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Interaction of the human insulin receptor with the ras oncogene product p21

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Autophosphorylation of the purified human insulin receptor tyrosyl kinase was found to be inhibited by the ras oncogene product p21 in a concentration- and GDP-dependent manner. GDP-β-S but not Gpp(NH)p could substitute for GDP in eliciting the ras-dependent inhibition. The inhibition was seen with both normal or mutant (Lys-61) $p21^{N\text{-}ras}$ and normal or mutant (Val-12) $p21^{Ha\text{-}ras}$. Inhibition occurred at 23°C but not 4°C and was unaffected by the presence or absence of insulin although insulin stimulated the autophosphorylation rate of the receptor β-subunit some 2-fold. The insulin receptor did not phosphorylate native $p21^{Ha\text{-}ras}$ in the presence or absence of added guanine nucleotide. After denaturation of $p21^{Ha\text{-}ras}$ with urea it became a substrate, but then failed to inhibit receptor autophosphorylation even in the presence of added GDP.

Insulin; Phosphorylation; Receptor; Kinase; Guanine nucleotide; *ras* Gene

1. INTRODUCTION

The receptors for insulin, EGF and PDGF as well as a variety of kinases expressed by oncogenes exhibit the ability to phosphorylate proteins on tyrosine residues [1–3]. However, there is, as yet, no clear indication of the physiological targets for these activities. Many cell surface receptors appear to generate intracellular signals by modulating the activity of an appropriate effector system via distinct guanine nucleotide regulatory proteins (G-proteins). Thus stimulation and inhibition of adenylate cyclase by a variety of hormone receptors are mediated via G_s and G_i, respectively [5–8]. Although no G-proteins have yet been identified that couple growth factor receptors to intracellular effectors, there is some evidence that the ras gene product p21 may function in this way [9].

Recently it has been shown that the insulin receptor tyrosyl kinase can phosphorylate the holomeric, GDP-bound forms of the guanine nucleotide binding proteins G_i, G_o and transducin [10,11]. In addition, the tyrosyl kinase activity of the insulin receptor has been shown to be attenuated following phosphorylation by protein kinase C [12] and protein kinase A [13], while p21 ras proteins have been shown to modify the phosphorylation of mitochondrial-associated proteins [14]. Moreover, protein kinase C has been shown to phosphorylate $p21^{Ki\text{-}ras}$ [15]. The physiological significance of these phosphorylation events is not known.

In the present study, we demonstrate a functional interaction between the insulin receptor and p21 ras proteins, where the GDP-bound form of p21 ras proteins attenuates the autophosphorylation of the insulin receptor β-subunit.

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2. MATERIALS AND METHODS

Protease inhibitors, Hepes, PMSF, DTT and all reagents for SDS-polyacrylamide gel electrophoresis were purchased from Sigma, Poole, England. GDP, GDP-β-S, Gpp(NH)p and ATP were from Boehringer Mannheim, Lewes, England. Magnesium chloride, manganese chloride and sodium metavanadate were from BDH Chemicals, Poole, England. Porcine monocomponent insulin was a generous gift from Novo Research Institute, Copenhagen, Denmark. [^{32}P]Phosphate (PBS-11) was from Amersham, England and [γ-^{32}P]ATP was synthesized as described [16].

Membranes from human placenta were prepared and then solubilised using Triton X-100 as described by Fujita-Yamaguchi et al. [17], except that 50 mM Hepes, pH 7.6, was used in place of the 50 mM Tris buffer. Furthermore, at the solubilisation step, the protease inhibitors pepstatin A, leupeptin and antipain were all added at 1 μg/ml as well as benzamidine (2.5 mM) and PMSF (0.1 mM). The insulin receptor kinase was purified using an immunoadsorption technique employing a monoclonal anti-receptor antibody as described in detail elsewhere [18,19].

Purification of p21$^{N\text{-}ras}$ and p21$^{Ha\text{-}ras}$ has been described [20]. The proteins were essentially 95% pure with only one band on Coomassie blue stained gels. Where applicable p21$^{Ha\text{-}ras}$ was denatured by addition of 7 M urea, dialysed against 7 M urea to remove endogenous GDP for 24 h at 23°C and then renatured by dialysing against 10 mM Tris, pH 7.5 [21].

For the phosphorylation assay approx. 20 ng insulin receptor, immobilized on an immunoadsorbent, was employed per assay (total volume 40 μl). This is equivalent to a concentration of 2 nM insulin receptor. The final Triton X-100 concentration in the assay was 0.1%. Unless stated otherwise, insulin (100 nM) was present.

The experiments involved a series of different pre-incubations prior to the actual phosphorylation assay. The receptor was pre-incubated in a total volume of 20 μl containing insulin receptor (4 nM) together with 24 mM $MgCl_2$, 4 mM $MnCl_2$, 0.2 mM $NaVO_3$ and 2 mM DTT in the presence or absence of 200 nM insulin. These were incubated for 15 min at 23°C prior to mixing with ras p21 together with guanine nucleotide analogues, both at concentrations defined in the text. This mixture (total volume 30 μl) was incubated for a further 10 min at 23°C. After 10 min this material (30 μl) was mixed with 10 μl of 0.4 mM [γ-^{32}P]ATP (2 μCi/nmol) which was then incubated for 60 min at 23°C unless stated otherwise.

Reactions were terminated by addition of 3 × concentrated electrophoresis sample buffer (20 μl) containing 0.19 M Tris, pH 6.8, 6% (w/v) SDS, 30% (v/v) glycerol, 15 mM EDTA, 300 mM DTT and 0.02% (w/v) bromophenol blue. After heating for 5 min at 100°C samples were analysed by electrophoresis on SDS-polyacrylamide gels (150 × 150 × 1.5 mm) containing 15% (w/v) acrylamide [22]. Stained and dried gels were subjected to autoradiography. Results were quantified by excision of the appropriate regions from the gel and determination of ^{32}P by counting Cerenkov radiation.

Analysis of the phosphoamino acid content of ^{32}P-labelled proteins eluted from SDS-polyacrylamide gels was performed using chromatographic separation of phosphoamino acids as described [23].

3. RESULTS

Purification of p21 ras proteins from an *E. coli* expression system yielded a preparation containing one major band (21 kDa) on Coomassie staining [20]. The normal and mutant (Lys-61) forms of p21$^{N\text{-}ras}$ and the normal and mutant (Val-12) forms of p21$^{Ha\text{-}ras}$ were all assayed as possible substrates of the insulin receptor tyrosyl kinase, over a range of concentrations up to 40 μM. However, none of the ras p21 proteins were phosphorylated. Addition of GDP (1 mM) or Gpp(NH)p (1 mM) did not cause ras p21 phosphorylation by the receptor kinase preparation. Under the conditions employed, histones and casein were both phosphorylated efficiently by the insulin receptor. However, when normal p21$^{Ha\text{-}ras}$ was first denatured in 7 M urea and subsequently renatured by dialysis against 10 mM Tris, pH 7.5, it was found to provide a better substrate for the insulin receptor tyrosyl kinase. The K_m for phosphorylation of denatured/renatured p21$^{Ha\text{-}ras}$ by the insulin receptor kinase was estimated to be 30 μM and 4 × 10^{-3} mol P_i were incorporated per mol p21$^{Ha\text{-}ras}$

after 60 min at 23°C (fig.1A). The addition of either GDP (1 mM) or Gpp(NH)p (1 mM) had no effect on the kinetics of phosphorylation by the insulin receptor kinase. Furthermore, as expected phosphoamino acid analysis showed that both insulin receptor β-subunit and denatured/renatured $p21^{Ha\text{-}ras}$ were phosphorylated on tyrosine residues (fig.1B).

Although native $p21^{Ha\text{-}ras}$ was not a substrate for phosphorylation by the insulin receptor tyrosyl kinase it was found to inhibit insulin receptor autophosphorylation in the presence of GDP (fig.2). This effect was not seen with either GDP or $p21^{Ha\text{-}ras}$ alone. Gpp(NH)p could not substitute for GDP in eliciting the inhibition (fig.3). $p21^{Ha\text{-}ras}$ yielded a dose-dependent inhibition with a half-maximal effect at approx. 10–20 μM at saturating GDP concentrations, and GDP gave a dose-dependent inhibition with a half-maximal effect at approx. 100 μM in the presence of 20 μM $p21^{Ha\text{-}ras}$ (fig.4).

The mutant (Val-12) form of $p21^{Ha\text{-}ras}$ and both the normal and mutant (Lys-61) forms of $p21^{N\text{-}ras}$ also inhibited receptor autophosphorylation in their GDP-bound forms and not Gpp(NH)p-bound forms, with similar GDP and p21 concentration dependencies. We found, however, that denatured/renatured normal $p21^{Ha\text{-}ras}$ failed to inhibit receptor autophosphorylation in the presence of GDP (not shown), showing that such an action was exerted only by the native form of p21 ras.

The inhibitory effect of p21 ras proteins on insulin receptor autophosphorylation was found to

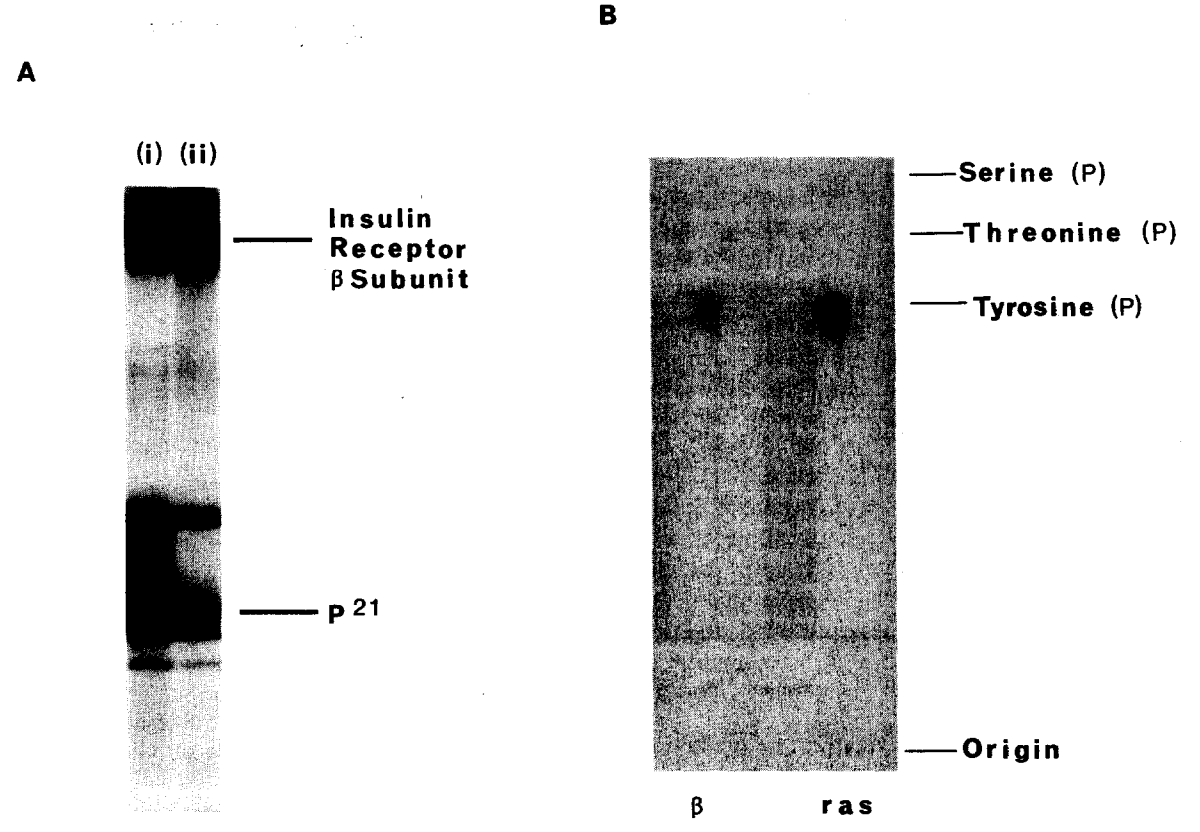


Fig.1. Phosphorylation of denatured/renatured normal $p21^{Ha\text{-}ras}$ by the insulin receptor tyrosyl kinase. (A) Autoradiograph of proteins separated on a 15% SDS-polyacrylamide gel. $p21^{Ha\text{-}ras}$ (10 μM) and insulin receptor (2 nM) were incubated for 60 min at 23°C with [γ-^{32}P]ATP (100 μM) in the phosphorylation assay; (i) denatured/renatured $p21^{Ha\text{-}ras}$; (ii) native $p21^{Ha\text{-}ras}$. (B) Phosphoamino acid analysis of labelled proteins from the above experiment.

be temperature dependent since the inhibition of autophosphorylation was not seen at 4°C, yet was apparent at 23°C (fig.5A). The rate of insulin receptor autophosphorylation was reduced at 4°C compared to 23°C, but after incubating with [γ-^{32}P]ATP for 60 min at either 4°C or 23°C the insulin receptor β-subunit became maximally phosphorylated. Thus no effect of temperature was seen on the extent of autophosphorylation, in

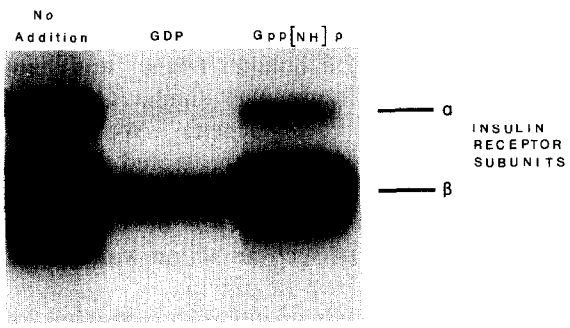


Fig.2. Inhibition of insulin receptor autophosphorylation by native p21$^{Ha\text{-}ras}$. Autoradiograph shows insulin receptor β-subunit. Insulin receptor (2 nM) was incubated with 20 μM p21$^{Ha\text{-}ras}$ for 60 min at 23°C with [γ-^{32}P]ATP (100 μM) in the presence of either (i) no added guanine nucleotide, (ii) 1 mM GDP or (iii) 1 mM Gpp(NH)p.

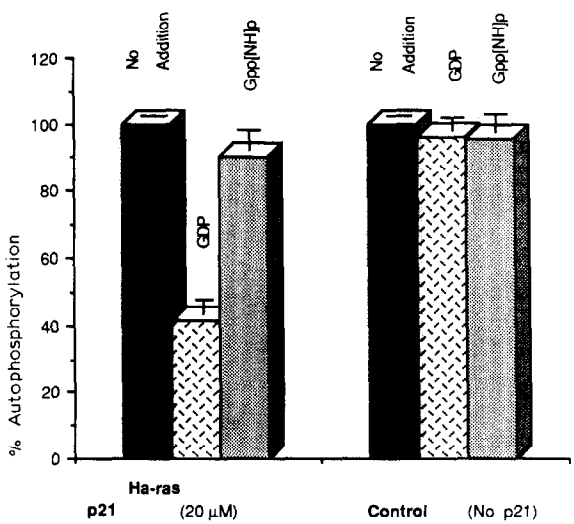
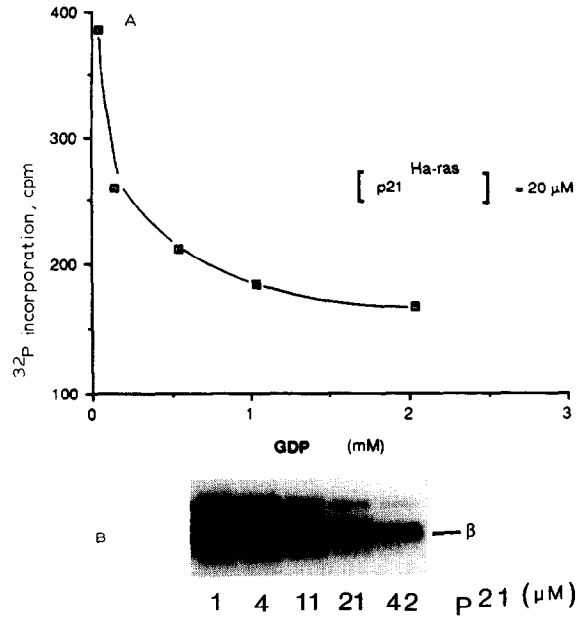


Fig.3. Inhibition of insulin receptor autophosphorylation by native p21$^{Ha\text{-}ras}$. The histograms show data from three experiments. Autophosphorylation is expressed as a percentage of the phosphorylation in the absence of added guanine nucleotide. Insulin (2 nM) was incubated in the presence or absence of 20 μM p21$^{Ha\text{-}ras}$ for 60 min at 23°C with [γ-^{32}P]ATP (100 μM) with either (i) no added guanine nucleotide, (ii) 1 mM GDP or (iii) 1 mM Gpp(NH)p.



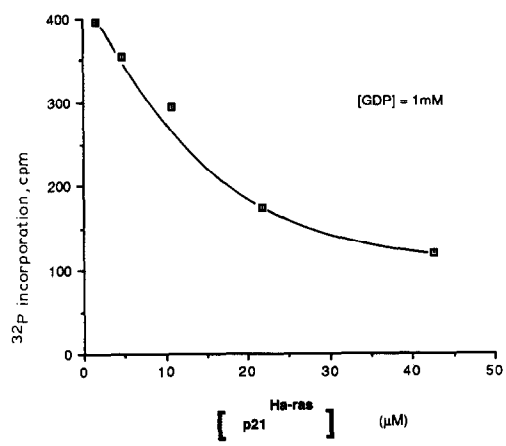


Fig.4. Dependence of p21$^{Ha\text{-}ras}$ mediated inhibition of insulin receptor tyrosine kinase on GDP and p21$^{Ha\text{-}ras}$ concentration. Results show a typical experiment. (A) Insulin receptor (2 nM) was incubated in the presence of 20 μM p21$^{Ha\text{-}ras}$ for 60 min at 23°C with [γ-^{32}P]ATP (100 μM) with varying concentrations of GDP (0–2 mM). (B) Insulin receptor (2 nM) was incubated with 1 mM GDP and varying concentrations of p21$^{Ha\text{-}ras}$ (0–42 μM) in the presence of [γ-^{32}P]ATP (100 μM) for 60 min at 23°C.

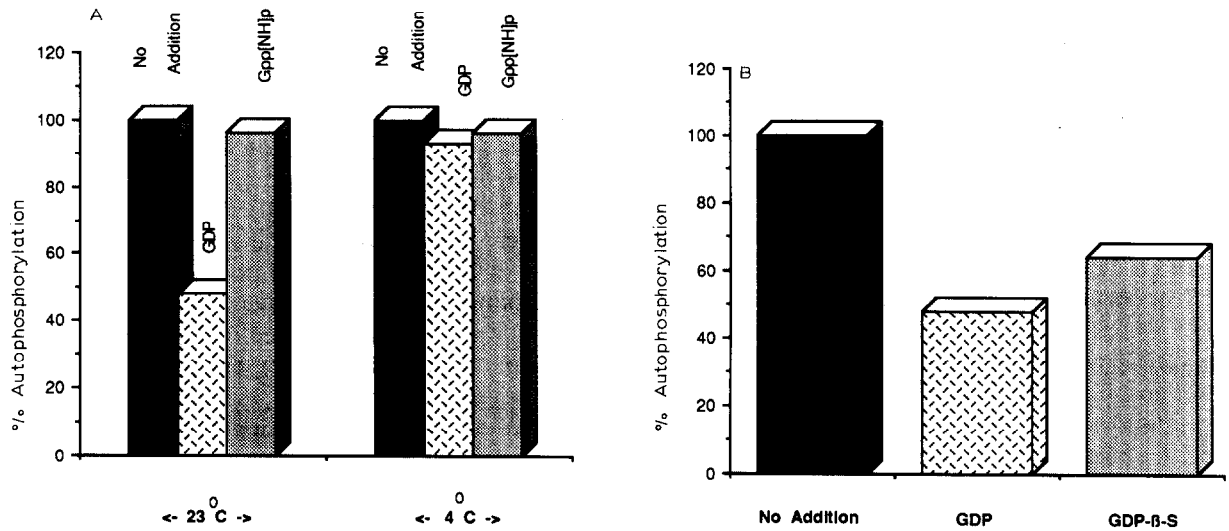


Fig.5. Effect of temperature (A) and GDP-β-S (B) on inhibition of insulin receptor autophosphorylation by normal $p21^{Ha\text{-}ras}$. Autophosphorylation is expressed as a percentage of the phosphorylation in the absence of added guanine nucleotide. (A) Insulin receptor (2 nM) was incubated with 20 μM $p21^{Ha\text{-}ras}$ for 60 min at either 23°C or 4°C with [γ-^{32}P]ATP (100 μM) with either (i) no added guanine nucleotide, (ii) 1 mM GDP or (iii) 1 mM Gpp(NH)p. (B) Insulin receptor (2 nM) was incubated with 20 μM $p21^{Ha\text{-}ras}$ for 60 min at 23°C with [γ-^{32}P]ATP (100 μM) with either (i) no added guanine nucleotide, (ii) 1 mM GDP or (iii) 1 mM GDP-β-S.

the absence of p21 ras, in this experiment. We also found that replacement of GDP by GDP-β-S maintained the ability of the various p21 ras proteins to inhibit receptor autophosphorylation (fig.5B). This shows that the reaction is specific for GDP and obviates any possible conversion of bound GDP to GTP on ras p21.

The presence or absence of insulin in the receptor kinase assay appeared to have little effect on p21 ras-mediated inhibition of receptor autophosphorylation. Thus in the presence of GDP (1 mM) and $p21^{Ha\text{-}ras}$ (20 μM), inhibition of phosphorylation during a 5 min assay period at 23°C was 60 and 40% in the absence and presence of insulin, respectively.

4. DISCUSSION

Guanine nucleotide-binding proteins (G-proteins) provide a means whereby receptors can couple to and regulate distinct effector systems. It would appear, however, that their functioning can be attenuated during certain desensitization processes (see for example [24]) or following their ribosylation by various toxins [5–8]. Recent evidence suggests that they are subject to control by phosphorylation/dephosphorylation reactions. Thus protein kinase C can phosphorylate and inactivate G_i [25] and the insulin receptor tyrosyl kinase has been shown to be capable of phosphorylating the holomeric, GDP-bound forms of G_i, G_o [10] and transducin [11].

Here we show that native p21 ras proteins, which are G-proteins, are not substrates for the insulin receptor tyrosyl kinase. However, after a cycle of denaturation and renaturation then $p21^{Ha\text{-}ras}$ provides a better substrate for this tyrosyl kinase activity. (The K_m for phosphorylation of $p21^{Ha\text{-}ras}$ by the insulin receptor kinase was estimated to be 30 μM in the presence of insulin. This compares with values of around 10–100 μM for histones, 10–20 μM for casein, 5–10 μM for poly Glu 4:Tyr 1, and 2–4 mM for angiotensin and the src-related peptide [26,27].) This suggests that the urea denaturation/renaturation cycle used by a number of workers in the purification of ras p21 [21] may profoundly disturb the structure of this molecule. As such our observations question the advisability of using such a protocol to prepare p21 ras proteins for either structural or functional analysis.

Although we found that native ras p21 did not provide a substrate for the insulin receptor tyrosyl kinase we did observe that the native, but not denatured forms of the normal and mutant (Lys-61) $p21^{N\text{-}ras}$ species as well as the normal and mutant (Val-12) species of $p21^{Ha\text{-}ras}$ could all inhibit the ability of the insulin receptor to undergo autophosphorylation. Such an inhibitory effect was dose dependent upon p21 ras and interestingly, dependent upon GDP being present. It did not occur when the non-hydrolysable GTP analogue, Gpp(NH)p, was present but was evident when the non-phosphorylatable analogue of GDP, GDP-β-S was employed. Inhibition by p21 ras was exerted on the basal kinase activity as well as that stimulated by insulin and whilst observed at 23°C was not evident at 4°C.

The mechanism of ras/GDP-mediated inhibition of receptor autophosphorylation is unclear. The effect cannot be explained by depletion of ATP in the assay mixture (not shown). $p21^{Ha\text{-}ras}$ is not itself phosphorylated under the conditions in which receptor phosphorylation is inhibited, so it does not act as a competitive substrate for the tyrosyl kinase. It is interesting that the extent of inhibition is very similar whether the initial rate of phosphorylation is measured after 5 min or the plateau of incorporation after 60 min. This suggests that ras might be acting to block phosphorylation sites on the receptor rather than to inhibit the kinase activity per se. Experiments with exogenous substrates will help to resolve this issue.

Normal cellular p21 ras proteins are found in most if not all cell types, with oncogenic forms being identified as having single point mutations. Evidence has been provided which indicates that such proteins may act as regulatory G-proteins able to couple growth factor receptors to phospholipase C in order to stimulate inositol phospholipid metabolism in certain cells where such responses are unaffected by pertussis toxin [9]. Our observations here indicate that the unstimulated, GDP-bound form of p21 ras can inhibit insulin receptor autophosphorylation. As autophosphorylation can lead to increased kinase activity [28,29] and is believed to be crucial to receptor functioning [4,30], it is possible that p21 ras might exert a tonic inhibitory effect on insulin receptor functioning. One might expect this to be relieved only when an appropriate occupied receptor was able to interact with p21 ras, converting it to an active GTP-bound form, hence relieving the tonic inhibition. This might offer an explanation for the synergising action of insulin and various growth factors in stimulating growth [31,32]. It will thus be of interest to attempt to demonstrate whether such interactions can occur in intact cells where diffusion of such components is confined to the 2-dimensions of the bilayer, such that the concentration of p21 ras required for half-maximal inhibition and its GDP sensitivity may be significantly reduced, and to define the consequences of such an interacting system.

Interestingly, recent work using *Xenopus* oocytes has demonstrated a possible, if indirect, interaction between the insulin receptor and p21 ras proteins since insulin induction of oocyte maturation is inhibited by a monoclonal antibody to p21 [33].

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