these effects can be investigated by introducing the dominant inhibitory mutants as stably transfected genes in the A14 cells. Due to the growth-suppressing character of the mutants, conditionally inducible mutant genes are necessary. Thus far we have not been able to stably express these mutants in the A14 cells. The conclusion that p21^{ras} mediates insulin-induced gene expression does not exclude an involvement of p21^{ras} in the signal transduction pathways of other growth factors. Indeed, using similar experiments, Cai et al. showed that p21^{ras} mediates the induction of *fos* promoter activity by epidermal growth factor and fibroblast growth factor (4). However, it should be noted that the direct involvement of p21^{ras} in signal transduction is more evident for the insulin pathway than for the epidermal growth factor pathway, since insulin causes a considerable increase in the amount of p21^{ras}-GTP (3), whereas this increase is relatively small in response to epidermal growth factor (3, 22). Finally, it is interesting that we found that in HeLa cells p21^{ras} does not mediate TPA-induced gene expression, in agreement with

the observation that in PC12 cells, p21^{ras} mediates nerve growth factor-induced gene expression but not TPA-induced gene expression (28).

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CHAPTER 4

Insulin-induced p21ras activation does not require protein kinase C, but a protein sensitive to phenylarsine oxide.

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Insulin-induced p21ras Activation Does Not Require Protein Kinase C, but a Protein Sensitive to Phenylarsine Oxide*

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Insulin treatment of fibroblasts overexpressing the insulin receptor causes a rapid accumulation of the GTP-bound form of p21ras. We have studied the involvement of protein kinase C (PKC) in, and the effect of phenylarsine oxide (PAO), a putative inhibitor of tyrosine phosphatase activity on, this process. Activation of p21ras was not observed when the cells were stimulated with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) and pretreatment with TPA for 16 h, sufficient to down-regulate PKC activity, did not abolish p21ras activation by insulin. These results show that PKC is not involved in the insulin-induced activation of p21ras.

Pretreatment of the cells with PAO for 5 min completely blocked insulin-induced p21ras activation. Addition of 2,3-dimercaptopropanol prevented this inhibition by PAO. Also, addition of PAO after insulin stimulation could reverse the activation of p21ras. Since PAO did not affect overall phosphorylation of the insulin receptor β-chain, we conclude that a PAO-sensitive protein is involved in the induction of p21ras activation by insulin.

The ras genes encode closely related 21-kDa proteins, p21ras, that can bind GTP and GDP and may function as signal transducing molecules (1, 2). ras proteins cycle between an active (GTP-bound) and an inactive (GDP-bound) conformation (3, 4). This ras-cycle is thought to be controlled by at least two different activities. First, activation of p21ras is facilitated by nucleotide exchange factors that promote the exchange of GDP for GTP (5–7). Second, p21ras can be inactivated by GTPase activating proteins (GAP,[1] NF-1) that stimulate the intrinsic GTPase activity of p21ras (8–12). Oncogenic mutations in the ras gene, found in a large number of human tumors (13), give rise to a ras protein that is constitutively in the GTP-bound form, and therefore active (14–17).

Using a variety of experimental approaches it has been shown that p21ras may function in several signal transduction pathways directed by membrane-associated tyrosine kinase receptors, to mediate growth factor stimulated mitogenicity and gene expression (18–21). Indeed in several cases growth factor stimulation leads to an increase in the GTP-conformation of p21ras (22–26). In particular, stimulation of the T cell receptor (TCR) in T lymphocytes (25), and stimulation of the insulin receptor (IR) in fibroblasts expressing elevated levels of the human insulin receptor (24) resulted in a dramatic increase in the GTP-bound state of p21ras.

In T lymphocytes, p21ras activation is suggested to occur via the activation of protein kinase C (PKC), resulting in the inactivation of GAP (or NF-1) activity (25). Furthermore, proper TCR-signaling seems to require the function of two tyrosine kinases, lck (27) and fyn (28), and a tyrosine phosphatase, CD45 (29). The mechanism by which insulin triggers the activation of p21ras, however, is still elusive. Similarities between TCR and IR signaling suggest that the mechanism of p21ras activation by insulin may resemble that of TCR-induced p21ras activation. For instance, signal transduction initiated by either one of these receptors requires tyrosine kinase activity (lck and fyn for TCR, IR-β-chain for IR), and in both pathways tyrosine phosphatases are involved that are sensitive to phenylarsine oxide (CD45 for TCR (30), HA1 and HA2 for IR (31)). Also, like in TCR-signaling, in insulin-mediated signal transduction PKC may play an important role as well. First, insulin can stimulate the production of diacylglycerol from various sources, resulting in the subsequent activation of PKC (32, 33). Secondly, PKC-activating agents, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), mimic insulin action in some cases (34–36).

In this report we have investigated the involvement of PKC in, and the effect of PAO on insulin-induced p21ras activation. We found that in contrast to TCR-induced p21ras activation, PKC is not involved in insulin-induced activation. However, insulin-induced p21ras activation is sensitive to PAO.

MATERIALS AND METHODS

Materials—Phenylarsine oxide (PAO), 2,3-dimercaptopropanol (DMP), 12-O-tetradecanoylphorbol-13-acetate (TPA), insulin, protease inhibitors, and sodium vanadate were obtained from Sigma. Monoclonal antibody against the insulin receptor (RPN 538) and radiochemicals were from Amersham, and cell culture media were from GIBCO. Stock solutions for PAO and DMP were made in dimethyl sulfoxide (DMSO).

Cell Culture—A14 cells are NIH3T3 cells expressing 3×10^5 high affinity human insulin receptors ($K_d \leq 10^{-9}$ M) per cell (24). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 8% fetal calf serum (FCS, GIBCO). For serum starvation, subconfluent dishes were cultured in DMEM containing 0.5% FCS for 16 h.

[^{32}P]Orthophosphate Labeling of Cell Cultures—Cells were plated on 5-cm tissue culture dishes, at least 24 h prior to labeling, to exclude residual activation of the insulin receptor caused by trypsinization. After serum starvation for 16 h, cells were labeled for 3 h in phosphate-free/serum-free medium (GIBCO) supplemented with 200 μCi of [^{32}P]orthophosphate per 5-cm dish (24).

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[1] The abbreviations used are: GAP, GTPase-activating protein; TCR, T cell receptor; IR, insulin receptor; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; PAO, phenylarsine oxide; DMP, 2,3-dimercaptopropanol; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Determination of GTP/GDP Ratio—^{32}P-Labeled cells were treated with different compounds as indicated, and lysates were made using a phase-split purification as described (37). Subsequently, p21ras was immunoprecipitated with monoclonal antibody Y13-259, and GTP/GDP nucleotides were eluted and separated by thin-layer chromatography as described (25). After autoradiography, GTP/GDP ratios were determined by counting the separated nucleotides in a scintillation counter.

80-kDa Phosphorylation—During serum starvation cells were either left untreated or treated with 100 ng/ml TPA for 16 h, and cells were labeled for an additional 3 h with [^{32}P]orthophosphate. Both untreated and TPA pretreated cells were either left unstimulated or stimulated with 100 ng/ml TPA for 5 min. Total protein lysates were separated on a 7.5% SDS-polyacrylamide gel. Gels were fixed and dried prior to autoradiography.

Insulin Receptor β-Chain Phosphorylation—After treatment with PAO, DMSO, and/or insulin, [^{32}P]orthophosphate-labeled cells were lysed in a buffer containing: 50 mM HEPES buffer, pH 7.4, 1% Triton X-100, 0.05% SDS, 0.5% deoxycholic acid, 100 mM NaCl, 5 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 10 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium vanadate. The insulin receptor was immunoprecipitated using a monoclonal antibody coupled to protein A beads. Immunoprecipitates were analyzed on a 7.5% SDS-polyacrylamide gel.

RESULTS

PKC Is Not Involved in Insulin-induced Activation of p21ras—We have previously shown a rapid accumulation of the GTP form of p21ras induced by insulin in NIH3T3 fibroblasts overexpressing the human insulin receptor (A14 cells) (24). To analyze an upstream function for PKC activation in this process, we measured the effect of TPA treatment on the activation state of p21ras. As shown in Fig. 1, a 5-min

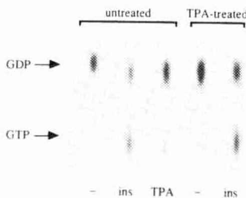

FIG. 1. **The role of protein kinase C in p21ras activation.** Cells were serum-starved for 16 h on 0.5% FCS, in the absence (untreated), or presence of 100 ng/ml TPA (TPA-treated). The cells were labeled with [^{32}P]orthophosphate for 3 h, and stimulated with growth factors for 5 min. Cells were lysed and p21ras was collected by immunoprecipitation with monoclonal Y13-259. Bound nucleotides were eluted and separated by thin-layer chromatography. A14 cells: unstimulated (–), stimulated insulin for 5 min (*ins*, 10 μg/ml), or stimulated with TPA for 5 min (*TPA*, 100 ng/ml).

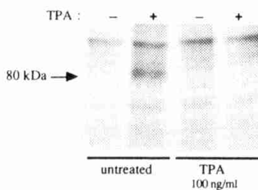

FIG. 2. **PKC depletion by prolonged exposure to TPA.** Cells were serum-starved for 16 h on 0.5% FCS, in the absence or presence of TPA (100 ng/ml). Cells were labeled with [^{32}P]orthophosphate for 3 h, and stimulated with TPA for 5 min (100 ng/ml). Cells were lysed and proteins were separated on polyacrylamide gel electrophoresis. Gels were fixed and dried before autoradiography. A14 cells: untreated (untreated), pretreated with TPA (TPA, 100 ng/ml); either unstimulated (–), or stimulated with TPA (+) for 5 min. The *arrow* indicates the position of the 80-kDa protein.

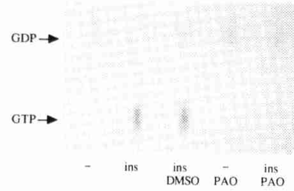

FIG. 3. **Inhibition of insulin-induced p21ras activation by PAO.** Cells were serum-starved for 16 h on 0.5% FCS, and labeled for 3 h with [^{32}P]orthophosphate. Cells were preincubated with, or without PAO, or DMSO for 5 min. After preincubation, insulin was added to the cells for 5 min, and GTP/GDP ratios were determined as described in Fig. 1. A14 cells: unstimulated (–), or stimulated with insulin (*ins*, 10 μg/ml); without pretreatment, pretreated with DMSO (*DMSO*, 1 μl/ml), or pretreated with PAO (*PAO*, 25 μM).

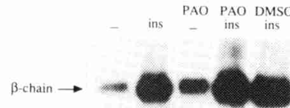

FIG. 4. **Effect of PAO on autophosphorylation of the insulin receptor β-chain.** Cells were serum-starved and labeled with [^{32}P] orthophosphate as described in the legend of Fig. 1. After pretreatment with PAO, or DMSO for 5 min, cells were stimulated with insulin (5 min, 10 μg/ml). Cells were lysed and the insulin-receptor was precipitated using monoclonal antibody RPN538. The precipitates were analyzed on a 7.5% SDS-polyacrylamide gel. A14 cells: unstimulated (–) or stimulated with insulin (*ins*); without pretreatment, pretreated with PAO (*PAO*, 25 μM), or pretreated with DMSO. The *arrow* indicates the position of the insulin receptor β-chain.

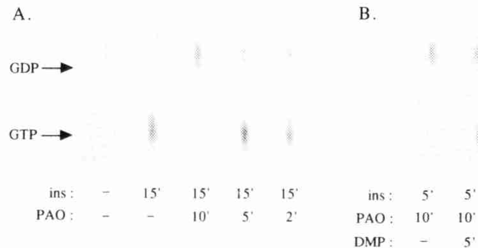

FIG. 5. **Reversion of p21ras activation and PAO inhibition.** Cells were serum-starved and labeled with [^{32}P]orthophosphate as described in the legend of Fig. 1. The cells were then treated with insulin (10 μg/ml), PAO (25 μM), and/or DMP (50 μM) for various lengths of time prior to lysis as indicated. Cell lysates were made and GTP/GDP ratios on p21ras determined as described under "Materials and Methods." *A*, A14 cells: untreated (–), or treated with insulin for 15 min; without addition of PAO (–), or with addition of PAO for 10, 5, or 2 min prior to lysis. *B*, A14 cells: pretreated with PAO for 5 min (25 μM), and stimulated with insulin (10 μg/ml) in the absence (–) or presence of DMP for 5 min prior to lysis.

TPA stimulation of A14 cells did not result in a shift of the p21ras GTP/GDP ratio. Next, we pretreated the A14 cells with 100 ng/ml TPA for a period of 16 h, to deplete the cells of PKC activity (38), and measured the p21ras GTP/GDP ratio after insulin stimulation. This TPA pretreatment did not influence the insulin-induced shift in the GTP/GDP ratio on p21ras. As a control for PKC down-modulation, we analyzed the phosphorylation of the 80-kDa MARCKS protein, a major substrate of PKC often used as a marker for PKC activity in intact cells (38–40). A 5-min stimulation with TPA was sufficient to stimulate 80-kDa phosphorylation (Fig. 2).

Induction of 80-kDa phosphorylation by a short stimulation with TPA was abolished after pretreatment with 100 ng/ml TPA for 16 h, indicating that cells are depleted of TPA-sensitive PKC activity using this protocol. From these results we conclude that PKC is not involved in the activation of p21ras by insulin.

PAO Inhibits the Activation of p21ras by Insulin—Phenylarsine oxide has been found to interfere with signal transduction from the insulin receptor (41, 42). To test its effect on the shift in the p21ras GTP/GDP ratio induced by insulin, we added PAO 5 min prior to insulin stimulation. PAO completely blocked activation of p21ras at a concentration of 25 μM (Fig. 3). Addition of solvent alone (DMSO) had no effect on the insulin-induced activation of p21ras. To ascertain that addition of PAO does not influence insulin-induced phosphorylation of the β-chain of the insulin receptor, thereby inhibiting p21ras activation, we immunoprecipitated the insulin receptor from ^{32}P-labeled cell lysates. Consistent with previous findings (42), PAO treatment resulted in a small increase in phosphorylation rather than an inhibition of insulin-induced β-chain phosphorylation (Fig. 4). This suggests that PAO inhibition does not affect the insulin receptor itself, but interacts with a protein that functions somewhere between the activated receptor and p21ras.

Reversion of p21ras Activation and PAO Inhibition—To gain further insight in the process affected by PAO, we investigated the kinetics of inhibition. For this, we stimulated the cells 15 min prior to lysis with insulin. Full activation of p21ras (~70% GTP-bound) is achieved within 2 min, and remains at this level for at least 15 min (24). PAO was added at different time points during a 15-min insulin stimulation, starting at 10 min prior to lysis. We did no longer observe an insulin-induced shift in the GTP/GDP ratio on p21ras when PAO was added for 10 min, but activation was hardly affected when PAO treatment lasted only 5 min (Fig. 5*A*).

Inhibition of insulin-induced responses by PAO can be prevented by addition of DMP, a compound containing vicinal sulfhydryls, thus capable of competing for PAO binding. We observed complete reversion of PAO inhibition when 50 μM DMP was added simultaneously with insulin (Fig. 5*B*), similar to findings with insulin-induced glucose uptake (41).

DISCUSSION

We have studied the role of PKC in the insulin-induced activation of p21ras, since PKC mediates p21ras activation in T lymphocytes (25). Depletion of PKC by prolonged TPA treatment in A14 cells did not abolish the ability of insulin to induce a shift in the p21ras GTP/GDP ratio. Such a pretreatment did result in the loss of TPA-sensitive PKC activity as judged by the ability of TPA to induce phosphorylation of the 80-kDa substrate (MARCKS protein) of PKC. Likewise, PKC activation by a short incubation with TPA did not mimic the effect of insulin on the activation state of p21ras. Although not all PKC isozymes are sensitive to TPA (43–45), the PKC isozyme that mediates activation of p21ras in T cells is activated by TPA (25). Furthermore, in NIH3T3 cells the PKC-α isozyme seems to be the major form that is expressed, and this PKC isozyme is sensitive to TPA down-modulation (46–48). We conclude that (a TPA-sensitive) PKC is not involved in insulin-induced activation of p21ras, and that the mechanism of p21ras activation by insulin in A14 cells differs from the activation of p21ras by TCR stimulation.

The finding that PAO can inhibit both insulin-induced, as well as TCR-induced signal transduction, prompted us to evaluate the effect of PAO on insulin-induced p21ras activation. A pretreatment of 5 min with PAO resulted in a complete inhibition of p21ras activation, although the overall insulin receptor phosphorylation remained unaffected. Addition of 2,3-dimercaptopropanol to cells preincubated with PAO resulted in restoration of the insulin-induction of p21ras activation. Thus, PAO seems to exhibit its inhibitory effect on p21ras activation through binding of vicinal sulfhydryls (41). The kinetics and characteristics of inhibition by PAO are similar to that found for the inhibition of insulin-induced glucose uptake by PAO (42). This may indicate that the same PAO-sensitive protein is involved in the generation of both effects. Inhibition of insulin-induced glucose uptake by PAO is thought to occur through inactivation of a tyrosine phosphatase, since several proteins, phosphorylated in response to insulin, were shown to accumulate in the presence of PAO (49). PAO had no effect on proteins phosphorylated in response to serum or platelet-derived growth factor, indicating that there is some specificity in the inhibiting effect of PAO. One of the proteins that could be detected after insulin stimulation in the presence of PAO is a 15-kDa phosphoprotein (50). This protein was shown to be the fatty acid-binding protein 422(aP2) that can be phosphorylated by the insulin receptor in vitro, in a fatty acid-dependent manner (51). Recently, it was shown that the dephosphorylation of this protein can be mediated by two different tyrosine phosphatases, HA1 and HA2, both associated with the cell membrane (31). These two phosphatases were purified from 3T3 adipocytes, and shown to be inactive in the presence of PAO. In T lymphocytes PAO inhibits CD45 phosphatase activity, having no effect on the kinase activity of both lck and fyn, again indicating that PAO may be a specific tyrosine phosphatase inhibitor (30). However, PAO binds to vicinal sulfhydryl groups (52) and may interact with other proteins as well, so clearly a more detailed analysis is needed before a definite conclusion concerning the function of the PAO-sensitive component in this mechanism can be drawn.

Accumulation of the GTP-bound form of p21ras in A14 cells is maximal within 2 min after insulin addition, and remains at this elevated level (~70% GTP bound) for at least 15 min (24). Thus we were able to study whether PAO could not only prevent, but also reverse the activation of p21ras induced by insulin. When cells were stimulated with insulin for 15 min and PAO was present during the last 10 min, we observed complete reversion of insulin-induced p21ras activation, whereas addition of PAO 5 min prior to lysis had almost no effect. The implications of these results are severalfold. First, the fact that we indeed observe reversion points to a mechanism in which constitutive activation of upstream elements is necessary for sustained p21ras activation. Secondly, while a 5-min treatment with PAO is still without effect on the insulin stimulation, 10 min after addition of PAO all p21ras is converted to the GDP form. Thus, complete inactivation takes place in 5 min. The fact that we find no effect of PAO during the first 5 min of treatment is probably due to the time PAO needs to cross the cellular membrane (53). Since hydrolysis of GTP bound to p21ras *in vitro* is rather slow (54, 55), this implies that the activity of a GTPase activating protein (GAP or NF-1) is considerable in these cells, at least in the absence of an insulin stimulus.

Thus far, the nature of the PAO-sensitive protein is still unknown. It could be one of the tyrosine phosphatases as discussed above. This would imply that tyrosine dephosphorylation is an essential step in insulin induction of p21ras activation, alternatively, PAO might inhibit another protein activity. In both cases, PAO can be used as a tool for deciphering the pathway between insulin stimulation and p21ras activation.

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CHAPTER 5

Ras activation by insulin and epidermal growth factor through enhanced exchange of guanine nucleotides on p21ras.

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Ras Activation by Insulin and Epidermal Growth Factor through Enhanced Exchange of Guanine Nucleotides on $p21^{ras}$

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A number of growth factors, including insulin and epidermal growth factor (EGF), induce accumulation of the GTP-bound form of $p21^{ras}$. This accumulation could be caused either by an increase in guanine nucleotide exchange on $p21^{ras}$ or by a decrease in the GTPase activity of $p21^{ras}$. To investigate whether insulin and EGF affect nucleotide exchange on $p21^{ras}$, we measured binding of [α-^{32}P]GTP to $p21^{ras}$ in cells permeabilized with streptolysin O. For this purpose, we used a cell line which expressed elevated levels of p21 H-*ras* and which was highly responsive to insulin and EGF. Stimulation with insulin or EGF resulted in an increase in the rate of nucleotide binding to $p21^{ras}$. To determine whether this increased binding rate is due to the activation of a guanine nucleotide exchange factor, we made use of the inhibitory properties of a dominant negative mutant of $p21^{ras}$, $p21^{ras}$(Asn-17). Activation of $p21^{ras}$ by insulin and EGF in intact cells was abolished in cells infected with a recombinant vaccinia virus expressing $p21^{ras}$(Asn-17). In addition, the enhanced nucleotide binding to $p21^{ras}$ in response to insulin and EGF in permeabilized cells was blocked upon expression of $p21^{ras}$(Asn-17). From these data, we conclude that the activation of a guanine nucleotide exchange factor is involved in insulin- and EGF-induced activation of $p21^{ras}$.

The products of the *ras* proto-oncogenes are small proteins of 21 kDa that bind guanine nucleotides and are located on the inner side of the plasma membrane (2). These proteins play an important role in signal transduction from a variety of receptors that belong to the tyrosine kinase family (25). The *ras* proteins are activated through conversion of the inactive GDP-bound form into the active GTP-bound form. When bound to GTP, *ras* proteins can induce a number of cellular responses, such as activation of serine-threonine kinases (29), expression of early response genes (44), and mitogenicity (21, 43). Oncogenic mutations in the *ras* proto-oncogene that enable $p21^{ras}$ to transform cells in culture lead to accumulation of the GTP-bound form, either through enhanced nucleotide exchange or through decreased intrinsic GTPase activity (34).

Evidence that activation of $p21^{ras}$ plays a crucial role in the signal transduction of external signals comes from experiments in which the function of normal $p21^{ras}$ has been inhibited. Microinjection of neutralizing antibodies to $p21^{ras}$ results in the inhibition of growth factor-induced mitogenicity (37) and gene expression (44). Also, the expression of dominant negative mutants of $p21^{ras}$ blocks the induction of extracellular signal-regulated kinases (16, 47, 51), gene expression (14, 35), and differentiation (45) by several of these growth factors. A number of external signals that control the activity of $p21^{ras}$ have been characterized. First, in T cells, stimulation of the T-cell receptor leads to a rapid activation of $p21^{ras}$ (18). Secondly, in fibroblasts, stimulation with a variety of growth factors results in *ras* activation; of these, insulin and epidermal growth factor (EGF) seem to have the most profound effect on the activation state of $p21^{ras}$ (12, 23, 38, 40, 41).

The GTP/GDP cycle of normal $p21^{ras}$ is controlled by two classes of regulatory proteins (7, 8). The activation state of $p21^{ras}$ is negatively regulated by GTPase-activating proteins (GAPs), which accelerate the hydrolysis of the bound GTP to GDP. Two GAPs have been identified in mammalian cells: p120GAP (48) and the product of the neurofibromatosis type 1 gene, neurofibromin (1, 33, 52). Deletion of the neurofibromin gene leads to increased levels of *ras*-GTP (3), and overexpression of p120GAP decreases the amount of *ras*-GTP in NIH 3T3 cells (23), indicating that these proteins play an important role in regulating the activation state of $p21^{ras}$. Apart from regulating the GTP/GDP balance of $p21^{ras}$, both proteins have previously been proposed to be mediators of signal transduction from *ras*-GTP (4). In *Saccharomyces cerevisiae*, two proteins have been previously shown to negatively regulate the activity of the yeast RAS proteins IRA1 and IRA2 (46). Deletion of IRA1 or IRA2 results in a phenotype similar to that of the oncogenic RAS2(Val-19) mutant (46). More recently, a putative GAP with similarity to mammalian p120GAP was cloned from *Drosophila melanogaster* (22). Inactivation of this Gap1 locus mimics constitutive activation of the sevenless receptor, which has previously been suggested to activate the *Drosophila* Ras1 protein (22). Positive regulation, i.e., activation of $p21^{ras}$ by the replacement of GDP for GTP, requires the action of an exchange factor that facilitates dissociation of GDP. This will result in the subsequent binding of GTP, since levels of GTP predominate over those of GDP in the cytoplasm of intact cells. In cell and tissue extracts, both cytoplasmic and membrane-bound exchange activities have previously been identified, and these factors have been partially purified (19, 49, 50). Recently, the cloning of mammalian exchange factors for $p21^{ras}$ from a mouse brain cDNA library (32) and from a rat brain cDNA library (42) has been reported. In lower eukaryotes, exchange proteins have been characterized in more detail. In *S. cerevisiae*, the nucleotide exchange on RAS is controlled

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by CDC25 (9) as well as by a C-terminal fragment of SDC25 (15). In *D. melanogaster*, the product of son of sevenless has been indicated as the exchange factor for $p21^{ras}$ on the basis of its homology to CDC25 (6). However, it is still unclear whether the activity of these exchange factors is regulated by external signals, although indirect evidence suggests that EGF might enhance the affinity of $p21^{ras}$ for guanine nucleotides (27). Therefore, we investigated whether the exchange activity of guanine nucleotides on $p21^{ras}$ is altered in response to growth factors that activate $p21^{ras}$.

The rate of nucleotide exchange on $p21^{ras}$ in permeabilized cells expressing elevated levels of p21H-*ras* was measured. We observed an increase in the rate at which $p21^{ras}$ binds [α-^{32}P]GTP in these permeabilized cells in response to insulin and EGF. Furthermore, in this article we show that this enhanced binding rate is inhibited by a dominant negative mutant of $p21^{ras}$, $p21^{ras}$(Asn-17). In addition, expression of *ras*(Asn-17) inhibited the activation of $p21^{ras}$ in intact cells by insulin and EGF. From these data, we conclude that insulin- and EGF-induced activation of $p21^{ras}$ is, at least in part, mediated by the increased activity of an exchange factor for $p21^{ras}$.

MATERIALS AND METHODS

Cell culture. The H13 cell line is a Rat-1-derived cell line overexpressing H-*ras* (approximately 100-fold), and it was a kind gift from J. Downward (17). H13 cells were cultured in Dulbecco's modified eagle medium (DMEM) (GIBCO) supplemented with 10% fetal calf serum (FCS) (GIBCO)–0.05% glutamine. The H13IR2000 cell line was constructed by transfecting H13 fibroblasts with an expression vector containing the human insulin receptor cDNA and a dihydrofolate reductase selection marker. After stepwise amplification with methotrexate up to 2,000 nM, a clonal line, H13IR2000, was obtained. This cell line contains about 8×10^5 insulin receptors per cell (38). H13IR2000 cells were routinely grown in DMEM supplemented with 10% dialyzed FCS–0.05% glutamine plus 2 μM methotrexate. Prior to in vivo labeling and permeabilization experiments, H13IR2000 cells were transferred to DMEM–10% FCS without methotrexate for at least 18 h. A14 cells are NIH 3T3-derived cells expressing 7×10^5 human insulin receptors per cell (12). RR3 cells are Rat-1-derived cells transformed with oncogenic $p21^{ras}$, *ras*(Val-12), and they have been described previously (13). RR3, A14, and Rat-1 cells were grown in DMEM–10% FCS supplemented with 0.05% glutamine.

In vivo labeling of $p21^{ras}$. Subconfluent cultures of A14, Rat-1, H13, and H13IR2000 were grown in DMEM–0.5% FCS for 18 h prior to in vivo labeling. Cells were washed three times with phosphate-free DMEM (Sigma) and labeled with 0.1 Ci of $^{32}P_i$ per ml (3,000 Ci/mmol; Amersham) for 3 to 5 h. After being stimulated with the appropriate growth factor, cells were lysed as described previously (12). Fully processed, membrane-bound $p21^{ras}$ was separated from nonprocessed $p21^{ras}$ by a Triton X-114 phase-split as described previously (12, 24). From the detergent phase, processed $p21^{ras}$ was immunoprecipitated with the monoclonal antibody Y13-259. Alternatively, immunoprecipitation was carried out with another *ras*-specific antibody (Y13-238) or with a nonrelated antibody directed against simian virus 40 large T (kT3) as a control. After extensive washing of the immunoprecipitates, bound guanine nucleotides were eluted as described previously (12). Nucleotides eluted from $p21^{ras}$ were separated by ascending thin-layer chromatography (TLC) as described previously (12), and spots corresponding to GTP and GDP were cut out. The amount of ^{32}P present in each spot was determined by scintillation counting.

Cell permeabilization. Subconfluent cultures of H13, H13IR2000, and RR3 (5-cm-diameter dishes) were serum starved for 18 h prior to permeabilization. Cells were stimulated with the appropriate growth factor, the medium was aspirated, and then the dishes were washed once with warm phosphate-buffered saline (PBS) (37°C). To each dish, 0.8 ml of freshly prepared permeabilization buffer (150 mM KCl, 37.5 mM NaCl, 6.25 mM $MgCl_2$, 0.8 mM $CaCl_2$, 1 mM EGTA, 1.25 mM ATP, 12.5 mM PIPES [piperazine-*N,N′*-bis(2 ethanesulfonic acid)] [pH 7.4]) was added and diluted with 0.2 ml of streptolysin O in water (2 U/ml; Wellcome Diagnostics). To each dish, 1 μl of [α-^{32}P]GTP (3,000 Ci/mmol; Amersham) was added immediately (time point zero), and at discrete time points, cells were lysed and $p21^{ras}$ was immunoprecipitated with the antibody Y13-259 as described in "In vivo labeling of $p21^{ras}$." In experiments in which binding of [α-^{32}P]GTP to $p21^{ras}$ was compared with binding to total cellular proteins, [α-^{32}P]GTP was diluted with 10 μM unlabeled GTP to minimize variations in the specific activity. A chase of the labeled nucleotides bound to $p21^{ras}$ after a 10-min exposure to 3 nM [α-^{32}P]GTP was performed by adding 1 mM unlabeled GTP to the permeabilized cells. At discrete time points, cells were lysed and the amount of labeled nucleotide remaining on $p21^{ras}$ was determined.

Filter binding of total lysates on nitrocellulose. Permeabilized cells exposed to [α-^{32}P]GTP in the presence of 10 μM unlabeled GTP were washed once with cold PBS. Cells were lysed in 500 μl of lysis buffer as described in "In vivo labeling of $p21^{ras}$." After phase separation, 100 μl of the aqueous phase was filtered through a nitrocellulose filter (Millipore, type HA, 0.45-μm pore size). Filters were washed four times with 10 ml of ice-cold wash buffer (20 mM Tris-Cl [pH 7.5], 20 mM NaCl, 5 mM 2-mercaptoethanol, 3 mM $MgCl_2$) and counted in 4 ml of scintillation liquid.

Construction and expression of $p21^{ras}$(Asn-17) recombinant vaccinia virus. The dominant negative mutant of $p21^{ras}$, *ras*(Asn-17), was introduced into a viral growth factor minus strain of vaccinia virus to avoid autocrine stimulation of the infected cells by the viral growth factor (10). This vaccinia virus strain was a kind gift from B. Moss, and the *ras*(Asn-17) mutant gene was inserted through homologous recombination as described previously (16). Six additional histidine codons were inserted after the AUG start codon of the H-*ras*(Asn-17) gene (16) to allow binding to Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) agarose (Qiagen) (26). Wild-type and recombinant viruses were grown in HeLa cells (titer, 10^9 virus particles per ml). H13IR2000 cells were infected with 10 PFU of recombinant or wild-type virus per cell in serum-free medium. After 60 min, the medium was replaced with DMEM–0.5% FCS and 10 mM hydroxyurea to block DNA synthesis and late viral expression. Sixteen to 18 h after infection, cells were used for in vivo labeling or permeabilization experiments. Analysis of the guanine nucleotides bound to $p21^{ras}$ was performed as described under "In vivo labeling of $p21^{ras}$," with one minor modification: all lysates were precleared with Ni^{2+}-NTA agarose (15-μl bead volume, 15 min at 4°C), instead of being precleared with 50 μl of protein A agarose as usual. Expression of *ras*(Asn-17) was checked by binding of *ras*(Asn-17) recombinant protein to Ni^{2+}-NTA agarose through 6 histidine residues at the N terminus. After binding of recombinant *ras*(Asn-17), wild-type *ras* was immunoprecipitated with Y13-259. Samples were separated on a 15% polyacrylamide gel, blotted onto nitrocellulose, and incubated with Y13-259. Immune com-

plexes were detected first by horseradish peroxidase second antibodies and then by enhanced chemiluminescence.

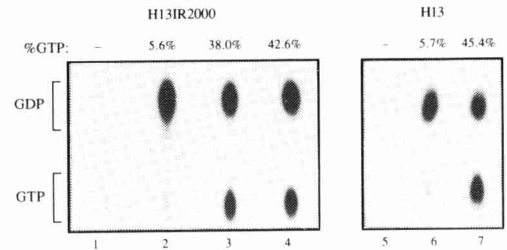
RESULTS

Insulin- and EGF-induced activation of p21^{ras}. We have constructed a cell line (H13IR2000) which expresses elevated levels of both p21 H-*ras* and the human insulin receptor. To test whether the activation state of p21^{ras} is regulated by insulin in H13IR2000 cells, these cells were labeled with $^{32}P_i$, and then the guanine nucleotide ratio bound to p21^{ras} was analyzed. Stimulation of H13IR2000 cells with insulin for 5 min resulted in an increase in the level of GTP-bound p21^{ras}, from ~7 to ~40% of total bound guanine nucleotides (Fig. 1A). This increase is similar to that observed previously with NIH 3T3 cells overexpressing the human insulin receptor (A14 cells) (12). Treatment of H13IR2000 cells with EGF for 5 min resulted in an activation of p21^{ras} similar to that observed with insulin (Fig. 1A). This is not a consequence of overexpression of the insulin receptor, since EGF stimulation resulted in *ras* activation in the H13 cell line (Fig. 1A) and the parental Rat-1 cell line as well (Fig. 1C). Furthermore, we compared the time course of *ras* activation in A14 cells with that in H13IR2000 cells in response to insulin (Fig. 1B) as well as the time course in Rat-1 cells with that in H13IR2000 cells in response to EGF (Fig. 1C). Activation of p21^{ras} follows a pattern in H13IR2000 cells similar to that in A14 or Rat-1, with the minor difference being that the time required to reach half-maximal stimulation of *ras* activation in H13IR2000 cells is somewhat longer than that in A14 cells (Fig. 1B) and in Rat-1 cells (Fig. 1C). Considering the approximately 100-fold-higher expression of p21^{ras} in H13IR2000 cells, this suggests that the activation of p21^{ras} by insulin or EGF is not affected by the high levels of p21 H-*ras* in the H13IR2000 cell line.

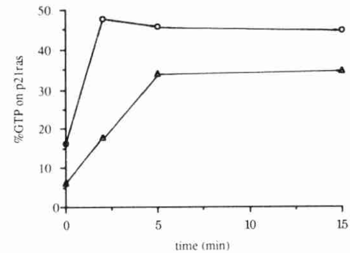
Increased nucleotide exchange on p21^{ras}. To determine whether the activation of p21^{ras} by insulin and EGF in H13IR2000 cells is caused by an increase in the rate of nucleotide exchange on p21^{ras}, we measured binding of [α-^{32}P]GTP to p21^{ras} in permeabilized H13IR2000 cells. Cells were stimulated with either EGF for 2 min or insulin for 5 min, permeabilized with streptolysin O, and incubated with [α-^{32}P]GTP. At different time points, cells were lysed and p21^{ras} was collected by immunoprecipitation. Nucleotides bound to p21^{ras} were eluted and separated by TLC. Nucleotide binding to p21^{ras} in H13IR2000 cells was enhanced approximately threefold in response to insulin and EGF compared with that in untreated cells (Fig. 2). Stimulation with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate had no effect on nucleotide binding to p21^{ras}, which is consistent with the finding that 12-*O*-tetradecanoylphorbol-13-acetate has no effect on the activation state of p21^{ras} in intact H13IR2000 cells (data not shown). The same difference in nucleotide binding in response to insulin could also be observed after immunoprecipitation with another monoclonal antibody directed against p21^{ras}, Y13-238 (data not shown).

No significant shift in the GDP/GTP ratio bound to p21^{ras} was observed in permeabilized cells after stimulation with insulin or EGF, whereas similar stimulation leads to accumulation of GTP on p21^{ras} of up to 50% of the total bound nucleotide in intact cells. This difference could be caused by hydrolysis of GTP on p21^{ras} or due to hydrolysis of GTP prior to binding to p21^{ras} in permeabilized cells. To discriminate between these two possibilities, we used the RR3 cell

A.



B.



C.

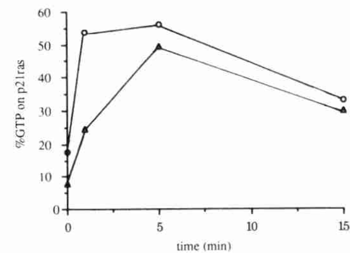


FIG. 1. Activation of p21^{ras} by insulin and EGF in fibroblasts. A14, Rat-1, H13, and H13IR2000 cells were labeled with $^{32}P_i$ as described in Materials and Methods. Cells were lysed, and p21^{ras} was immunoprecipitated. Nucleotides bound to p21^{ras} were eluted and separated by TLC. Following autoradiography, spots corresponding to GTP and GDP were cut out and counted by scintillation counting. (A) TLC of the guanine nucleotides eluted from p21^{ras} immunoprecipitates from H13IR2000 cells (lanes 1 to 4) or H13 cells (lanes 5 to 7). Immunoprecipitation was performed with the monoclonal antibody Y13-259 (lanes 2, 3, 4, 6, and 7) or with the nonrelated antibody kT3, which is unable to recognize p21^{ras} (lanes 1 and 5). Cells were unstimulated (lanes 1, 2, 5, and 6), stimulated with 10 μg of insulin per ml for 5 min (lane 3), or stimulated with 20 ng of EGF per ml for 5 min (lanes 4 and 7). Indicated are the positions at which GTP and GDP standards ran. At the top of each lane, the amount of GTP (as a percentage of total nucleotide bound to p21^{ras}) is indicated. (B) Time course of *ras* activation in response to insulin in A14 and H13IR2000 cells. A14 cells (○) or H13IR2000 cells (△) were stimulated with 10 μg of insulin per ml for 2, 5, or 15 min. (C) Time course of *ras* activation in response to EGF in Rat-1 and H13IR2000 cells. Rat-1 (○) or H13IR2000 cells (△) were stimulated with 20 ng of EGF per ml for 2, 5, or 15 min. The percentage of GTP is expressed as relative to the total amount of labeled nucleotide bound to p21^{ras}.

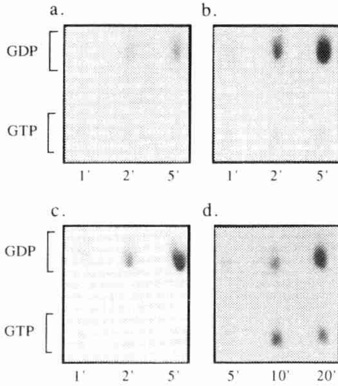


FIG. 2. Enhanced rate of nucleotide binding to p21^{ras} in response to insulin and EGF. Following growth factor stimulation, H13IR2000 and RR3 cells were permeabilized as described in Materials and Methods. H13IR2000 cells (panels a, b, and c) were exposed to [α-^{32}P]GTP for 1, 2, or 5 min, and RR3 cells (panel d) were exposed to [α-^{32}P]GTP for 5, 10, or 20 min as indicated at the bottom of each lane. After cell lysis, p21^{ras} was immunoprecipitated and the nucleotides were eluted. GTP and GDP were separated by TLC, and the positions at which GTP and GDP standards ran are indicated. H13IR2000 cells were left unstimulated (panel a), stimulated with 10 μg of insulin per ml for 5 min prior to permeabilization (panel b), or stimulated with 20 ng of EGF per ml for 2 min prior to permeabilization (panel c). RR3 cells were left unstimulated (panel d).

line expressing p21^{ras}(Val-12), a ras mutant defective in GTP hydrolysis. Since these cells express relatively small amounts of p21^{ras} compared with those by the H13IR2000 cells, longer exposures to [α-^{32}P]GTP were necessary to obtain significant amounts of labeled nucleotides on p21^{ras}. As shown in Fig. 2d, we did observe significant amounts of [α-^{32}P]GTP bound to p21^{ras} in RR3 cells (~50% at all time points in RR3, in contrast to ~15% in H13IR2000 cells). Thus, it seems that hydrolysis of GTP on p21^{ras} is responsible for the observed absence of GTP bound to p21^{ras} in H13IR2000 cells.

Increased nucleotide binding to p21^{ras} could be the consequence of an increased rate of binding or increased overall binding. We therefore compared the kinetics of binding of nucleotides to p21^{ras} in stimulated cells with that in unstimulated cells. To minimize variations in the specific activities in these experiments, 10 μM unlabeled GTP was added to the permeabilization buffer. After stimulation with insulin, a clear twofold increase was consistently observed in the initial rate of nucleotide binding to p21^{ras} (Fig. 3A). Nucleotide binding to p21^{ras} in insulin-stimulated cells approached plateau levels after 20 min, whereas binding in unstimulated cells was still submaximal. This indicates that increased binding is due to an increased rate of binding and is not a consequence of an increased number of binding sites available for [α-^{32}P]GTP. To exclude the possibility that insulin stimulation might affect the efficiency of permeabilization or the uptake of [α-^{32}P]GTP into permeabilized cells, we measured binding of [α-^{32}P]GTP to total cellular proteins, which was determined by filter binding on nitrocellulose filters. Total binding of [α-^{32}P]GTP in permeabilized cells was unaltered in the presence of insulin compared with that in unstimulated cells (Fig. 3B).

In addition, we measured the rate at which labeled guanine nucleotides bound to p21^{ras} are replaced by unlabeled nucleotide after the addition of excess unlabeled GTP (1 mM). For this purpose, permeabilized cells were exposed to 3 nM [α-^{32}P]GTP for 10 min, followed by a cold chase with 1 mM unlabeled GTP. Eighty percent of the labeled nucleotide bound to p21^{ras} in cells stimulated with insulin was released within 1 min, showing the specificity of the binding (Fig. 3C). However, a certain proportion of the labeled nucleotide has a much slower off rate. This could either result from a slow intrinsic exchange of guanine nucleotides on p21^{ras} or represent aspecific binding.

Inhibition of *ras* activation by expression of *ras*(Asn-17). To examine the involvement of guanine nucleotide exchange factors in more detail, we used a dominant negative mutant of p21^{ras}, *ras*(Asn-17). This mutant has a low affinity for GTP, and as a consequence it will predominantly bind GDP (20). Inhibition of *ras* function by this mutant presumably takes place through competition for activators of p21^{ras}. Therefore, we investigated whether the activation of p21^{ras} in intact cells can be blocked by the expression of this mutant p21^{ras}. For this purpose, cells were infected with a recombinant vaccinia virus containing the *ras*(Asn-17) mutant gene (16). After infection with *ras*(Asn-17) recombinant vaccinia virus, approximately 100% of the cells express *ras*(Asn-17) protein, since the activation of extracellular signal-regulated kinase 2 (ERK2) by insulin is completely inhibited (16). Sixteen to 18 h after infection, cells were labeled with $^{32}P_i$ for 4 h, and labeling was followed by an analysis of the GTP/GDP ratio bound to p21^{ras}. As a control for the effects of viral infection, cells were infected with wild-type vaccinia virus. The lysates were incubated with Ni^{2+}-NTA agarose to remove recombinant *ras*(Asn-17) protein prior to being immunoprecipitated. Incubation with Ni^{2+}-NTA agarose for 15 min removed all recombinant *ras*(Asn-17) protein, since a second incubation with Ni^{2+}-NTA agarose could not detect any remaining *ras*(Asn-17) protein (Fig. 4). Therefore, *ras*(Asn-17) protein will not contribute to the nucleotide ratio bound to p21^{ras} immunoprecipitates. In addition, the expression level of p21^{ras}(Asn-17) was estimated to be at least 20-fold lower than that of endogenous wild-type p21^{ras} in H13IR2000 cells (Fig. 4). Insulin- and EGF-induced activation of p21^{ras} occurred normally in H13IR2000 cells infected with wild-type virus (Fig. 5). However, the activation of p21^{ras} by both insulin and EGF was inhibited (85 and 50% inhibition, respectively) in cells infected with the recombinant *ras*(Asn-17) vaccinia virus (Fig. 5). Autophosphorylation of the insulin receptor occurred normally in these cells, as judged from immunoprecipitates with an anti-phosphotyrosine antibody (data not shown).

Inhibition of increased nucleotide binding by expression of *ras*(Asn-17). If activation of p21^{ras} by insulin or EGF occurs through activation of an exchange factor for p21^{ras}, as suggested by our findings, then the activation of nucleotide exchange by these growth factors should be sensitive to expression of *ras*(Asn-17). We investigated the effects of insulin and EGF on the binding rate of labeled nucleotides to p21^{ras} in cells infected with the *ras*(Asn-17) recombinant vaccinia virus. The increased binding rate observed in response to insulin was inhibited in cells expressing *ras*(Asn-17) (77 to 100% inhibition; Fig. 6). The increased binding rate induced by EGF was also inhibited by *ras*(Asn-17) (55% inhibition) (not shown). This indicates that the enhanced binding rate in response to insulin and EGF is due to the activation of a guanine nucleotide exchange factor. How-

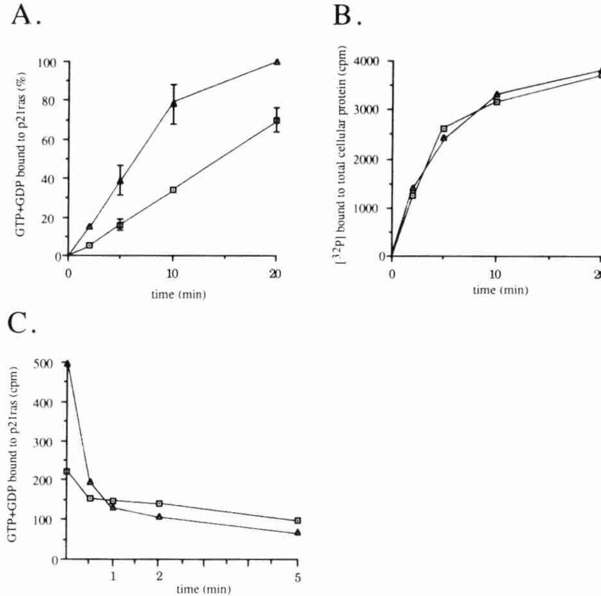


FIG. 3. Binding of [α-^{32}P]GTP to p21^{ras} and total cellular proteins. H13IR2000 cells were left unstimulated (□) or were stimulated with 10 μg of insulin per ml for 5 min (△). Cells were permeabilized and exposed to [α-^{32}P]GTP for various lengths of time. [α-^{32}P]GTP was diluted with unlabeled GTP to 10 μM for measurement of binding to p21^{ras} and total cellular proteins. Following lysis and Triton X-114 phase separation, p21^{ras} was immunoprecipitated from the detergent phase as usual, and a 100-μl fraction of the aqueous phase was used for filter binding. (A) Binding of α-^{32}P-labeled guanine nucleotides to immunoprecipitates of p21^{ras} expressed as the percentage of the amount of binding to p21^{ras} observed at 20 min in stimulated cells (set at 100%). Each point represents the average of four separate measurements, and error bars indicate standard deviations. (B) Binding of [α-^{32}P]GTP to total cellular proteins in lysates from unstimulated or insulin-stimulated H13IR2000 cells as determined in a filter-binding assay. (C) Unstimulated or insulin-stimulated H13IR2000 cells were permeabilized and exposed to 3 nM [α-^{32}P]GTP for 10 min. A chase was performed by the addition of excess unlabeled GTP (1 mM). At different time points after the addition of unlabeled GTP, cells were lysed and p21^{ras} was immunoprecipitated. The amount of α-^{32}P-labeled nucleotide retained on immunoprecipitates of p21^{ras} was determined as described in the legend to Fig. 2.

ever, the unstimulated binding rate was almost unaltered in the presence of p21^{ras}(Asn-17) (Fig. 6), suggesting that a large fraction of this binding occurs through a different mechanism.

DISCUSSION

Stimulation of tyrosine kinase receptors, including EGF and insulin receptors, activates p21^{ras} as measured by an increase in GTP-bound p21^{ras}. In this report, we demonstrate that this increase in the GTP-bound form of p21^{ras} is, at least in part, due to enhanced guanine nucleotide exchange. To study guanine nucleotide exchange on p21^{ras}, we have used a Rat-1-derived cell line expressing elevated levels of p21 H-*ras* (H13) as well as an H13 cell line transfected with the human insulin receptor (H13IR2000). Both insulin and EGF induce activation of p21^{ras} in this latter cell line. Treatment of H13IR2000 cells with insulin or EGF caused an increase of GTP-bound p21^{ras}, from ~7% of the total nucleotide bound to p21^{ras} in unstimulated cells to ~40% in insulin- or EGF-stimulated cells (Fig. 1A). Since the time course of *ras* activation in response to insulin or EGF followed similar patterns in H13IR2000 cells and A14 or Rat-1 cells, respectively (Fig. 1B and C), it seems unlikely

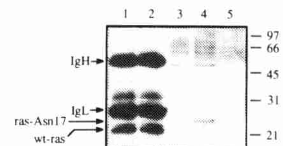


FIG. 4. Expression of wild-type p21^{ras} and recombinant *ras*(Asn-17) proteins in vaccinia virus-infected cells. H13IR2000 cells were infected with wild-type vaccinia virus (lanes 1 and 3) or *ras*(Asn-17) recombinant vaccinia virus (lanes 2, 4, and 5), and 16 to 18 h after infection, cells were harvested. Lysates were precleared with Ni^{2+}-NTA agarose to bind recombinant *ras*(Asn-17) protein (lanes 3 and 4), and preclearance was followed by immunoprecipitation of the remaining p21^{ras} with Y13-259 bound to protein A agarose beads (lanes 1 and 2) or by a second incubation with Ni^{2+}-NTA agarose (lane 5). Ni^{2+}-NTA agarose beads and p21^{ras} immunoprecipitates were boiled in sample buffer and loaded onto a 15% polyacrylamide gel. Ras proteins were detected as described in Materials and Methods. Exposure of the blot for detection of the *ras*(Asn-17) protein bound to Ni^{2+}-NTA agarose beads was 10 times longer than that for detection of p21^{ras} bound to Y13-259. Due to the presence of 6 histidine residues at the amino-terminal end, *ras*(Asn-17) has a slightly slower mobility than wild-type p21^{ras}. The positions of molecular size markers (in kilodaltons) are indicated on the right.

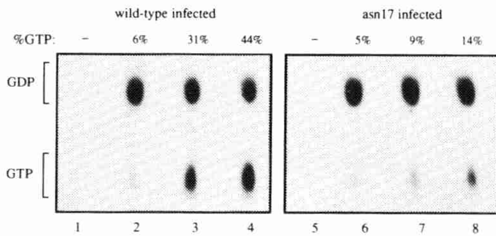


FIG. 5. Activation of p21^{ras} inhibited by *ras*(Asn-17). H13IR2000 cells were infected with wild-type vaccinia virus (left panel) or with the *ras*(Asn-17) recombinant vaccinia virus (right panel). Eighteen hours after transfection, cells were labeled with $^{32}P_i$. Following growth factor stimulation, cells were lysed, *ras*(Asn-17) recombinant protein was removed with Ni^{2+}-NTA agarose, and p21^{ras} was immunoprecipitated. Nucleotides were eluted from immunoprecipitates of p21^{ras} and separated by TLC. The cells were left unstimulated (lanes 1, 2, 5, and 6), stimulated with 10 μg of insulin per ml for 5 min (lanes 3 and 7), or stimulated with 20 ng of EGF per ml for 5 min (lanes 4 and 8). Control immunoprecipitations were performed with the nonrelated antibody kT3 (lanes 1 and 5). The percentage of GTP bound to p21^{ras} relative to the total amount of bound nucleotide (GTP plus GDP) is indicated at the top of each lane.

that overexpression of p21 H-*ras* has a profound effect on the mechanism of *ras* activation.

To study the effect of growth factor stimulation on nucleotide exchange on p21^{ras}, H13IR2000 cells were used to measure GTP binding to p21^{ras} in permeabilized cells. Binding of [α-^{32}P]GTP to p21^{ras} in permeabilized H13IR2000 cells was enhanced in response to insulin and EGF (Fig. 2), but not by 12-O-tetradecanoylphorbol-13-acetate, consistent with the effect of each of these growth factors on the activation state of p21^{ras} in intact cells. An increase of approximately two- to threefold was consistently observed

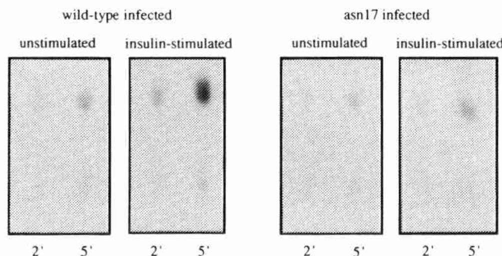


FIG. 6. Inhibition of enhanced nucleotide binding by *ras*(Asn-17). H13IR2000 cells were infected with wild-type (left panel) or *ras*(Asn-17) recombinant (right panel) vaccinia virus. Sixteen to 18 h after transfection, cells were permeabilized with streptolysin O and exposed to [α-^{32}P]GTP. After 2 or 5 min, cells were lysed, *ras*(Asn-17) recombinant protein was removed with Ni^{2+}-NTA agarose, and p21^{ras} was immunoprecipitated. Cells were left unstimulated or were stimulated with 10 μg of insulin per ml for 5 min prior to permeabilization, where indicated. The amount of total labeled nucleotide bound to p21^{ras} was determined as described in the legend to Fig. 3. Inhibition of insulin-induced nucleotide binding in cells expressing p21^{ras}(Asn-17) was reproduced in three separate experiments, with 77, 100, and 100% inhibition, respectively. We also observed similar inhibition (55%) in the rate of EGF-induced nucleotide binding in two independent experiments.

in the initial rate of guanine nucleotide binding to p21^{ras} after stimulation with insulin (Fig. 3A). Since growth factor stimulation had no effect on the binding of labeled nucleotides to total cellular proteins (Fig. 3B), we conclude that the increased rate of nucleotide binding in cells treated with insulin or EGF is not the consequence of a difference in uptake of [α-^{32}P]GTP or efficiency of permeabilization. Therefore, the observed difference reflects a real difference in the binding rate of guanine nucleotides to p21^{ras}, presumably through activation of a guanine nucleotide exchange factor.

This latter notion was further strengthened by the experiments performed with a dominant negative mutant of p21^{ras}, p21^{ras}(Asn-17). This mutant has a 40-fold-reduced affinity for GTP, but its affinity for GDP is unaltered (20). Inhibition of endogenous *ras* function by this mutant has been proposed to occur through competition with normal *ras* for regulatory proteins which normally promote nucleotide exchange (20). Indeed, we found that the introduction of p21^{ras}(Asn-17) almost completely inhibited insulin- and EGF-induced activation of p21^{ras} in intact cells (Fig. 5). This finding constitutes the first direct proof that p21^{ras}(Asn-17) actually inhibits p21^{ras} activation. Remarkably, up to 85% inhibition can be accomplished, although the expression level of p21^{ras}(Asn-17) in H13IR2000 cells infected with *ras*(Asn-17) recombinant virus is at least 20-fold lower than that of wild-type p21^{ras} (Fig. 4). Clearly, p21^{ras}(Asn-17) is a very efficient inhibitor of p21^{ras}, suggesting a very high affinity of *ras*(Asn-17) for the exchange factor.

The increased rate of nucleotide binding in response to insulin and EGF was also inhibited by expression of *ras*(Asn-17) (Fig. 6). In contrast, unstimulated nucleotide exchange was only slightly affected by p21^{ras}(Asn-17). Remarkably, the extent of inhibition of increased nucleotide binding in response to insulin (77 to 100%) or EGF (55%) is similar to the level of inhibition of insulin- (85%) and EGF-induced (50%) activation of p21^{ras} in intact cells. In conclusion, our findings that p21^{ras}(Asn-17) inhibits both *ras* activation in intact cells and growth factor-induced nucleotide binding to p21^{ras} in permeabilized cells to similar extents suggest that the activation of a nucleotide exchange factor is involved in the activation of p21^{ras} by insulin and EGF.

Virtually all of the labeled nucleotide bound to p21^{ras} after stimulation with insulin could be released by the addition of excess unlabeled GTP (Fig. 3C), stressing the specificity of the nucleotide binding. However, a certain proportion of the labeled nucleotide is released very slowly during the chase experiment. This fraction can be more than 50% of the total amount of labeled nucleotide recovered from *ras* immunoprecipitates in the unstimulated cells (Fig. 3C). The slow release could be caused by a fraction of p21^{ras} which is not in contact with an exchange factor and thus represent intrinsic exchange of nucleotides on p21^{ras}, or it could be due to aspecific binding. This would suggest that a certain proportion of labeled nucleotide is bound to p21^{ras} in a manner independent of a guanine nucleotide exchange factor. In turn, this would explain why unstimulated binding of nucleotides to p21^{ras} is hardly affected by expression of *ras*(Asn-17). If so, then the guanine nucleotide exchange factor per se is activated more than two- to threefold by EGF or insulin.

Although the release of labeled nucleotides from p21^{ras} is virtually complete within 1 min after the addition of 1 mM GTP in insulin-stimulated cells (Fig. 3C), it takes at least 20 min for binding of labeled nucleotides to p21^{ras} to reach

steady-state levels in these cells in the presence of 10 μM GTP (Fig. 3A). Thus, the measured off rate of nucleotides bound to p21^{ras} is higher than the measured on rate. Possibly, either the release of GDP or the binding of GTP is dependent on the concentration of GTP, and the observed difference in on and off rates is due to the different concentrations of GTP used in each experiment (10 μM for binding, 1 mM for release).

Unlike with intact cells, no accumulation of GTP-bound p21^{ras} is observed in permeabilized cells after stimulation with insulin or EGF (compare Fig. 1 and 2). However, [α-^{32}P]GTP is sufficiently stable in the permeabilized cells to obtain significant amounts of *ras* GTP in cells that express a mutated *ras* protein which is unable to hydrolyze GTP (RR3) (Fig. 2). Thus, it seems that hydrolysis of GTP on p21^{ras} is responsible for the observed absence of GTP bound to p21^{ras} in H13IR2000 cells. In our view, this difference between intact and permeabilized cells is best explained by the forementioned observation that the on rate might be limited by the availability of GTP in the experiments using permeabilized cells. A submaximal exchange rate in permeabilized cells would result in the absence of GTP bound to p21^{ras}. This also explains why our results differ from those of experiments using permeabilized T cells, in which a clear increase in the level of GTP bound to p21^{ras} after T-cell receptor stimulation has been previously observed (18). In these cells, regulation of the activation state of p21^{ras} has been previously proposed to occur through inhibition of GAP activity (18), which is in contrast to the mechanism proposed here for the regulation of *ras* activation in fibroblasts. This inhibition of GAP activity may permit the detection of GTP bound to p21^{ras} from permeabilized T cells. In fibroblasts, a reduction in GTPase stimulatory properties in response to insulin or EGF has not been observed thus far (5, 11). However, a role for p120GAP in the regulation of p21^{ras} in fibroblasts cannot be excluded at this point, considering that p120GAP interacts with all tyrosine kinase receptors known to activate p21^{ras} (28, 31, 39). Moreover, EGF stimulation has been previously reported to induce association of p120GAP with a 190-kDa phosphoprotein, which results in a fourfold reduction in GAP activity (36). Similarly, in T cells the additional involvement of induced exchange cannot be excluded. It could be that both the rate of nucleotide exchange on p21^{ras} and the rate of GTP hydrolysis are changed upon growth factor stimulation. Therefore, the apparent difference in regulation of the activation state of p21^{ras} in T lymphocytes and fibroblasts may be a consequence of the relative contribution of each of the two mechanisms. Regulation of both nucleotide exchange on p21^{ras} as well as GTPase activity of p21^{ras} has recently been suggested to occur in PC12 cells stimulated with nerve growth factor. Remarkably, an increase in GTPase stimulatory activity appears to antagonize the measured increase in activity of the nucleotide exchange factor (30). Also, by using a more indirect approach with a number of mutants of p21^{ras} introduced in NIH 3T3 cells, it was previously shown that serum- and platelet-derived growth factors most likely stimulate guanine nucleotide exchange, whereas the p21^{ras} GTPase activity seems to be affected by cell density (53). Taken together, these data strongly suggest that the activity of the exchange factor(s) for p21^{ras} is regulated by growth factors, leading to the activation of p21^{ras}. The mechanism by which these growth factors activate the exchange factor(s) remains to be elucidated.

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CHAPTER 6

GTPase-activating protein SH2-SH3 domains induce gene expression in a ras-dependent fashion.

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GTPase-Activating Protein SH2-SH3 Domains Induce Gene Expression in a Ras-Dependent Fashion

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The p21^{ras} GTPase-activating protein (GAP) is thought to function as both a negative regulator and a downstream target of p21^{ras}. Here, we have investigated the role of GAP by using a transient expression assay with a *fos* luciferase reporter plasmid. We used GAP deletion mutants that lack the domain involved in interaction with p21^{ras} and encode essentially only the SH2-SH3 domains. When these GAP deletion mutants were expressed, we observed a marked induction of *fos* promoter activity similar to induction by activated p21^{ras}. Expression of a full-length GAP construct had no effect on the activity of the *fos* promoter. Activation of the *fos* promoter by these GAP SH2-SH3 regions was inhibited by cotransfection of a dominant inhibitory mutant of p21^{ras}, Ras(Asn-17). Thus, the induction of gene expression by GAP SH2-SH3 domains is dependent on p21^{ras} activity. Moreover, induction of *fos* promoter activity by GAP SH2-SH3 domains is increased severalfold after cotransfection of an activated mutant of p21^{ras}, Ras(Leu-61), or insulin stimulation of A14 cells, both leading to an increase in the levels of GTP-bound p21^{ras}. The combined effect of Ras(Leu-61) and the GAP deletion mutants was not inhibited by Ras(Asn-17), indicating that GAP SH2-SH3 domains do not function to activate endogenous p21^{ras} but cooperate with another signal coming from active p21^{ras}. These data suggest that GAP SH2-SH3 domains serve to induce gene expression by p21^{ras} but that additional signals coming from p21^{ras} are required for them to function.

The products of the *ras* genes play an important role in the regulation of cellular growth and differentiation. Their potential to induce transformation and their role in signal transduction have been studied extensively (3, 5, 6). So far, it seems well established that p21^{ras} proteins are involved in signaling from a number of receptors of the tyrosine kinase family (16). This notion is supported by the fact that mitogenicity induced by a number of growth factors can be blocked by a neutralizing anti-Ras antibody (27) and by the action of a dominant inhibitory mutant of p21^{ras} (9). Also, activation of p21^{ras} by a shift to the GTP-bound form has been reported to take place upon activation of a number of tyrosine kinase receptors (8, 11, 14, 30, 31). The mechanism by which this activation takes place is still unknown. However, a possible link between p21^{ras} and tyrosine kinase signaling is provided by the p21^{ras} GTPase-activating protein (GAP).

GAP can negatively regulate p21^{ras} activity by increased hydrolysis of GTP bound to p21^{ras} (33, 36). The COOH-terminal domain of GAP is responsible for this catalytic effect on p21^{ras} GTPase activity (20). In addition, through its *src*-homology domains (SH2-SH3), GAP can associate with tyrosine-phosphorylated proteins (2, 25) such as the platelet-derived growth factor receptor (18), epidermal growth factor receptor (19), the insulin receptor (28), v-*src* (7, 29), and two proteins of 190 and 62 kDa (12). Also, GAP is phosphorylated on tyrosine in cells that are stimulated by platelet-derived growth factor (24), epidermal growth factor (12), or insulin (28) and in cells expressing v-*src* (12). Interestingly, all of these tyrosine kinases have been reported to activate p21^{ras} to some extent (8, 14, 30, 31), but at present, no effect of tyrosine phosphorylation on the catalytic activity of GAP on p21^{ras} has been observed. Only GAP associated with the 190-kDa protein seems to have a reduced activity (26).

Apart from negatively regulating p21^{ras}, GAP has been proposed to serve as a downstream target of p21^{ras}, since it interacts with a region of p21^{ras} implicated in effector function (1, 10). Direct proof for such a function of GAP was obtained with isolated atrial cell membranes, in which interaction between p21^{ras} and GAP can inhibit the carbachol-induced opening of K^+ channels (38). This inhibition most likely occurs through an uncoupling of the heterotrimeric G protein (G_k) and the muscarinic receptor. The SH2-SH3 domains of GAP are responsible for this effect (21). These domains can inhibit the opening of potassium channels in a *ras*-independent manner when the region of GAP that interacts with p21^{ras} is deleted. From these data, a model in which GAP is enabled to interact with its target through a conformational change induced upon binding of p21^{ras} was proposed (21).

Here, we have examined the role of GAP in the induction of gene expression. Activation of p21^{ras} has been shown to lead to the induction of *fos* promoter activity in a transient assay system (23). We tested whether transfection of full-length GAP or GAP SH2-SH3 domains would result in the induction of gene expression. The deletion mutants encoding GAP SH2-SH3 domains strongly induce of *fos* promoter activity when transiently expressed, whereas full-length GAP has no effect. In addition, the effect of GAP SH2-SH3 domains is dependent on the activity of p21^{ras} and cooperates with increased levels of active p21^{ras}.

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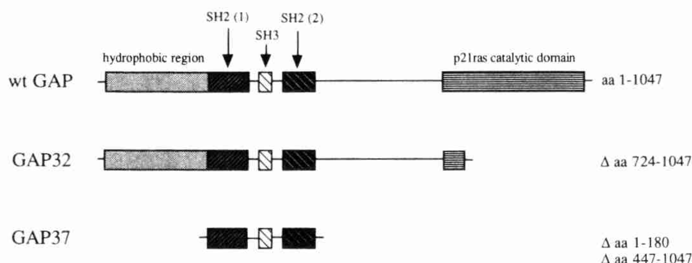

FIG. 1. Structure of GAP expression plasmids. The wild-type GAP clone (wt GAP) contains the full-length human type 1 GAP cDNA from clone 101 (34). At the NH2 terminus, it has a hydrophobic stretch of about 180 amino acids and the *src*-homology regions, SH2 (1), SH3, and SH2 (2), adjacent to it. The region important for the activation of $p21^{ras}$ GTPase activity is located at the COOH-terminal part of the protein (amino acids 714 to 1047). The GAP32 deletion mutant lacks amino acids 724 to 1047, a major part of the catalytic domain. GAP37 lacks amino acids 1 to 180, the hydrophobic stretch, and amino acids 447 to 1047, which are all amino acids COOH terminal to SH2 (2), and encodes almost only the SH2-SH3 regions.

MATERIALS AND METHODS

Cell culture and transfection. Cells were cultured in Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% fetal calf serum (GIBCO) and 0.05% L-glutamine. A14 cells are NIH 3T3-derived cell lines, expressing approximately 3×10^5 insulin receptors per cell, that have been described previously (8). Transient transfections in A14 cells were carried out by using the DEAE-dextran method (15). Chinese hamster ovary cells (CHO9) were transfected by using the calcium phosphate method (35) in combination with a glycerol shock as described previously (23). After being transfected, cells were cultured for 48 h in Dulbecco's modified Eagle medium supplemented with 0.5% fetal calf serum. Where indicated, cells were stimulated with insulin (10 μg/ml) 24 h prior to harvesting. Cells were lysed at room temperature in a buffer containing 15% glycerol, 1% Triton X-100, 0.1 M potassium phosphate buffer (pH 7.8), 1 mM dithiothreitol, and 8 mM $MgCl_2$ for 10 min and collected by scraping with a rubber policeman. Lysates were cleared by centrifugation in an Eppendorf centrifuge for 10 min. Luciferase activity was determined as described before (23).

Recombinant plasmids. The *fos* luciferase construct and Rous sarcoma virus (RSV)-driven constructs, RSV.neo, RSV.Ras(Asn-17), and RSV.Ras(Leu-61), were described previously (23). The full-length wild-type GAP expression vector was constructed by insertion of the cDNA from pUC101 (34), containing the complete human cDNA of GAP, into pRSV.H20 (23). The construction of cDNAs encoding GAP37 and GAP32R was described previously (21). For transient expression, these cDNAs were cloned into the pcDNA I vector (Invitrogen) under control of the cytomegalovirus promoter and enhancer.

RESULTS

Induction of c-*fos* expression by GAP deletion mutants. Two different GAP deletion mutants were constructed from a full-length human GAP cDNA. The structure of each of the GAP constructs is shown in Fig. 1. The wild-type GAP cDNA contains the complete coding sequence of human type 1 GAP. It contains a hydrophobic NH2 terminus of about 180 amino acids, the *src*-homology regions (SH2-SH3) directly adjacent to it, and the catalytic domain responsible for activation of the $p21^{ras}$ GTPase activity in the COOH-terminal part of the protein (20). GAP32 lacks a substantial part of the region for $p21^{ras}$ interaction. GAP37 lacks both the hydrophobic stretch and the catalytic domain and encodes essentially only the SH2-SH3 regions. Both proteins were shown to function in a manner independent of $p21^{ras}$ in the uncoupling of the muscarinic receptor from its G protein in a previous study (21). We used Chinese hamster ovary cells (CHO9) and an NIH 3T3-derived cell line expressing high levels of the human insulin receptor (A14) to test the effect of the GAP deletion mutants. Transfection of the full-length GAP construct did not affect the expression driven by the *fos* promoter (Fig. 2). In contrast, a severalfold induction was observed after the transfection of GAP32 and GAP37 clones in both cell lines (Fig. 2). Transfection of the GAP37 construct especially resulted in a strong induction (5- to 10-fold) of *fos* promoter activity.

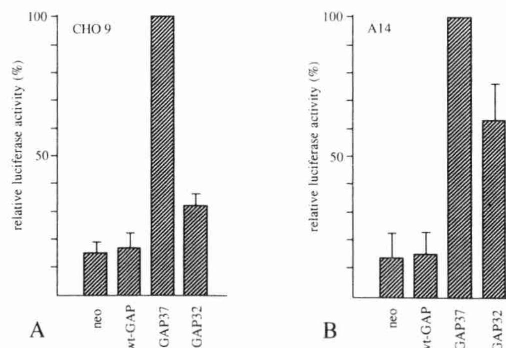

FIG. 2. Induction of gene expression by GAP SH2-SH3 domains. Cells were transfected with 4 μg of *fos* luciferase in combination with 6 μg of RSV.neo, RSV.GAP, CMV.GAP32, or CMV.GAP37 per 9-cm dish. The results for CHO9 cells transfected by calcium phosphate precipitation of DNA (A) and A14 cells transfected by DEAE-dextran-mediated DNA uptake (B) are shown. After being transfected, cells were grown on Dulbecco's modified Eagle medium supplemented with 0.5% fetal calf serum. Lysates were made 48 h after transfection and tested for luciferase activity. Activities are expressed relative to the activity found in cells transfected with GAP37. Error bars represent the standard deviation for each value (n = 6).

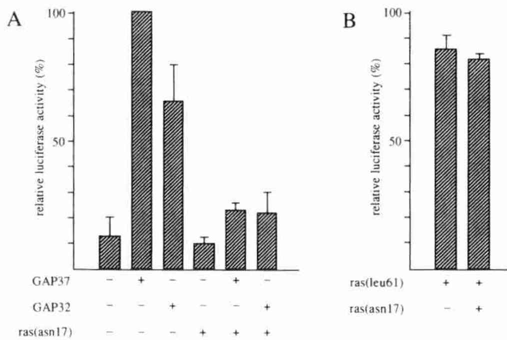

FIG. 3. Ras dependence of induction by GAP SH2-SH3 domains. A14 cells were transfected with 4 μg of *fos* luciferase in combination with 6 μg of CMV.GAP32 or CMV.GAP37 or 4 μg of RSV.Ras(Asn-17) where indicated (A). As a control for the specificity of inhibition by Ras(Asn-17), A14 cells were transfected with 4 μg of *fos* luciferase in combination with 2 μg of RSV.Ras(Leu-61) [ras(leu61)] with or without 4 μg of RSV.Ras(Asn-17) [ras(asn17)] (B). The total amount of DNA in all precipitates was adjusted to 14 μg with RSV.neo. After being transfected, cells were grown on Dulbecco's modified Eagle medium plus 0.5% fetal calf serum, and lysates were made after 48 h. Luciferase activity in these lysates is expressed relative to the activity found in cells transfected with GAP37. Error bars represent the standard deviation in each value ($n = 4$).

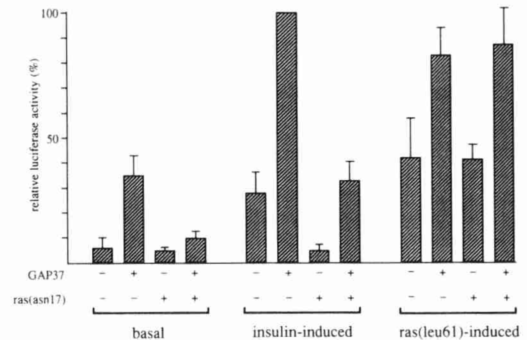

FIG. 4. Cooperation between active $p21^{ras}$ and GAP37. A14 cells were transfected with 4 μg of *fos* luciferase in combination with 6 μg of CMV.GAP37 (GAP37) in the presence or absence of 4 μg of RSV.Ras(Asn-17) [ras(asn17)] where indicated. Cells were left untreated (basal) or treated with insulin 24 h after transfection (insulin-induced), or 2 μg of Ras(Leu-61) was cotransfected [ras(leu61)-induced]. The amount of DNA in each precipitate was normalized to 16 μg with RSV.neo. Lysates were made 48 h after transfection, and luciferase activity was determined. Luciferase activity is expressed relative to the activity observed in cells transfected with GAP37 and stimulated with insulin. Error bars represent the standard deviation for each value ($n = 3$).

Ras dependence of gene induction by GAP32 and GAP37. We examined the Ras dependence of induction by GAP32 and GAP37 in A14 cells by using cotransfection with a dominant inhibitory mutant of $p21^{ras}$. This mutant, Ras(Asn-17), has been shown to specifically inhibit the induction of gene expression by normal $p21^{ras}$ (23), most likely through competition for the exchange factor of $p21^{ras}$ necessary for activation (13). Cotransfection of Ras(Asn-17) with either GAP32 or GAP37 resulted in an inhibition of *fos* promoter activity (Fig. 3). The residual *fos* promoter activity after the cotransfection of GAP SH2-SH3 domains and Ras(Asn-17) may suggest that induction of gene expression by GAP SH2-SH3 domains can also take place by a route independent of $p21^{ras}$. Also, in CHO9 cells, cotransfection of Ras(Asn-17) inhibited GAP32- and GAP37-induced activation of the *fos* promoter (data not shown). As a control for inhibition with Ras(Asn-17), we transfected this construct together with an activated mutant of $p21^{ras}$, Ras(Leu-61). The induction of *fos* promoter activity observed with Ras(Leu-61) was not affected by Ras(Asn-17) (Fig. 3), demonstrating that Ras(Asn-17) specifically inhibits activation of normal $p21^{ras}$.

Cooperation between GAP37 and activated $p21^{ras}$. The Ras dependence of gene induction by GAP32 and GAP37 suggests that the SH2-SH3 regions function upstream of $p21^{ras}$, since inhibition at the level of $p21^{ras}$ blocks their activity. However, another possible explanation for these findings could be that SH2-SH3 domains require another signal from $p21^{ras}$ in order to function. We therefore examined whether an increase in the level of active $p21^{ras}$ would lead to an increased activation of the *fos* promoter by the SH2-SH3 regions. In fibroblasts that overexpress the insulin receptor, like the A14 cells (approximately 3×10^5 receptors per cell), insulin stimulation leads to a rapid activation of $p21^{ras}$ (8). As a consequence, the luciferase expression is induced about sixfold in A14 cells after insulin treatment (Fig. 4), similar to the level obtained with GAP32 or GAP37 alone. When GAP37 and insulin were combined, we observed a marked increase in insulin-induced luciferase activity (Fig. 4), suggesting a cooperation between active $p21^{ras}$ and GAP37 in the induction of gene expression. However, this combined effect could be caused by a synergism between insulin and GAP SH2-SH3 domains at a different level. In order to exclude this possibility, we cotransfected GAP37 with an activated mutant of $p21^{ras}$, Ras(Leu-61). Transfection of Ras(Leu-61) with *fos* luciferase also gave rise to an induction of luciferase activity, and cotransfection of GAP37 resulted in an even stronger induction (Fig. 4), which shows that GAP37 actually cooperates with active $p21^{ras}$. The effect of GAP37 and insulin together could be inhibited with Ras(Asn-17) (Fig. 4), as was expected, since both insulin- and GAP37-induced gene expression is abolished by this mutant. The activity of Ras(Leu-61) is not affected by Ras(Asn-17) (Fig. 3 and 4); also, the combined effect of GAP37 and Ras(Leu-61) was unaltered in the presence of Ras(Asn-17) (Fig. 4). This excludes the possibility that the additional effect of GAP37 on Ras(Leu-61) induction is caused by the activation of normal endogenous $p21^{ras}$, since this effect would be inhibited by Ras(Asn-17). Therefore, it is very unlikely that GAP37 functions through activation of endogenous $p21^{ras}$ to activate the *fos* promoter.

DISCUSSION

We examined the role of $p21^{ras}$ GAP in the induction of gene expression by $p21^{ras}$. By using a transient expression assay with a *fos* luciferase reporter plasmid, we found that fragments of GAP that encode the SH2-SH3 regions but lack the catalytic domain (GAP32 and GAP37) can elevate the activity of the *fos* promoter to a level similar to that observed after transfection of activated $p21^{ras}$, whereas the full-length GAP construct was inactive. Assuming that the full-length

GAP construct was expressed properly, this result suggests that the catalytic domain of GAP inhibits the GAP effector function. This would be in agreement with the results obtained by Martin et al. (21). They reported that GAP32 and GAP37 can inhibit the carbachol-induced opening of potassium channels in isolated membranes from chicken atrial cells in a p21*ras*-independent manner, whereas full-length GAP can only do so in the presence of active p21*ras*. Apparently, activated p21*ras* overcomes the inhibitory effect of the catalytic domain of GAP, probably by binding to it.

In a manner analogous to that of the model proposed by Martin et al., the activation of *fos* promoter activity by GAP32 and GAP37 was expected to be independent of p21*ras*. However, the induction by GAP SH2-SH3 domains was found to be largely dependent on the activity of p21*ras*, since we could inhibit the activation of gene expression with a dominant inhibitory mutant of p21*ras*, Ras(Asn-17). The Ras dependency of gene induction by GAP SH2-SH3 domains suggests that GAP SH2-SH3 domains can cause the activation of normal endogenous p21*ras*, resulting in the induction of *fos* promoter activity. This would imply that the mechanism by which GAP SH2-SH3 domains activate the *fos* promoter is different from the mechanism by which GAP SH2-SH3 domains inhibit the carbachol-induced opening of potassium channels. An alternative explanation is that GAP SH2-SH3 domains trigger a p21*ras*-independent pathway similar to that observed for the inhibition of the opening of potassium channels, but that they need an additional signal from p21*ras* to activate the *fos* promoter. To discriminate between these two possibilities, we have introduced active p21*ras* into this system by two methods, i.e., stimulation with insulin in A14 cells or transfection with activated p21*ras*, Ras(Leu-61). If GAP SH2-SH3 domains cause an activation of normal endogenous p21*ras*, it is expected that high levels of introduced active p21*ras* would overrule the GAP32 and GAP37 effect. We observed a marked increase in *fos* promoter activity after the transfection of GAP37 in A14 cells treated with insulin. Also, GAP37 cooperated with Ras(Leu-61) in the activation of the *fos* promoter. The possibility that this additive effect is due to an extra increment in GTP-bound p21*ras* induced by GAP37 is unlikely, since insulin (which activates endogenous p21*ras*) does not enhance the effect of Ras(Leu-61) on *fos* promoter activity in A14 cells (data not shown). Moreover, the fact that the additional effect of GAP37 on Ras(Leu-61)-induced gene expression is insensitive to Ras(Asn-17) excludes the possibility that GAP SH2-SH3 domains function through the activation of endogenous p21*ras*. Therefore, our results strongly suggest that the GAP SH2-SH3 domains function separately from p21*ras* to induce *fos* promoter activity but require an additional signal coming from p21*ras*.

The cooperation of a GAP SH2-SH3-induced signal with a signal coming from p21*ras* implies that in unstimulated A14 cells, sufficient p21*ras* is in the GTP-bound state for downstream signaling, since GAP37 alone is able to activate the *fos* promoter. Sufficient indications are available for such a sustained effect of p21*ras* to be postulated. For instance, Downward et al. (11) reported constitutive exchange activity in permeabilized T lymphocytes and fibroblasts, suggesting that at least a fraction of p21*ras* is complexed to GTP for a certain time. Also, in normal fibroblasts or resting T cells, a small but significant (7 to 15%) fraction of p21*ras* is in the GTP-bound state (8, 11). Finally, the fact that inhibition of the opening of potassium channels by full-length GAP can be inhibited with Y13-259 indicates that active p21*ras* is present in isolated membranes (38).

Our results cannot distinguish whether the GAP SH2-SH3 domains function in a pathway completely independent of p21*ras* or the GAP SH2-SH3 domains mimic the full-length GAP-p21*ras* GTP complex, as postulated in the model of Martin et al. (21). However, the fact that full-length GAP is known to interact with p21*ras* and the proposed role of GAP as the effector molecule of p21*ras* suggest that GAP SH2-SH3 domains mimic this effector molecule and function immediately downstream from p21*ras*. Therefore, our current model is that p21*ras* generates two signals: one mediated by full-length GAP, which is mimicked by the GAP SH2-SH3 domains, and one whose nature is still elusive. A candidate protein that could mediate this unknown additional signal is the product of the neurofibromatosis type 1 gene, neurofibromin, which binds to the effector domain of p21*ras*. Alternatively, it could be that regions of GAP other than the SH2-SH3 domains are responsible for the additional signal and that the GAP SH2-SH3 domains might not fully mimic the full-length GAP-p21*ras* GTP complex. However, Zhang and colleagues concluded from their observations on the effect of full-length GAP on transformation of cells by activated p21*ras* that it is unlikely that GAP alone is the target for p21*ras* (39).

Assuming that GAP SH2-SH3 domains play a role downstream from p21*ras*, one would not expect an additional effect of GAP37 on mutant p21*ras* and insulin-induced expression of the *fos* luciferase reporter, since activated p21*ras* would unfold endogenous full-length GAP, making the presence of additional GAP SH2-SH3 domains unnecessary. It could be that the level of endogenous GAP which can interact with activated p21*ras* is not sufficient for the full effect or that competition for binding to active p21*ras* by other proteins displaces GAP from p21*ras*. In fact, neurofibromin has been shown to have an affinity for GTP-bound p21*ras* 30-fold higher than that of GAP (4). If indeed the amount of full-length GAP is limiting with respect to the induction of gene expression, one would expect that the introduction of full-length GAP would potentiate *fos* promoter activity under circumstances in which excess active p21*ras* is available. Such an effect was observed several times in the case of Ras(Leu-61)-induced gene expression (up to fivefold extra induction [22a]). For insulin-induced gene expression, we did not observe such a cooperativity with full-length GAP. This may be due to additional negative effects of the catalytic domain of GAP on normal p21*ras* (39).

In addition to GAP, a large number of proteins which contain SH2-SH3 domains have been identified, all of which interact with tyrosine-phosphorylated proteins. For instance, the *crk* oncogene is analogous to GAP37 in that it contains merely SH2-SH3 domains (22). This raises the question of whether the observed effects of the GAP SH2-SH3 domains are specific. From the work of Kashishian et al., it is clear that the SH2 domains of phosphatidylinositol-3 kinase, phospholipase C-γ, and GAP bind to different phosphotyrosines on the platelet-derived growth factor receptor, indicating selectivity in the binding of SH2-containing proteins (17). Furthermore, we observe only a very minor induction of *fos* promoter activity with the *crk* SH2-SH3 domains (unpublished observation). We therefore conclude that the observed effects of the GAP SH2-SH3 domains reflect specific interactions. It is attractive to speculate that the GAP SH2-SH3 domains interact specifically with a phosphotyrosine-containing protein which serves as the downstream target of the full-length GAP p21*ras*-GTP complex. Candidate targets are the GAP-associated tyrosine phosphoproteins of 62 and 190 kDa. In this respect, the

recent findings that the 62-kDa GAP-associated protein might function as a heterogeneous nuclear RNA binding protein (37) and the 190-kDa GAP-associated protein might function as a transcriptional repressor (32) are provocative.

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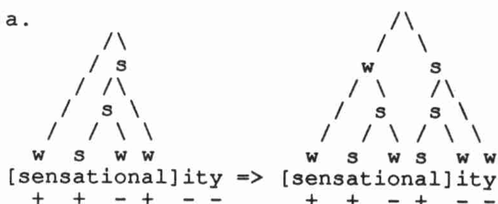
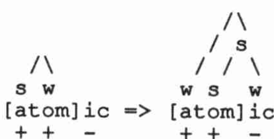
(201) a.

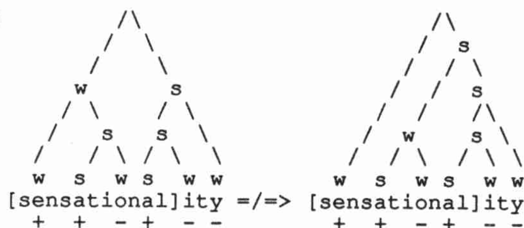


b.



In contrast, existing structure cannot be reorganized at the supra-foot level (202a):

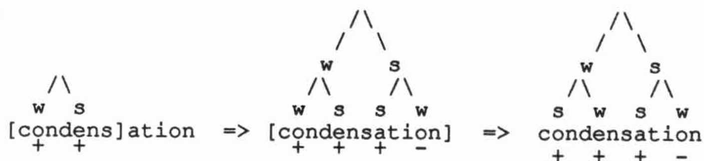
(202)



Accordingly, Kiparsky proposes to restrict metrical structure assignment so that it cannot destroy supra-foot structure.

Second, the uniqueness of the 4-3-1 contour in the *sensationality* class can be explained by the Rhythm Rule (125), reversing the word-internal prominence relations in all other classes, and producing 3-4-1 contours:

(203)



The Rhythm Rule extends to words with different stress sequences such as *ìnstrumèntality* and *àrtificiálity*. Since these derivations run precisely as those of underived words, we refer to (126) by way of illustration. Crucially its rhythmic condition singles out *sensàtionálity* as the only type which preserves its embedded prominence relations.

Third, destressing in *explanation* is fed by the Rhythm Rule essentially as in (203). And as required, it also bleeds destressing of the initial prefixes in *condensation* (203), as this becomes strong.

However, phrasal applications of the Rhythm Rule are in their turn bled by prefix destressing: **èxact chánge*, **bènign túmor*, cf. section 3.1, by the removal of [+stress] from non-prominent vowels. This classical ordering paradox is resolved by assuming the Rhythm Rule to be *cyclic*: word-level prefix destressing is sandwiched in between its word-internal and phrasal applications. In the framework of those days this implied cyclicity of metrical structure assignment as well, since no non-cyclic (word-level) rules were ordered before the block of cyclic rules.

6.6 Hayes (1981)

Hayes (1981) attacks the problems which are posed to Kiparskyan analysis when the feature [+stress] is eliminated in favor of foot structure. A purely metrical foot-based

CHAPTER 7

General discussion

The role of p21ras in insulin signal transduction.

Ras oncogenes belong to the most potent and prominent oncogenes encountered in human tumors. To understand the mechanism by which oncogenic p21ras can transform cells, researchers initially introduced oncogenic forms of p21ras in a variety of cells. However, using this approach, it was hard to distinguish direct effects of p21ras from effects that are a more general consequence of transformation. This has made investigators turn to the function of p21ras in its normal context. This thesis describes data that have been obtained concerning the role of normal p21ras in insulin signaling.

The protein products of the ras genes are small proteins of ~21 kDa that bind guanine nucleotides with high affinity. Normal ras proteins have an intrinsic GTPase activity and are located at the inner side of the plasma membrane. These characteristics have led to the hypothesis that ras proteins can function as molecular switches, that, in analogy with known G proteins, cycle between an active GTP-bound conformation, and an inactive GDP-bound conformation (see fig.1). Interestingly, most transforming mutations in the ras proto-oncogenes preclude cycling of p21ras through a loss of GTPase activity. This results in a protein that will not hydrolyze GTP and is therefore locked in its active conformation. The general idea behind the transforming potential of these mutants is that the continuous presence of active p21ras causes a never ceasing signal output from p21ras and, consequently, a cell that will continue to divide (see fig.1.). These transforming mutations have been found in a large variety of human tumors, indicating that ras proteins play an important role in the formation of these tumors.

When the work described in this thesis was first initiated, it was generally accepted that p21ras plays an important role in the regulation of cellular growth and differentiation. First of all, because introduction of oncogenic forms of p21ras had been found to cause aberrant cellular growth or differentiation, depending on the cell type used. Second, neutralizing anti-ras antibodies, as well as inhibitory mutants of p21ras, can block mitogenesis or differentiation induced by various growth or differentiation factors. How-

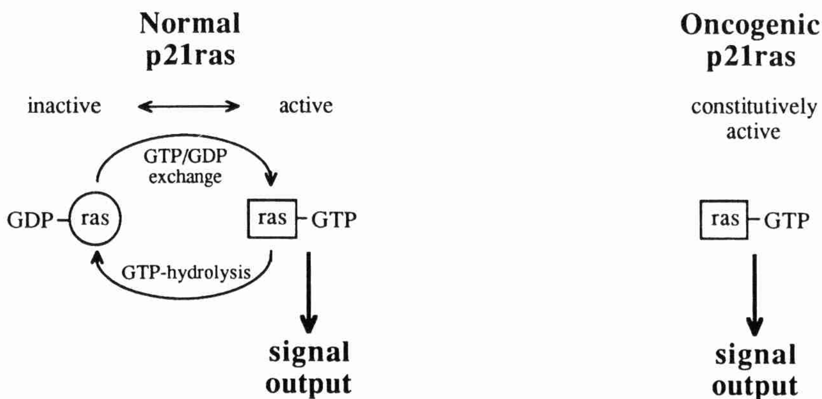


Figure 1. Ras cycling. Normal p21ras cycles between an inactive GDP-bound conformation and an active GTP-bound conformation, the latter giving rise to transduction of a signal. Activation of normal p21ras occurs through exchange of GTP for GDP, whereas inactivation requires hydrolysis of GTP bound to p21ras. Oncogenic p21ras can no longer hydrolyze GTP and is therefore constitutively activated.

ever, at that time it was still unclear whether normal p21ras is actually involved in the signaling pathways that are triggered by these factors, or if (basal) cycling of p21ras is a general requirement for cellular growth and differentiation. In other words, is p21ras activated upon stimulation with these factors, or is the activity of p21ras required in a separate synergizing pathway?

We therefore set out to investigate the effect of several growth factors on the activation state of p21ras (the actual GTP/GDP balance on p21ras in a living cell). Our initial studies (chapter 2) showed that insulin could cause a very dramatic and rapid activation of p21ras in cells that express sufficient insulin receptors. This activation of p21ras is crucial to stimulation of c-fos and collagenase expression by insulin (chapter 3), which indicates that p21ras plays a pivotal role in signal transduction from the insulin receptor.

As for the mechanism of ras activation by insulin, we found that this signaling pathway is sensitive to phenylarsine oxide (PAO), a putative inhibitor of tyrosine phosphatase activity (chapter 4). This inhibition suggests that an intermediate of this pathway could be a tyrosine phosphatase. Remarkably, glucose uptake induced by insulin is also inhibited by PAO, and might be part of the same pathway. In addition, although protein kinase C (PKC) has been proposed to be an important mediator of insulin action by some investigators (but contradicted by others), we can rule out the possibility that (phorbol ester-sensitive) PKC is involved in the pathway from the insulin receptor to p21ras, since depletion of PKC by prolonged treatment with phorbol esters does not prevent activation of p21ras by insulin (chapter 4). Finally, activation of p21ras by insulin (and epidermal growth factor) is at least in part mediated by enhanced exchange of guanine nucleotides on p21ras (chapter 5). This suggests that insulin can stimulate the activity of a guanine nucleotide releasing protein that specifically enhances the dissociation of GDP from p21ras, enabling GTP to bind.

We also investigated the mechanism by which p21ras can transmit the signal once it has been activated. One potential target of p21ras is p120GAP, which functions to increase hydrolysis of GTP on p21ras. Our studies with deletion mutants of p120GAP that lack the catalytic domain required for interaction with p21ras showed that the SH2/SH3 domains of p120GAP alone can stimulate fos-expression (chapter 6). SH2/SH3 domains are known to be involved in interaction with other proteins and could, through specific association with a downstream target, transmit a signal from p21ras (see fig.2.). This finding (and others) suggest a dual role for p120GAP in ras signaling, that is; termination of the signal (through stimulation of inactivation of

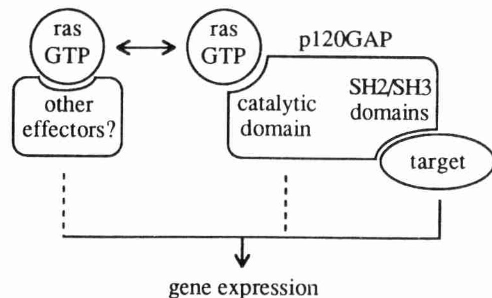


Figure 2. p120GAP as effector of p21ras. p120GAP interacts with p21ras through its catalytic domain and stimulates inactivation of p21ras by activating the GTPase activity of p21ras. This interaction is thought to enable the SH2/SH3 domains of p120GAP to interact with possible downstream targets of p21ras, contributing to signal transduction which eventually leads to stimulation of gene expression.

p21ras) and transmission of the signal. Nevertheless, other signals coming from p21ras seem to be required for activation of gene expression. Possibly domains of p120GAP distinct from the SH2/SH3 domains, or alternatively, other proteins that bind p21ras (such as neurofibromin) can transmit these additional signals (see fig.2.).

Recently, the activation of p21ras by insulin was also shown to be responsible for activation of the extracellular signal-regulated kinases, erk1 and erk2. These kinases are part of a kinase cascade, in which raf-1 kinase and a recently identified kinase (MEK-1) play a role as well (see fig.3.). In addition, upstream of p21ras several potential intermediates have been identified that contain SH2/SH3 domains, such as shc and grb2 (see fig.3.). These proteins have no enzymatic activity themselves and are thought to function through specific interaction with other proteins. For instance, SH2 domains were shown to specifically bind tyrosine phosphorylated proteins. Therefore, signal transduction from the insulin receptor to p21ras seems to involve a number of specific protein interactions, that are partly governed by the activity of tyrosine kinases as well as tyrosine phosphatases. The exact mechanism by which these proteins transmit the signal from the insulin receptor to p21ras is

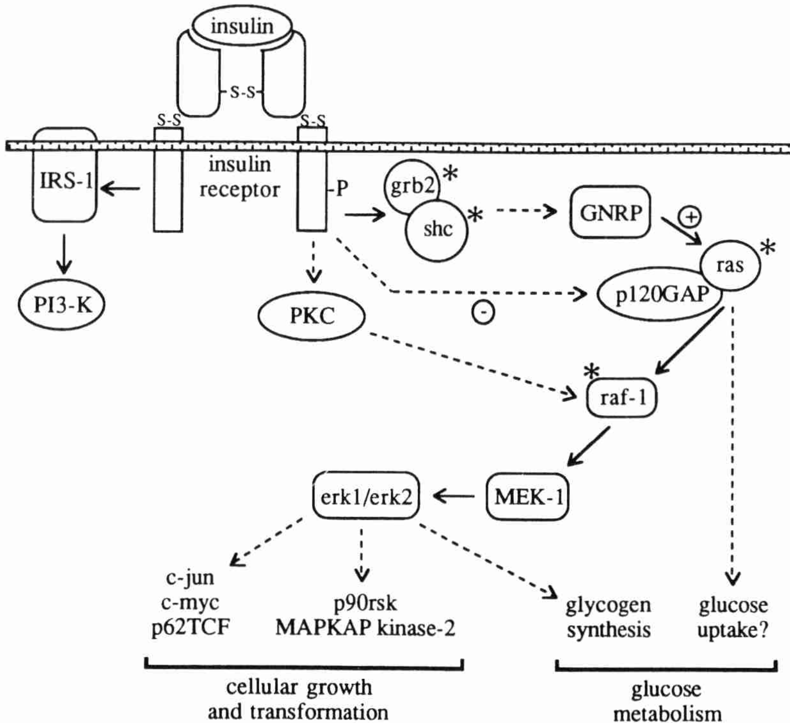


Figure 3. Signaling pathway initiated by insulin. Insulin-induced activation of p21ras is mediated by stimulation of the guanine nucleotide releasing protein specific for p21ras. This stimulation could be mediated by shc and grb2, through SH2/phosphotyrosine interactions and is likely to involve the action of tyrosine kinases and phosphatases. However, regulation of the activity of p120GAP by the insulin receptor could also play a role in activation of p21ras. Activation of p21ras results in activation of raf-1 kinase and consequently stimulation of the activity of erk1 and erk2. These pathways possibly control cellular growth, as well as glucose metabolism. Asterisks indicated proto-oncogene products.

presently under investigation and preliminary data point to a link with the guanine nucleotide releasing protein for p21ras (see fig.3.). Thus, insulin signaling via p21ras involves a number of proto-oncogene products, namely shc, grb2 and raf-1 kinase (and of course p21ras itself). This signaling route has been conserved in many organisms and plays an important role in the control of cellular growth and development (see also chapter 1). The fact that this signaling pathway contains many proto-oncogene products clearly indicates that the unraveling of this pathway will be important for our understanding of the development of cancer.

We and others showed that many different growth factors can trigger activation of p21ras (chapter 5 and see chapter 1 for an overview). Therefore, p21ras appears to be a very common intermediate for growth factor signaling in control of cellular growth and differentiation. Nevertheless, insulin is one of the more potent agents among all factors tested for activation of p21ras until now. Remarkably, compared to growth factors such as the epidermal growth factor and the platelet-derived growth factor, insulin is not a very potent stimulatory agent with respect to cellular growth. Thus, the efficiency of ras activation does apparently not determine the extent of growth stimulation.

Insulin is better known for its important role in regulation of the glucose and lipid metabolism. Release of insulin in the blood stream will result in increased glycogen and lipid synthesis, as well as a rapid increase in glucose uptake by peripheral tissues. Possibly, these effects of insulin are also a consequence of activation of p21ras. As mentioned above, both the glucose uptake, as well as ras activation are sensitive to PAO. Moreover, glucose uptake is dramatically increased in cells expressing oncogenic p21ras, suggesting a link between p21ras and glucose transport. In addition, extracellular signal-regulated kinases may play a role in the regulation of glycogen synthesis as well. This would provide us with the intriguing possibility that this signaling pathway could be affected in diabetes mellitus type II patients. The cause of this type of diabetes has until now largely escaped identification. With the elucidation of the ras pathway, we are enabled to study malfunctioning of this route in diabetes. In particular, the possible effect of p21ras on the glucose metabolism will have to be investigated in more detail.

The finding that several growth factors can activate p21ras, indicates the importance of p21ras in cell signaling. The function of p21ras is not only required for regulation of cellular growth, but appears to be crucial for the regulation of differentiation, immune response and cellular metabolism as well. Thus far, defects in p21ras have been observed in many types of cancer. However, it would not surprise us if flaws in the function of p21ras or other constituents of the ras pathway are responsible for other types of disease, with diabetes type II as the prime candidate. In any case, the diverse function of p21ras will continue to surprise us in the coming years.

Summary

Samenvatting

Sintesi dei contenuti

Summary

Cell division (mitosis) is a tightly regulated process, involving many factors. A normal cell will not divide spontaneously, but requires a stimulus (growth factor or hormone) to enter mitosis and complete the cell division. The binding of a growth factor to its receptor, which is present on the cell-membrane, triggers several signaling pathways, the combination of which elicits the mitogenic response. Normally, these signals are of a transient nature and the cell will become quiescent after division. However, aberrant (constitutively activated) forms of the proteins that are involved in this signaling may result in constitutive signals, and consequently, a cell that divides continuously, independent of the presence of growth factors. This is what happens in many forms of cancer, where mutations in proto-oncogenes have resulted in constitutively activated proteins that partake in the mitogenic signaling pathways. One of the intermediates in mitogenic signaling is p21ras. Ras proteins function as molecular switches, cycling between an active and an inactive conformation. Mutations in the ras gene that result in a protein that is locked in the active conformation are often found in human tumors, consistent with its positive role in mitogenic signaling. This thesis addresses the function of ras proteins, in particular the role these proteins play in the normal signal transduction.

The first issue we addressed was to find growth factors that could drive p21ras in its active conformation. A survey of several growth factors demonstrated that both insulin (chapter 2) and epidermal growth factor (EGF) (chapter 5) can cause a rapid and dramatic activation of p21ras. This suggested that p21ras plays an important role in signaling from the insulin- and EGF receptor. Indeed, the activation of p21ras by insulin is essential for stimulation of expression of the c-fos and collagenase genes (chapter 3). This prompted us to investigate the mechanism by which insulin can cause activation of p21ras.

The activation of p21ras by insulin is not affected by depletion of phorbol ester-sensitive protein kinase C (PKC) from the cells and, contrary to T cells, stimulation of PKC in fibroblasts does not result in activation of p21ras (chapter 4). From these findings we concluded that (phorbol ester-sensitive) PKC is not involved in insulin-induced activation of p21ras. Furthermore, activation of p21ras by insulin is blocked by phenylarsine oxide (an inhibitor of tyrosine phosphatases), suggesting that one of the intermediates in the signaling pathway from the insulin receptor towards p21ras is a tyrosine phosphatase (chapter 4). This provides us with a useful tool to study the signaling pathway between insulin and p21ras.

The mechanism of ras activation in response to growth factors can theoretically involve an increased rate of activation (by an increase in nucleotide exchange), or alternatively a decreased rate of inactivation (through a decrease in GTP-hydrolysis). To investigate which of these mechanisms is employed by insulin and the EGF we measured the rate of nucleotide exchange on p21ras in the absence and presence of growth factor. Stimulation of cells with insulin or EGF resulted in an increased nucleotide exchange on p21ras, possibly by activation of a GNRP (guanine nucleotide releasing protein) (chapter 5).

However, the mechanism by which p21ras activates its downstream targets is still unknown.

One possible target of p21ras is p120GAP. This protein is thought to function both as a negative regulator and a downstream target of p21ras. Therefore, we tested p120GAP deletion mutants that lack the domain involved in inactivation of p21ras in a transient expression assay. These deletion mutants that encode essentially only the SH2/SH3 domains of p120GAP induced c-fos expression in a ras-dependent fashion. Moreover, these mutants enhanced the effect of insulin and active p21ras on c-fos expression, but the combined effect of the SH2/SH3 domains and active p21ras was not affected by inhibition of the function of normal p21ras (chapter 6). These findings suggest that the SH2/SH3 domains of p120GAP function to transmit signals from p21ras, but that additional signals coming from p21ras are required for stimulation of gene expression.

Taken together, the results presented in this thesis show that p21ras is pivotal to insulin signaling, and that activation of p21ras can occur through stimulation of nucleotide exchange. These findings roughly define an important signaling pathway. Elucidation of this pathway will help us to understand the function of p21ras and its role in cancer.

Samenvatting

Celdeling (mitose) is een nauw gereguleerd proces, waarbij vele faktoren betrokken zijn. Een normale cel zal niet spontaan gaan delen, maar is afhankelijk van een stimulus (groeifaktor of hormoon) om tot mitose over te gaan en deze ook te voltooien. De binding van een groeifaktor aan zijn receptor, die aanwezig is op de celmembraan, resulteert in de aktivatie van verschillende signaleringsroutes, die tezamen tot de mitogene response zullen leiden. Normaliter zijn deze signalen van korte duur en zal de cel na deling weer "tot rust" komen. Echter, afwijkingen in de eiwitten (t.g.v. mutaties) die betrokken zijn bij deze signalering kunnen een constitutief signal tot gevolg hebben, met als resultaat een voortdurend delende cel, onafhankelijk van de aanwezigheid van groeifaktoren. Dit is het geval in vele vormen van kanker, waar mutaties in proto-oncogenen een constitutief aktief eiwit tot gevolg hebben dat betrokken is bij de mitogene signaleringsroutes. Eén van de intermediairen in mitogene signalering is p21ras. Ras eiwitten funktioneren als molekulaire schakelaars, die zich afwisselend in de aktieve dan wel inaktieve vorm bevinden. Mutaties in het ras-gen die resulteren in een eiwit dat geblokkeerd is in zijn aktieve conformatie worden vaak aangetroffen in humane tumoren, consistent met hun positieve rol in mitogene signalering. Dit proefschrift behandelt de funktie van de ras eiwitten, in het bijzonder de rol die deze eiwitten spelen in de normale signaaltransductie.

Allereerst is gezocht naar groeifaktoren die de aktivatie van p21ras konden bewerkstelligen. Een studie met verscheidene groeifaktoren toonde aan dat insuline (hoofdstuk 2) en EGF (epidermal growth factor) (hoofdstuk 5) zeer snel en efficiënt ras konden aktiveren. Dit suggereerde dat p21ras een belangrijke rol speelt bij de overdracht van signalen van de insuline- en EGF-receptor. Het feit dat aktivatie van p21ras essentieël bleek te zijn voor stimulering van c-fos en collagenase expressie door insuline, bevestigde dit (hoofdstuk 3). Daarom besloten wij de aktivatie van p21ras door insuline nader te onderzoeken.

Remming van proteine kinase C (PKC) in fibroblasten door langdurige behandeling met phorbol-esters had geen effekt op ras aktivering door insuline. Ook had stimulering van PKC in fibroblasten geen aktivatie van p21ras tot gevolg, in tegenstelling tot wat er gerapporteerd was voor T cellen (hoofdstuk 4). Hieruit concludeerden wij dat (phorbol ester-gevoelige) PKC niet betrokken is bij insuline-geïnduceerde activatie van p21ras. Daarnaast was de aktivering van p21ras te blokkeren met phenylarseen oxide (een remmer van tyrosine-phosphatasen), wat suggereert dat één van de intermediairen in de signaleringsroute van de insuline-receptor naar p21ras mogelijk een tyrosine-phosphatase is (hoofdstuk 4).

Aktivatie van p21ras door een groei faktor kan theoretisch via twee verschillende mechanismen verlopen. Ofwel een toename in de snelheid waarmee p21ras geaktiveerd wordt (door een toename in de uitwisselingssnelheid van nucleotiden), ofwel door een afname in de snelheid waarmee p21ras geïnaktiveerd wordt (door een afname in GTP-hydrolyse). Om te onderzoeken welk mechanisme van toepassing is op aktivatie van p21ras door insuline en EGF, hebben we de uitwisselingssnelheid van nucleotiden op p21ras gemeten in aan- of afwezigheid van de groeifaktor in kwestie. Stimulatie met insuline of EGF leidde tot een versnelde uitwisseling van nucleo-

tiden op p21ras, mogelijk door aktivatie van een exchange faktor (in staat de dissociatie van nucleotiden te stimuleren) (hoofdstuk 5).

Het is nog onduidelijk hoe aktief p21ras aktivatie van verdere signalering bewerkstelligt. Eén mogelijke target van p21ras is p120GAP. Dit eiwit funktioneert in de eerste plaats als negatieve regulator van p21ras, maar wordt verondersteld tegelijk als transmitter van signalen te funktioneren. Daarom hebben wij gedeleteerde vormen van p120GAP, die het domein betrokken bij inaktivatie van p21ras missen, getest op stimulatie van gen-expressie. De deletie-mutanten, die de SH2/SH3 domeinen van p120GAP bevatten, zijn in staat c-fos-expressie te stimuleren op ras-afhankelijke wijze. Deze mutanten zijn tevens in staat de inductie van c-fos-expressie door insuline en aktief p21ras verder te stimuleren. Stimulering van c-fos-expressie door aktief p21ras en de p120GAP deletie mutanten samen is niet te remmen door blokkering van de funktie van normaal p21ras (hoofdstuk 6). Dit suggereert dat p120GAP als signaaloverdrager voor p21ras kan funktioneren, maar dat de SH2/SH3 domeinen op zich niet voldoende zijn voor stimulatie van gen-expressie.

De resultaten beschreven in dit proefschrift waarborgen een aantal conclusies. Ten eerste, p21ras speelt een belangrijke rol in de overdracht van signalen van verschillende groeifaktor-receptoren, waaronder de insuline- en EGF-receptor. Ten tweede, aktivatie van p21ras door verschillende groeifaktoren is mogelijk door stimulering van nucleotide uitwisseling op p21ras. Ten derde, p120GAP lijkt betrokken te zijn bij de overdracht van signalen van p21ras. Deze resultaten gunnen ons een eerste blik op het mechanisme van signalering via p21ras. Verdere ontrafeling van deze signaleringsroute zal ons helpen de funktie van p21ras en de rol die ras eiwitten spelen in het ontstaan van kanker beter te begrijpen.

Sintesi dei contenuti

La scissione delle cellule (mitosi) è un processo saldamente regolato, che coinvolge diversi fattori. Una cellula normale non si scinderebbe spontaneamente, ma richiederebbe uno stimolo (un ormone o fattore di crescita) per entrare in mitosi e completare la scissione. Il legame di un fattore di crescita al proprio recettore, che è sito sulla membrana cellulare, innesca diversi segnali, la combinazione dei quali causa la risposta mitogenica. Normalmente questi segnali sono di natura transitoria e la cellula diverrebbe quiescente dopo la scissione. Tuttavia, forme abberranti delle proteine (attivate costitutivamente) che sono implicate in questo stimolo, possono risolversi in segnali costitutivi, in seguito ai quali la cellula inizierebbe a dividersi ininterrottamente, indipendentemente dalla presenza di fattori di crescita. Questo è ciò che si verifica in molte forme di cancro, in cui mutazioni nei geni pre-tumorali hanno dato origine a proteine attivate costitutivamente, che prendono parte ai segnali di innesco della mitosi. Uno di questi intermediari nella segnalazione mitogenica è p21ras. Le proteine ras funzionano come interruttori molecolari, oscillando tra una comformazione attiva ed una inattiva. Mutazioni nel gene ras che si osservano in una proteina che è bloccata nella sua conformazione attiva, vengono spesso riscontrate in tumori umani, compatibili con il loro ruolo positivo nel segnale mitogenico. Questa tesi tratta la funzione delle proteine ras, in particolare il ruolo che le stesse giocano nella normale trasmissione del segnale.

Come prima cosa abbiamo cercato di trovare dei fattori di crescita che fossero in grado di condurre p21ras nella propria conformazione attiva. Lo studio di diversi fattori di crescita, ha dimostrato che entrambe, insulina (Capitolo 2) ed EGF (epidermal growth factor) (Capitolo 5), possono causare una rapida ed efficiente attivazione di p21ras. Questo ci ha suggerito che p21ras dovesse giocare un ruolo importante nella segnalazione di insulina e di EGF. Di fatto l'attivazione di p21ras provocata da insulina, appare essenziale per lo stimolo dell'espressione dei geni c-fos e collagenase (Capitolo 3). Questo ci ha indotto a ricercare il meccanismo con il quale l'insulina può causare l'attivazione di p21ras.

L'attivazione di p21ras per mezzo di insulina non è influenzata dall'inibizione della proteina kinasi C (PKC) sensibile a lungo trattamento con estere phorbol (12-O-tetradecanoylphorbol-13-acetate) nella cellula e, al contrario dei linfociti T, lo stimolo della PKC in fibroblasti non porta all'attivazione di p21ras (Capitolo 4). A partire da questi risultati abbiamo concluso che la PKC (sensibile a estere phorbol) non è coinvolta nell'attivazione di p21ras indotta da insulina. Inoltre, l'attivazione di p21ras tramite insulina, è bloccata da fenilarsenossido (un inibitore della tyrosine fosfatasi), il che suggerisce che uno degli intermediari nel percorso dal recettore dell'insulina verso p21ras può essere una tyrosine fosfatasi (Capitolo 4). Tutto questo ci fornisce un utile strumento per studiare il percorso tra insulina e p21ras.

Il meccanismo di attivazione di p21ras in risposta a fattori di crescita, può teoricamente implicare un aumento della velocità di attivazione (per mezzo di un incremento nello scambio di nucleotide), o in alternativa, una diminuzione della velocità di inattivazione (attraverso un decremento dell'idrolisi del GTP). Per scoprire quale di questi meccanismi viene impiegato da insulina e EGF, abbiamo misurato la velocità di

scambio di nucleotide su p21ras, sia in presenza che in assenza del fattore di crescita. Lo stimolo di cellule con insulina o EGF porta ad un aumentato scambio di nucleotide su p21ras, possibilmente tramite l'attivazione di una GNRP (guanine nucleotide releasing protein) (Capitolo 5). Così, l'attivazione di p21ras che si ottiene tramite stimolo di scambio di nucleotide, sopravviene su stimolo con diversi fattori di crescita, e provoca un incremento dell'espressione dei diversi geni.

Comunque, il meccanismo attraverso il quale p21ras attiva i suoi bersagli a valle è ancora sconosciuto. Un possibile bersaglio di p21ras è p120GAP. Questa proteina si pensa funzioni sia come regolatrice negativa di p21ras, sia come bersaglio a valle di p21ras. Per questo, abbiamo esaminato mutanti per delezione di p120GAP che portano alla mancanza del dominio coinvolto nella inattivazione di p21ras, per lo stimolo dell'espressione dei geni. Queste mutanti per delezione, che trascrivono essenzialmente solo per i domini SH2/SH3 di p120GAP, sono capaci di stimolare l'espressione di c-fos in un modo dipendente da p21ras. Inoltre, questi mutanti aumentano l'effetto dell'insulina e p21ras attiva sull'espressione di c-fos, ma l'effetto combinato dei domini SH2/SH3 e di p21ras attiva non è influenzato dall'inibizione della funzione di p21ras normale (Capitolo 6). Questi risultati ci suggeriscono che i domini SH2/SH3 di p120GAP servono per trasmettere segnali da p21ras, ma che per avere lo stimolo dell'espressione genica sono necessari anche ulteriori segnali provenienti da p21ras.

Considerati nel loro insieme, i risultati presentati in questa tesi, ci mostrano che p21ras è vitale nella segnalazione di insulina e che p21ras si può attivare attraverso stimolo di scambio di nucleotide. Queste conclusioni definiscono approssimativamente una importante traccia. Delucidazioni di questa traccia ci saranno d'aiuto per cercare di comprendere la funzione di p21ras ed il suo ruolo nell'insorgere del cancro.

Nawoord

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Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 22 december 1964 te Delft. In 1983 behaalde hij het eindexamen ongedeeld VWO aan het Erasmus College te Zoetermeer. Daarna studeerde hij Scheikunde aan de Rijksuniversiteit te Leiden. In januari 1989 behaalde hij het doctoraal examen, met als hoofdvak Medische biochemie (Dr(s). R. Offringa en Prof. dr. A.J. van der Eb, Sylvius Laboratorium, Rijksuniversiteit Leiden) en als bijvak Moleculaire biologie (Dr. J. Papkoff, Syntex Research, Palo Alto, California). Vanaf maart 1989 tot maart 1993 werkte hij aan het onderzoek beschreven in dit proefschrift. Gedurende de eerste twee jaar bij de vakgroep Medische biochemie op het Sylvius laboratorium aan de Rijksuniversiteit te Leiden, o.l.v. Prof. dr. A.J. van der Eb. Vervolgens was hij werkzaam bij de Rijksuniversiteit te Utrecht bij de vakgroep Fysiologische chemie, o.l.v. Prof. dr. J.L. Bos. Na zijn promotie zal hij onderzoek gaan verrichten naar de rol van het retinoblastoma eiwit in de regulatie van de cel cyclus, allereerst op het Whitehead institute (Cambridge, Massachusetts), o.l.v. Prof. dr. R.A. Weinberg, en daarna op het Nederlands Kanker instituut te Amsterdam o.l.v. Dr. R. Bernards. Hiertoe wordt hij financieël ondersteund door een KWF-fellowship.

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STELLINGEN

1. Aktivatie van p21ras is in korte tijd eigenlijk heel "gewoontjes" geworden, maar de gevolgen zijn nog altijd heel bijzonder.

   Dit proefschrift.

2. Als Mulder en Morris daadwerkelijk gecontroleerd hadden op welke plek GTP en GDP te detecteren zijn op de dunnelaag plaat, dan waren zij zeker niet tot de conclusie gekomen dat TGF-β in staat is p21ras te aktiveren in epitheliale cellen.

   Mulder and Morris (1992) J. Biol. Chem. **267**: 5029-5031.

3. Als Moscat en zijn medewerkers in eerste instantie rapporteren dat "microinjection of transforming ras p21 promotes a rapid and dramatic increase in phosphocholine levels which is detectable by 5 to 10 min and maximal by 1 h" en dezelfde groep laat in een volgende publicatie een identieke proef zien waarin phosphocholine release nog niet te detecteren is 1 uur na microinjectie, dan moeten zij daar wel een verklaring voor geven.

   Garcia de Herreros *et al.* (1991) J. Biol. Chem. **266**: 6825-6829.
   Diaz-Meco *et al.* (1992) Mol. Cell. Biol. **12**: 302-308.

4. Smeal *et al.* hebben hun observatie, dat co-expressie van ras(Asn17) de inductie van c-jun fosforylering en aktivering door ras(Leu61) kan remmen ten onrechte geïnterpreteerd als zou het hier een specifieke remming van de funktie van ras(Leu61) betreffen.

   Smeal *et al.* (1992) Mol. Cell. Biol. **12**: 3507-3513.

5. Goodrich en Lee spreken ten onrechte van een antagonisme, aangezien zij slechts bewijzen dat c-myc de funktie van Rb negatief kan beïnvloeden.

   Goodrich en Lee (1992) Nature **360**: 177-179.

6. Genetisch bewijs dat een signaleringsroute de funktie van een zeker eiwit nodig heeft om effektief te kunnen signaleren, wordt soms ten onrechte geïnterpreteerd als zou dit eiwit ook deel uit moeten maken van deze route.

7. Al is de transient nog zo snel, de controle is en blijft een hel.

8. Het feit dat de faculteiten scheikunde en biologie aan verschillende nederlandse universiteiten stages buiten de faculteit ontmoedigen, zal in de toekomst zijn weerslag hebben op de kwaliteit van het medisch biologisch onderzoek in Nederland.

9. Gezien het scherpe contrast tussen de voetbalprestaties van de Nederlanders in het roodzwarte tricot in vergelijking met de "prestaties" geleverd in het oranje shirt, zou de KNVB moeten overwegen de kleding van het Nederlands Elftal aan te passen.

11. Toepassing van het principe van "coöperatieve binding" op wetenschappelijke onderzoeksgroepen zal in de regel leiden tot verhoogde expressie, maar tevens tot competitie voor bepaalde sites.

12. Wetenschap is kunst, je moet je werk tenslotte ook verkopen.

René Medema

Leiden, februari 1993.