MOLECULAR AND CELLULAR BIOLOGY, Dec. 1991, p. 5963–5967
0270-7306/91/125963-05$02.00/0

Vol. 11, No. 12

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

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Received 15 July 1991/Accepted 22 September 1991

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

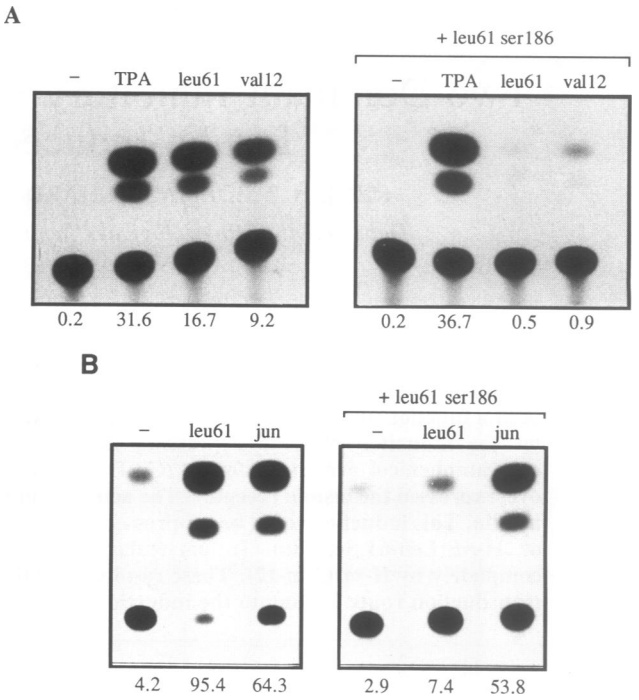

FIG. 1. Biological activity of *ras*(Leu-61,Ser-186). HeLa cells were transfected with 2 μg of Col-CAT in combination with RSV-*neo* (left panels) or 8 μg of RSV-*ras*(Leu-61,Ser-186) (right panels) per 9-cm dish. (A) Basal expression of the Col-CAT construct (–), TPA induction performed by adding 100 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

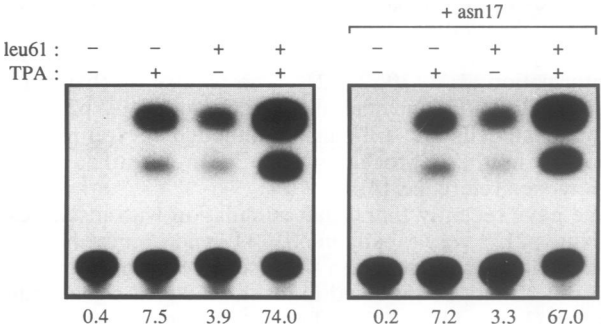

FIG. 2. Biological activity of *ras*(Asn-17). HeLa cells were transfected with 2 μg of Col-CAT in combination with RSV-*neo* (left panel) or 8 μg of RSV-*ras*(Asn-17) (right panel) per 9-cm dish. RSV-*ras*(Leu-61) (2 μg) was cotransfected where indicated above the lanes, and TPA (100 ng/ml) was added 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.

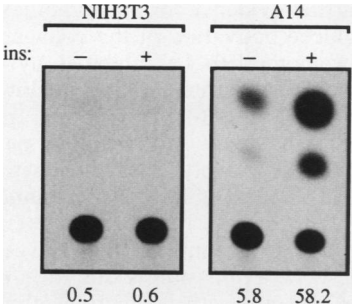

FIG. 3. Insulin induction of Col-CAT expression. NIH 3T3 (left panel) and A14 (right panel) cells were transfected with 2 μg of Col-CAT plus 8 μg of RSV-*neo*. Cells were treated with insulin (ins) (10 μg/ml) 24 h after transfection where indicated. CAT activity was determined 48 h after transfection.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

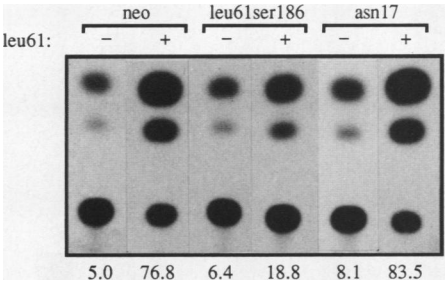

FIG. 4. Inhibition of *ras*-induced Col-CAT expression in A14 cells. A14 cells were transfected with 2 μg of Col-CAT, with or without 2 μg of RSV-*ras*(Leu-61) as indicated above the lanes, to test basal (–) and *ras*(Leu-61)-induced (+) Col-CAT expression. Eight micrograms of RSV-*neo* (neo), RSV-*ras*(Leu-61,Ser-186) (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-

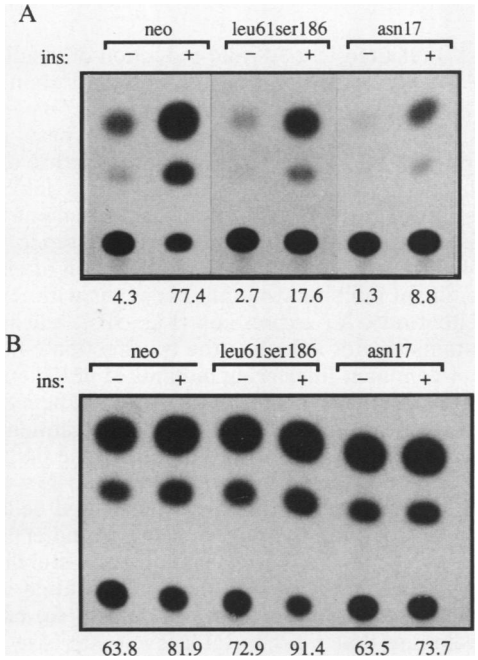

FIG. 5. Inhibition of insulin-induced Col-CAT expression. A14 cells were transfected with 2 μg of Col-CAT (A) or 2 μg of RSV-CAT (B) per 9-cm dish. We cotransfected the cells with 8 μg of RSV-*neo* (neo), RSV-*ras*(Leu-61,Ser-186) (leu61ser186), or RSV-*ras*(Asn-17) (asn17) as indicated. Cells were stimulated 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.

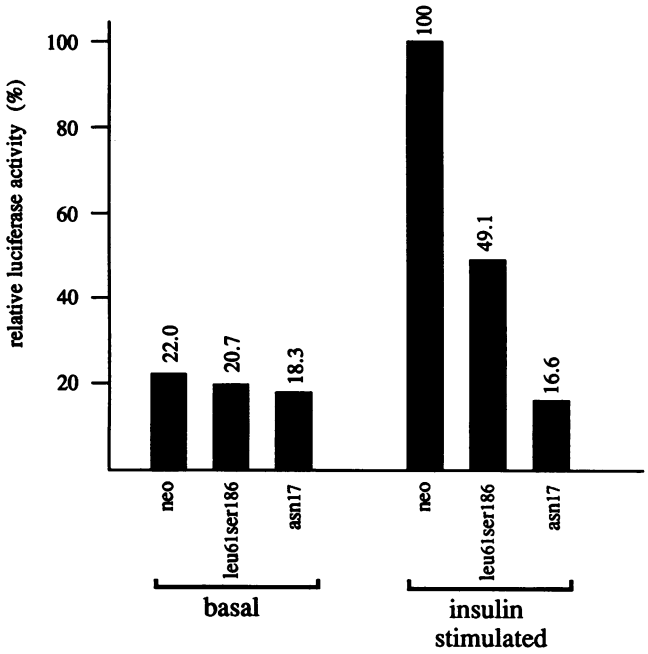

FIG. 6. Inhibition of insulin-induced Fos-luciferase expression. A14 cells were transfected with 2 μg of Fos-luciferase together with 8 μg of RSV-*neo* (neo), RSV-*ras*(Leu-61,Ser-186) (leu61ser186), or RSV-*ras*(Asn-17) (asn17). Cells were left unstimulated (basal) or stimulated with insulin (10 μg/ml) 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).

ACKNOWLEDGMENTS

We thank Channing Der for kindly providing the Leu-61 and Leu-61,Ser-186 mutant H-*ras* genes; Peter Angel for the Col-CAT and Fos-CAT expression vectors; Matty Verlaan-de Vries for constructing the *ras*(Asn-17) mutation; Anneke Kluin for excellent technical assistance; Boudewijn Burgering, Bert Pronk, and Helmy van Erp for critically reading the manuscript; and Frank McCormick for helpful discussions.

This investigation was supported by a grant from the Dutch Cancer Society.

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