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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

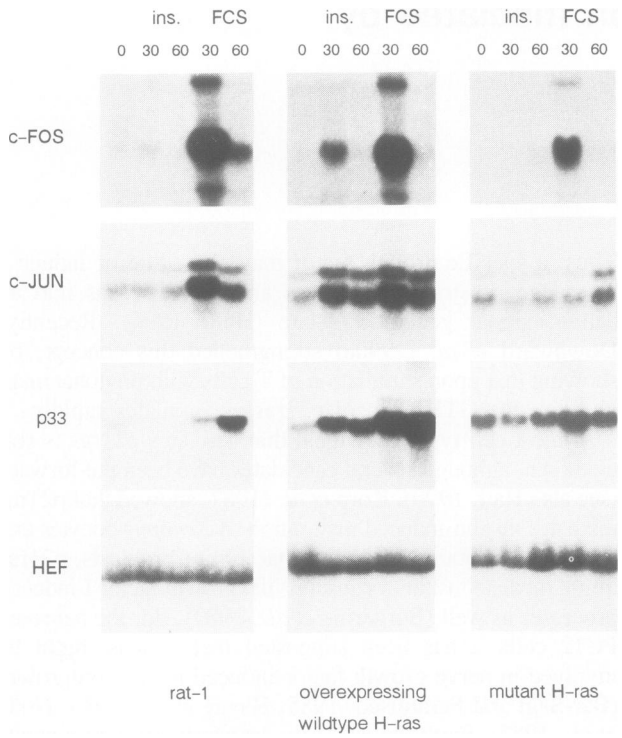

Fig. 1. Induction of c-*fos*, c-*jun* and *p33* mRNAs after growth factor treatment of rat-1 parental cells and p21Hras-transformed cell lines. Subconfluent cultures of different cell lines were serum-arrested for 24 h and stimulated with insulin (ins, 10 μg/ml) or fetal calf serum (FCS, 10%). Total 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.

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

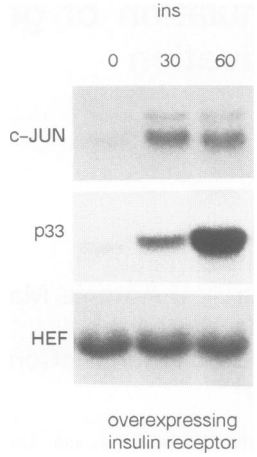

Fig. 2. Induction of c-*jun* and *p33* expression after insulin stimulation of insulin receptor overexpressing cells. Subconfluent cultures of A14 cells, overexpressing the insulin receptor, were serum-arrested for 24 h and stimulated with insulin (ins, 10 μg/ml). Total RNA was isolated at the indicated 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.

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

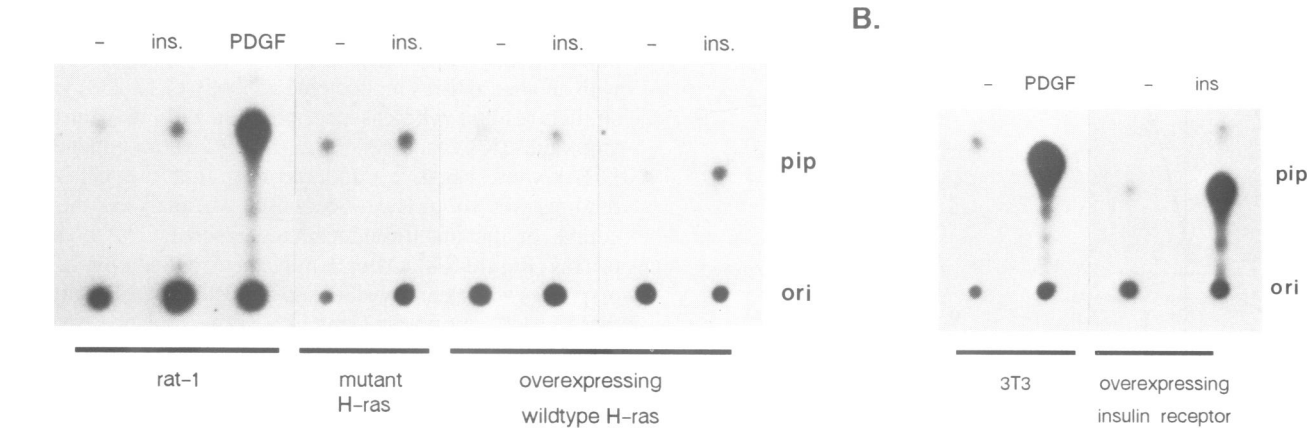

Fig. 3. Activation of PI-3-K after growth factor treatment. Cells were serum-arrested for 24 h and stimulated with insulin (ins, 10 μg/ml) or PDGF (40 ng/ml) for 15 min. Cells were lysed and 400 μg of protein was precipitated with polyclonal anti-phosphotyrosine serum. The precipitates bound to protein 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.

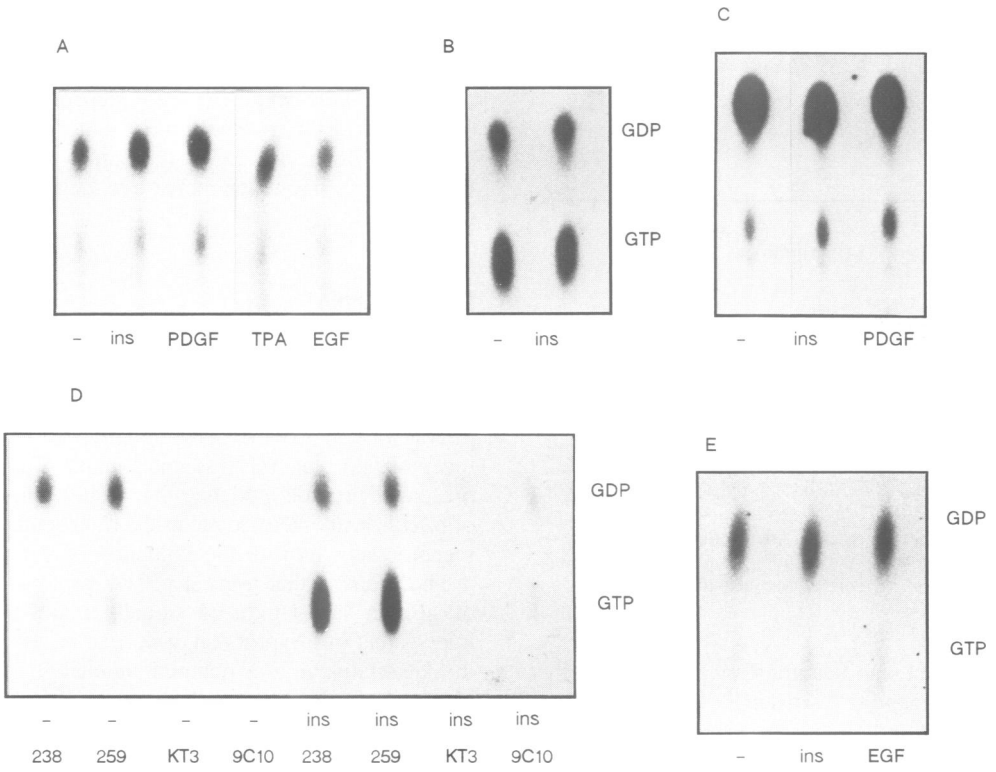

Fig. 4. GTP/GDP bound to p21ras. Autoradiographs after chromatographic separation of GTP and GDP eluted from p21ras. Cells were labelled with [^{3}P]orthophosphate for 3 h and stimulated with growth factors for 5 min. Cells were lysed and p21ras was collected by immunoprecipitation with the ras-specific 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

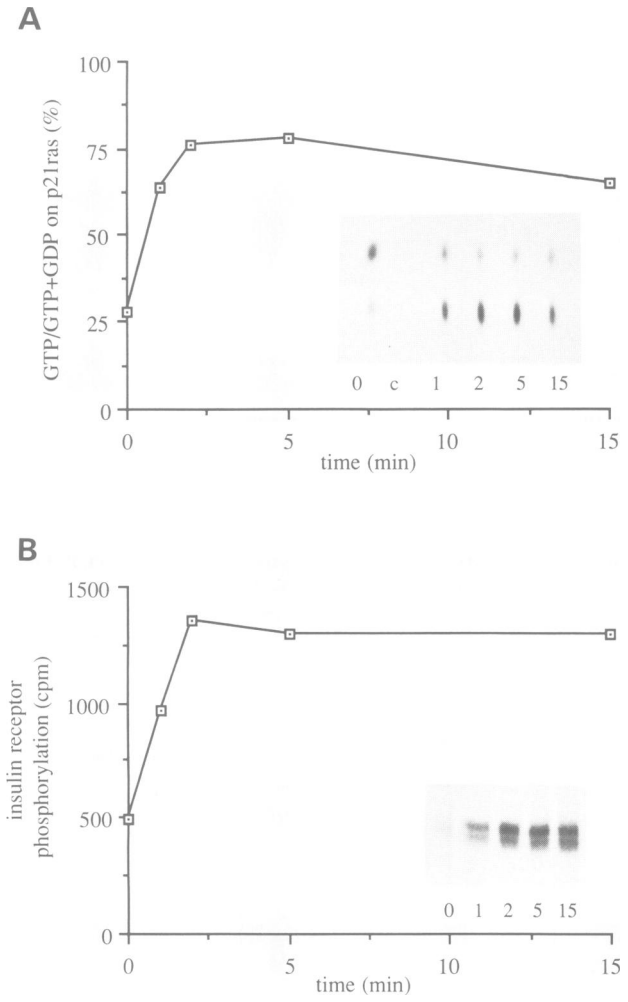

Fig. 5. Time course of insulin-induced increase in GTP bound to p21ras and increase in insulin receptor phosphorylation. **A**. Graphic presentation of the p21rasGTP/GDP ratio in A14 cells after stimulation with insulin for the indicated times. The insert shows the autoradiograph of the thin layer separation 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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