MOLECULAR AND CELLULAR BIOLOGY, Jan. 1993, p. 155–162
0270-7306/93/010155-08$02.00/0

Vol. 13, No. 1

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

RENÉ H. MEDEMA,[1] ALIDA M. M. DE VRIES-SMITS,[1] GERARD C. M. VAN DER ZON,[2] J. ANTONIE MAASSEN,[2] AND JOHANNES L. BOS[1]*

Laboratory for Physiological Chemistry, University of Utrecht, Vondellaan 24A, 3521 GG Utrecht,[1] and Laboratory of Protein Synthesis and Hormone Regulation, Sylvius Laboratory, 2333 AL Leiden,[2] The Netherlands

Received 21 August 1992/Returned for modification 21 September 1992/Accepted 23 October 1992

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

* Corresponding author.

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

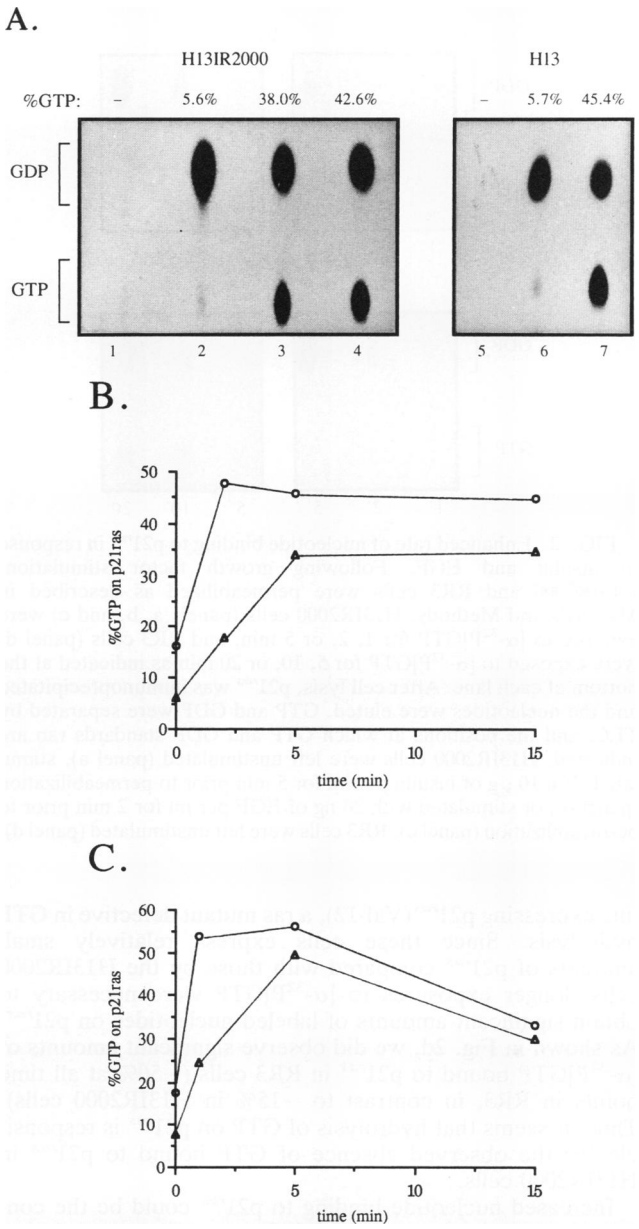

FIG. 1. Activation of p21^{ras} by insulin and EGF in fibroblasts. A14, Rat-1, H13, and H13IR2000 cells were labeled with $^{32}P_i$ as described in Materials and Methods. Cells were lysed, and p21^{ras} was immunoprecipitated. Nucleotides bound to p21^{ras} were eluted and separated by TLC. Following 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}.

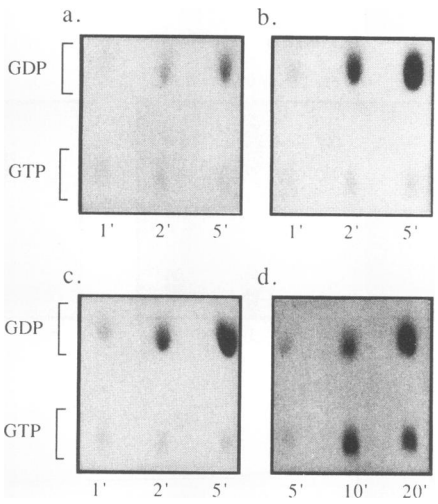

FIG. 2. Enhanced rate of nucleotide binding to p21^{ras} in response to insulin and EGF. Following growth factor stimulation, H13IR2000 and RR3 cells were permeabilized as described in Materials and Methods. H13IR2000 cells (panels a, b, and c) were exposed to [α-^{32}P]GTP for 1, 2, or 5 min, and RR3 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-

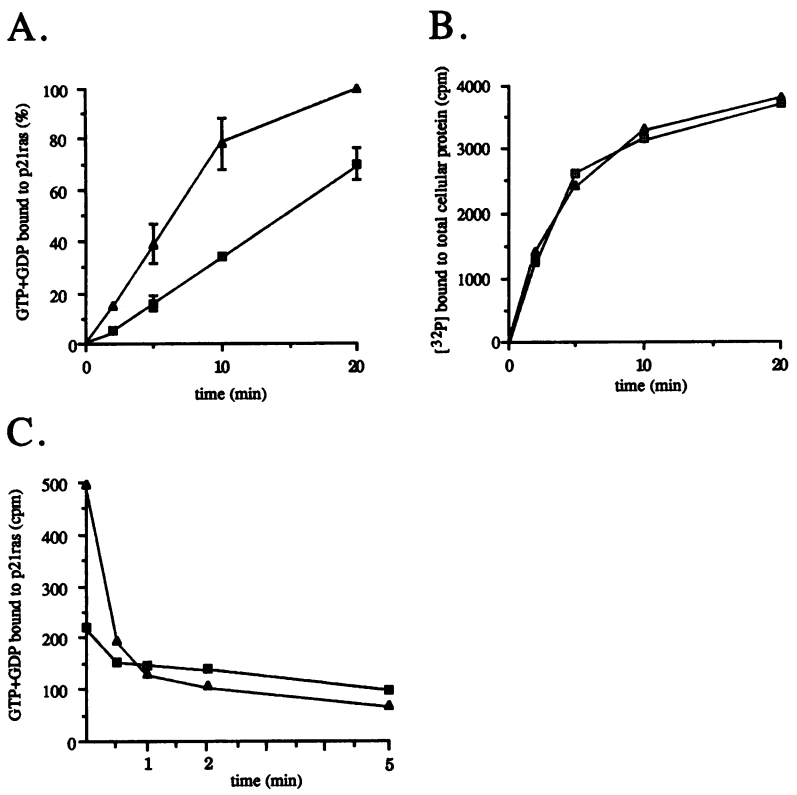

FIG. 3. Binding of [α-^{32}P]GTP to p21^{ras} and total cellular proteins. H13IR2000 cells were left unstimulated (□) or were stimulated with 10 μg of insulin per ml for 5 min (△). Cells were permeabilized and exposed to [α-^{32}P]GTP for various lengths of time. [α-^{32}P]GTP was diluted with unlabeled 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

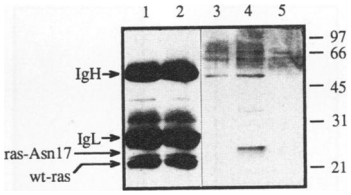

FIG. 4. Expression of wild-type p21^{ras} and recombinant *ras*(Asn-17) proteins in vaccinia virus-infected cells. H13IR2000 cells were infected with wild-type vaccinia virus (lanes 1 and 3) or *ras*(Asn-17) recombinant vaccinia virus (lanes 2, 4, and 5), and 16 to 18 h after infection, cells were harvested. 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.

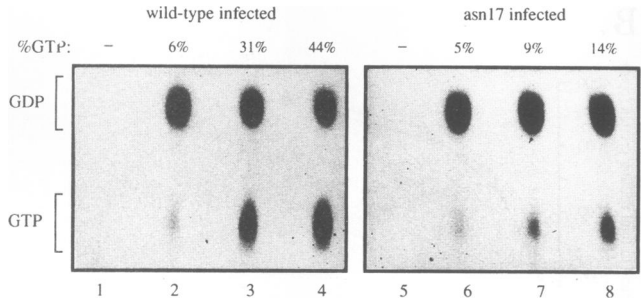

FIG. 5. Activation of p21^{ras} inhibited by *ras*(Asn-17). H13IR2000 cells were infected with wild-type vaccinia virus (left panel) or with the *ras*(Asn-17) recombinant vaccinia virus (right panel). Eighteen hours after transfection, cells were labeled with $^{32}P_i$. Following growth factor stimulation, 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

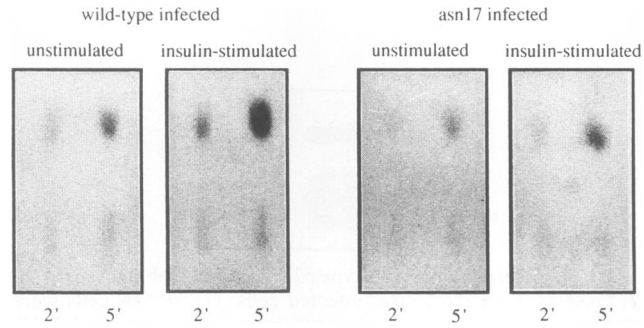

FIG. 6. Inhibition of enhanced nucleotide binding by *ras*(Asn-17). H13IR2000 cells were infected with wild-type (left panel) or *ras*(Asn-17) recombinant (right panel) vaccinia virus. Sixteen to 18 h after transfection, cells were permeabilized with streptolysin O and exposed to [α-^{32}P]GTP. After 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.

ACKNOWLEDGMENTS

We thank Julian Downward for discussions. Also, we thank our colleagues in the laboratory, Boudewijn Burgering, Jan Paul Medema, Bert Pronk, and Loesje van der Voorn, for helpful discussions and critically reading the manuscript.

This work was supported in part by the Dutch Cancer Society.

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