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Epidermal Growth Factor Regulates the Exchange Rate of Guanine Nucleotides on p21^{ras} in Fibroblasts

LÁSZLÓ BUDAY AND JULIAN DOWNWARD*

Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, United Kingdom

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Treatment of intact Rat-1 fibroblasts with epidermal growth factor (EGF) leads to rapid activation of cellular *ras*-encoded proteins. By using the bacterial toxin streptolysin O to permeabilize these cells, it was shown that the low basal rate at which guanine nucleotides bind to, and dissociate from, *ras*-encoded protein in quiescent fibroblasts was greatly accelerated by EGF treatment. Nucleotide binding to other proteins was not affected. Stimulation of nucleotide exchange on *ras*-encoded protein required tyrosine kinase but not phospholipase activity. EGF had no effect on total GTPase-activating protein activity. Regulation of *ras*-encoded protein in Rat-1 fibroblasts is therefore mediated by stimulation, either directly or indirectly, of *ras*-encoded protein-specific guanine nucleotide exchange factors by the EGF receptor tyrosine kinase.

The products of the *ras* family of proto-oncogenes are low-molecular-weight, guanine nucleotide-binding proteins that play essential roles in the control of cellular growth and differentiation (6). They possess intrinsic GTPase activity, being biologically active when bound to GTP and inactive when bound to GDP. The low basal rate of GTP hydrolysis on *ras*-encoded protein is stimulated by GTPase-activating proteins (GAPs), such as p120^{GAP} and neurofibromin, the product of the neurofibromatosis type I gene; these proteins therefore inactivate *ras*-encoded protein but may also have roles as downstream effectors.

The intrinsic rate at which guanine nucleotides exchange on and off *ras*-encoded protein is very low. A number of mammalian exchange activities which stimulate this reaction have been identified (8, 13, 17, 29, 30). Recently, mammalian homologs of yeast *ras* exchange factors CDC25, SDC25, and ste6 have been cloned (3, 16, 25, 28). Within a cell, the activation state of *ras*-encoded protein is controlled by the opposing action of GAPs and exchange factors, with the exchange factors acting to replace with fresh cytosolic GTP the GDP produced on *ras*-encoded protein in response to interaction with GAPs. Activation of *ras* could be achieved either by stimulation of exchange factors or by inhibition of GAPs.

A large number of extracellular stimuli have been shown to activate *ras*-encoded proteins within cells, as determined by measuring an increase in the amount of GTP relative to GDP bound to *ras*-encoded protein in cells metabolically labelled with $^{32}P_i$. These stimuli include phorbol esters, T-cell receptor agonists, CD2 antibodies, and interleukin 2 in T lymphocytes (7, 11, 12, 24); interleukin 3, granulocyte-macrophage colony-stimulating factor, and Steel factor in mast cells (9, 24); erythropoietin in erythroleukemia cells (27); transforming growth factor β in epithelial cells (18); the nerve growth factor NGF and epidermal growth factor (EGF) in PC12 cells (19, 20); and platelet-derived growth factor (PDGF), EGF, and insulin in various fibroblast cell lines (5, 10, 22, 23). In two instances, stimulation of *ras*-encoded protein has been linked to a decrease in GAP activity as measured in cell extracts: phorbol ester or T-cell receptor agonist treatment of T cells (7) and erythropoietin treatment of human erythroleukemia cells (27). In one case, PC12 cells treated with the differentiation-inducing factor NGF, an increase in nucleotide exchange activity has been associated with activation of *ras*-encoded protein (15); in this study, however, the activity measured in the cell lysates may not have been specific for *ras*-encoded protein but rather the entire superfamily of *ras*-encoded proteins.

The diverse nature of the factors that can give rise to activation of *ras*-encoded proteins suggests that several different mechanisms exist by which *ras*-encoded protein regulation may occur and that there are considerable differences in the pathways used in diverse cell types. We have set out to study the mechanism by which EGF causes stimulation of *ras*-encoded protein in Rat-1 fibroblasts. A cell permeabilization technique, previously used successfully with T cells (7), was employed to measure rates of guanine nucleotide exchange on *ras*-encoded protein. In this report, we show that EGF treatment greatly increased the rate of nucleotide exchange on *ras*-encoded protein in fibroblasts while not affecting GAP activity. Regulation of *ras*-encoded protein in fibroblasts therefore involves a mechanism markedly different from that found in T lymphocytes and HEL cells.

MATERIALS AND METHODS

Cell culture. Rat-1 cells were an early-passage culture from the original isolate and were a kind gift from Hans Bos (University of Utrecht). They were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum.

Metabolic labelling. Rat-1 cells were grown in 10-cm-diameter dishes until just confluent and then labelled without serum for 18 h in 5 ml of phosphate-free Dulbecco's modified Eagle's medium containing 1 mg of bovine serum albumin per ml and 1 mCi of $^{32}P_i$ per dish. Cells were lysed in 1% Triton X-114-containing buffer as previously described (1). p21^{ras} was immunoprecipitated from the detergent phase by using monoclonal antibody Y13-259. Amounts of GTP and GDP bound to *ras*-encoded protein were quantitated by thin-layer chromatography followed by direct scanning for β emission as described previously (1, 7). Backgrounds were determined by immunoprecipitation with a nonspecific anti-

* Corresponding author.

body and were subtracted to give figures for specific binding. All determinations were made in duplicate.

Cell permeabilization. Rat-1 cells were grown in 3-cm-diameter dishes until just confluent. They were then serum starved in Dulbecco's modified Eagle's medium plus 1 mg of bovine serum albumin for 18 h. Cells were washed with warm phosphate-buffered saline (PBS) and then placed in 0.8 ml of permeabilization buffer [150 mM KCl, 37.5 mM NaCl, 6.25 mM $MgCl_2$, 0.8 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM $CaCl_2$, 1.25 mM ATP, 12.5 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), pH 7.4] at 37°C. A 0.2-ml volume of streptolysin O (2 IU/ml in water; Wellcome) was then added. After 5 min, 5 μCi of [α-^{32}P]GTP (3,000 Ci/mmol; Amersham) or [^{35}S]GTPγS (1,500 Ci/mmol; Amersham) was added, along with growth factor where indicated. After the required incubation, the supernatant was removed, the cells were lysed in 1 ml of 1% Triton X-100 buffer plus 0.1 mM unlabelled GTP, and *ras*-encoded protein immunoprecipitated without phase partition, as previously described (7). Total specific radioactivity associated with *ras*-encoded protein immunoprecipitates was determined by Cerenkov counting for ^{32}P or liquid scintillation counting for ^{35}S. In all cases, samples were assayed in duplicate and values were averaged.

Nucleotide binding to total protein. Aliquots of 20 μl were removed from the total permeabilized cell lysates and applied to nitrocellulose filters. The filters were washed with 20 ml of PBS plus 5 mM $MgCl_2$, and then radioactivity was counted.

EGF receptor immunoprecipitation and immunoblotting for phosphotyrosine. Lysates were made from unlabelled cells as described above. The EGF receptor was immunoprecipitated with affinity-purified rabbit antibodies against the peptide PVPEYINQSVPKRPAGS, which corresponds to residues 1088 to 1104 of the human EGF receptor. These immunoprecipitates were run on a 7% sodium dodecyl sulfate–polyacrylamide gel which was then immunoblotted with antiphosphotyrosine antibody 4G10 (UBI, Lake Placid, N.Y.) by using the manufacturer's instructions.

GAP assays. Rat-1 cells were grown on 10-cm-diameter dishes until just confluent and then serum starved for 18 h before growth factor treatment. The cells were then washed with ice-cold PBS and lysed in low-salt 1% Triton X-100 lysis buffer, and total GAP content was assayed as described previously (7).

Peptide phosphorylation assays. Cells were grown and permeabilized as described above, except that a total volume of 0.5 ml was used. H1 peptide (AAASFKAKK) was then added at 0.5 mg/ml along with EGF or phorbol dibutyrate (100 ng of each per ml). After 5 min of prepermeabilization, 5 μCi of [γ-^{32}P]ATP was added (final ATP concentration, 200 μM). The cells were incubated at 37°C, and the reaction was stopped by addition of 200 μl of 25% trichloroacetic acid–2 M acetic acid. After 15 min at 0°C, the extract was spotted onto Whatman P81 paper squares in duplicate and washed in 30% acetic acid–0.5% phosphoric acid (4 × 10 min, 500 ml each). The washed squares were Cerenkov counted for radiation.

RESULTS

EGF stimulates *ras*-encoded protein in intact Rat-1 fibroblasts. Rat-1 fibroblasts were grown to confluence in serum-containing medium and then metabolically labelled with $^{32}P_i$ in the absence of serum for 16 h. The cells were then stimulated with a number of factors for 5 min prior to detergent lysis and immunoprecipitation of endogenous *ras*-encoded protein with monoclonal antibody Y13-259. The guanine nucleotide bound to the *ras*-encoded protein in the immune complex was determined by thin-layer chromatography. As shown in Fig. 1A, treatments such as phorbol ester, insulin, and serum caused no significant increase in the relative amount of GTP bound to the *ras*-encoded protein,

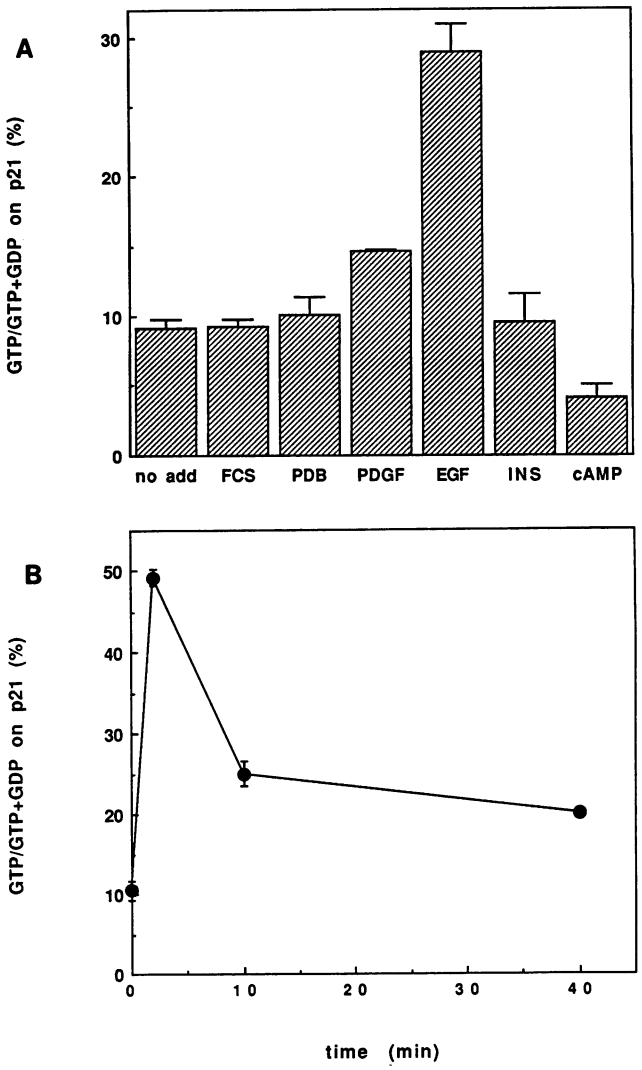


FIG. 1. Stimulation of *ras*-encoded protein by EGF in intact Rat-1 fibroblasts. (A) Rat-1 cells were metabolically labelled with $^{32}P_i$ in the absence of serum and stimulated as indicated for 5 min at 37°C. The cells were then lysed, and *ras*-encoded protein was immunoprecipitated. The amounts of GTP and GDP bound to *ras*-encoded protein were determined by thin-layer chromatography. FCS, 10% fetal calf serum; PDB, 100 ng of phorbol dibutyrate per ml; PDGF, 20 ng/ml; EGF, 100 ng/ml; INS, 10 μg of insulin per ml; cAMP, 100 μM dibutyryl cyclic AMP plus 100 μM isobutylmethylxanthine. Total nucleotide binding to Y13-259 immunoprecipitates was as follows: fetal calf serum, 281 cpm; phorbol dibutyrate, 310 cpm; PDGF, 298 cpm; EGF, 276 cpm; insulin, 303 cpm; cyclic AMP, 295 cpm. Total nucleotide binding to negative controls (nonspecific immunoglobulin G in place of Y13-259) was 7 ± 2 cpm, with 2 ± 1 cpm as GTP. (B) Time course of stimulation of *ras*-encoded protein in $^{32}P_i$-labelled Rat-1 cells in response to 100 ng of EGF per ml.

while PDGF caused a small increase and cyclic AMP analogs caused a small decrease. EGF, however, caused a high level of stimulation of *ras*-encoded protein, with the amount of GTP bound rising from about 10% to nearly 30%. When the time course of the EGF stimulation of *ras*-encoded protein was determined, stimulation was found to be greatest after just 2 min of treatment, at which time the GTP level rose to 50% (Fig. 1B). The stimulation peaked sharply and was transient, although it was still just detectable after 40 min.

EGF stimulates the rate of guanine nucleotide binding to *ras*-encoded protein in streptolysin O-permeabilized Rat-1 fibroblasts. Confluent, serum-starved Rat-1 cells were treated with the bacterial toxin streptolysin O, which binds to cholesterol in the plasma membrane to form pores through which exogenous molecules can be introduced into the cells. After 5 min of prepermeabilization with streptolysin O in a buffer designed to mimic the cytosol with 100 nM free calcium, [α-^{32}P]GTP was added to the cells along with EGF. After various lengths of time, aliquots of cells were lysed with detergent in the presence of excess unlabelled GTP. The *ras*-encoded protein, together with the bound nucleotide, was immunoprecipitated with antibody Y13-259. The amount of the labelled nucleotide specifically associated with the *ras*-encoded protein was plotted against time of incubation (Fig. 2A). Treatment with EGF caused a large increase in the rate at which the nucleotide bound to *ras*-encoded protein in this system, some sixfold over unstimulated cells. Nucleotide binding to *ras*-encoded protein in EGF-stimulated cells saturated by 10 min, while it still increased linearly in unstimulated cells at 15 min. Extrapolation of the unstimulated binding rate suggests that about 1 h is required to reach saturated binding in the absence of EGF; this is similar to the time course for GTP binding to purified *ras*-encoded protein under the same buffer conditions, suggesting that the basal activity of the exchange factor in quiescent cells is very low. The other stimuli shown in Fig. 1A were not able to stimulate nucleotide exchange on *ras*-encoded protein (data not shown).

To determine whether the nucleotide bound to *ras*-encoded protein was GTP or GDP, it was separated by thin-layer chromatography. As shown in Fig. 2B, the nucleotide bound to *ras*-encoded protein was almost entirely GDP in all cases (less than 2% GTP in all cases). This presumably reflects either the rapid hydrolysis of GTP on *ras*-encoded protein due to the influence of GAPs or hydrolysis of GTP within the cell prior to its binding to *ras*-encoded protein. Analysis of the labelled nucleotide present in total lysates from washed, permeabilized cells indicated that very little GTP relative to GDP was present within the cells at any time (data not shown); it is therefore likely that the failure to accumulate GTP on *ras*-encoded protein reflects the high level of general nucleotide triphosphatase activity in these cells. We have been unable to find a way of protecting the radiolabelled GTP so that it is not hydrolyzed prior to binding to *ras*-encoded protein. However, when the nonhydrolyzable GTP analog GTPγS was used, EGF-stimulatable accumulation of the triphosphate-bound form of *ras*-encoded protein resulted (Fig. 2C and D).

To ensure that the binding seen was reversible, permeabilized cells that had been incubated in [α-^{32}P]GTP for 5 min were subjected to a cold chase with 1 mM unlabelled GTP. The amount of the labelled nucleotide bound to *ras*-encoded protein rapidly diminished in EGF-treated cells; in untreated cells, the rate was much lower (Fig. 2E).

Since activation of *ras*-encoded protein in intact Rat-1 cells is transient, the effect of treating the cells with EGF for 5 min prior to their permeabilization was determined. In this assay, streptolysin O and [α-^{32}P]GTP were added at the same time; no prepermeabilization was carried out. As shown in Fig. 2F, there was a lag of some 5 min before any of the nucleotide was taken up by the cells. More significantly, cells that had been pretreated with EGF for 5 min before addition of streptolysin O and [α-^{32}P]GTP displayed a markedly reduced rate of exchange onto *ras*-encoded protein compared with cells which had been treated with EGF at the same time as streptolysin O and [α-^{32}P]GTP. This suggests that stimulation of the exchange rate is considerably attenuated after 5 min of treatment with EGF.

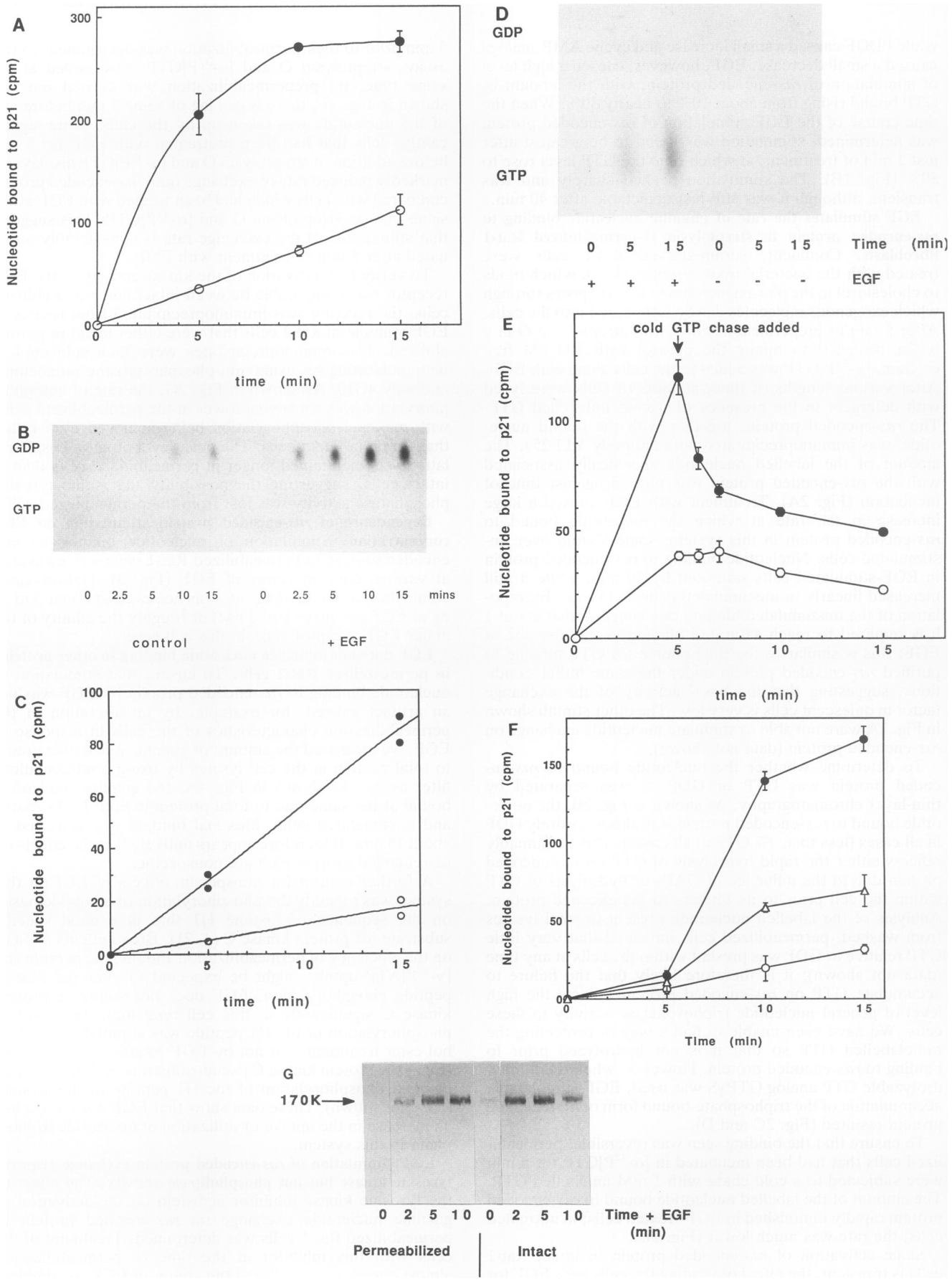
To verify that activation of the kinase activity of the EGF receptor was comparable between intact and permeabilized cells, the receptor was immunoprecipitated from lysates of EGF-stimulated Rat-1 cells that were either intact or permeabilized. The immunoprecipitates were then subjected to immunoblotting by using anti-phosphotyrosine monoclonal antibody 4G10. As shown in Fig. 2G, the rate of autophosphorylation was somewhat lower in the permeabilized cells, with maximal phosphorylation being achieved by 5 rather than 2 min in intact cells. The peak level of autophosphorylation was maintained longer in permeabilized cells than in intact cells, suggesting the possibility that some tyrosine phosphatase activity was lost from the permeabilized cells.

Dependence of *ras*-encoded protein stimulation on EGF concentration. Stimulation of nucleotide binding to *ras*-encoded protein in permeabilized Rat-1 cells was measured at various concentrations of EGF (Fig. 3). Half-maximal stimulation of the binding rate was achieved at about 5 to 10 ng of EGF per ml (~1 to 2 nM) or roughly the affinity of the major EGF receptor class in this cell type.

EGF does not influence nucleotide binding to other proteins in permeabilized Rat-1 cells. To ensure that stimulation of nucleotide binding to *ras*-encoded protein by EGF was not an artifact caused, for example, by an alteration in the permeabilization characteristics of the cells in response to EGF, we measured the amount of guanine nucleotide bound to total protein in the cell lysates by using a nitrocellulose filter assay. As shown in Fig. 4A, the guanine nucleotide bound at the same rate to total protein in both EGF-treated and unstimulated cells. Maximal binding was achieved at about 15 min. It therefore appears unlikely that the effects on *ras*-encoded protein seen are nonspecific.

A further control for nonspecific effects of EGF in this system was to study the phosphorylation of a peptide based on the sequence of histone H1 that is a good specific substrate for protein kinase C (4, 21). Broad effects of EGF on the efficiency of permeabilization and, hence, peptide and [γ-^{32}P]ATP uptake might be expected to alter the rate of peptide phosphorylation. EGF does not stimulate protein kinase C significantly in this cell type (6a). The level of phosphorylation of the H1 peptide was stimulated by phorbol ester treatment, but not by EGF treatment, of the cells (Fig. 4B). Protein kinase C pseudosubstrate inhibitor peptide blocked phosphorylation of the H1 peptide in this system (data not shown). These data show that EGF does not cause an increase in the uptake or utilization of nucleotide triphosphate in this system.

EGF stimulation of *ras*-encoded protein exchange requires tyrosine kinase but not phospholipase activity. The effect of the tyrosine kinase inhibitor genistein on the activation of guanine nucleotide exchange on *ras*-encoded protein in permeabilized Rat-1 cells was determined. Treatment of the cells with this inhibitor at the time of permeabilization almost completely abolished the ability of EGF to stimulate


A
Nucleotide bound to p21 (cpm)
300
200
100
0
0
5
10
15
time (min)
B
GDP
GTP
0 2.5 5 10 15 0 2.5 5 10 15 mins
control
+ EGF
C
Nucleotide bound to p21 (cpm)
100
80
60
40
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0
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time (min)
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0 5 15 0 5 15 Time (min)
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E
cold GTP chase added
Nucleotide bound to p21 (cpm)
100
50
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0
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time (min)
F
Nucleotide bound to p21 (cpm)
150
100
50
0
0
5
10
15
Time (min)
G
170K
0 2 5 10 0 2 5 10 Time + EGF (min)
Permeabilized
Intact

FIG. 2. Stimulation of guanine nucleotide exchange on *ras*-encoded protein in permeabilized Rat-1 cells. (A) [α-^{32}P]GTP was added to streptolysin O-permeabilized Rat-1 cells for the indicated times in the presence (●) or absence (○) of 100 ng of EGF per ml. The cells were then lysed, and *ras*-encoded protein was immunoprecipitated. Total nucleotide binding to *ras*-encoded protein was measured by scintillation counting. (B) Thin-layer chromatogram of nucleotide bound to *ras*-encoded protein from panel A. The positions at which GTP and GDP standards ran are indicated. (C) [^{35}S]GTPγS binding to *ras*-encoded protein in permeabilized cells with (●) or without (○) EGF treatment. (D) Thin-layer chromatogram of nucleotide bound to *ras*-encoded protein from panel C. (E) Labelled GTP was incubated with permeabilized cells as for panel A for 5 min prior to addition of a 1 mM unlabelled GTP chase for the indicated times. (F) Effect of pretreatment of Rat-1 cells with EGF on the rate of nucleotide binding to *ras*-encoded protein. Streptolysin O and [α-^{32}P]GTP were added at time zero (without prepermeabilization). EGF treatment was either at time zero (●) or 5 min earlier (Δ), or cells were unstimulated (○). (G) Tyrosine phosphorylation of the EGF receptor in permeabilized and intact Rat-1 cells after treatment with EGF. The EGF receptor was immunoprecipitated with antipeptide antibodies and immunoblotted with antiphosphotyrosine antibody.

nucleotide exchange on *ras*-encoded protein (Fig. 5A). As expected, the activity of the EGF receptor tyrosine kinase is required for the effects of EGF on the cells. Under these conditions, autophosphorylation of the EGF receptor in permeabilized cells was heavily inhibited by genistein (Fig. 5B).

In previous experiments, the permeabilization buffer used has been designed to maintain a free-calcium concentration of 100 nM, similar to that in quiescent cells. Under these conditions, growth factors can cause activation of a number of phospholipases, particularly PLA_2, PLC, and PLD in the case of EGF. When the free calcium is buffered to levels below 1 nM, no activation of these phospholipases can occur, although activation of tyrosine kinase activity is still normal (14). Normal stimulation of nucleotide binding to *ras*-encoded protein in response to EGF was still seen in the absence of calcium (Fig. 5C), so it is unlikely that any calcium-dependent steps, such as phospholipase activation, can lie on the signalling pathway between the EGF receptor and the *ras*-encoded protein guanine nucleotide exchange factor.

EGF does not affect GAP activity in Rat-1 cells. While the activity of the *ras*-encoded protein guanine nucleotide exchange factors appears to be stimulated by EGF in these cells, it is also possible that the activity of GAPs, such as $p120^{GAP}$ and neurofibromin, could also be regulated. To investigate this, the GAP activity in Triton X-100 lysates of Rat-1 cells was assayed after various lengths of treatment with EGF (Fig. 6). No change in the total assayable GAP activity in response to EGF was seen. These extracts were shown to contain both $p120^{GAP}$ and neurofibromin. Assay of the activity of these molecules in washed specific immune complexes from cells lysed either in nonionic detergent or in RIPA buffer also showed no effect of EGF treatment (data not shown). Furthermore, the amounts of total GAP activity in the plasma membrane fraction from EGF-treated and untreated cells were also identical, indicating that the activity of a subfraction of GAP with good access to $p21^{ras}$ was not subject to regulation by EGF (data not shown). We cannot, however, rule out the possibility that the activity of a relatively small subfraction of GAP or a GAP-related protein is regulated.

DISCUSSION

The data presented in this report imply that a guanine nucleotide exchange factor for *ras*-encoded protein is positively regulated by the EGF receptor tyrosine kinase in normal Rat-1 fibroblasts. The mechanisms by which *ras*-encoded protein is controlled by any stimuli in fibroblasts or by EGF in any cell type have previously been controversial. Our current study indicates that regulation of *ras*-encoded protein by EGF in fibroblasts is entirely due to modulation of exchange activity without involvement of changes in GAP activity; this is the exact opposite of the situation we previously characterized in T lymphocytes, in which *ras*-encoded protein-activating stimuli do not affect exchange activity but do strongly suppress GAP activity (7). Inhibition of GAP activity has also been described in human erythroleukemia cells in response to erythropoietin (27). By contrast, NGF treatment of PC12 cells causes a small increase in GAP activity along with a larger increase in an exchange activity measureable in cell lysates (15). While this stimulation of exchange could clearly account for the activation of *ras*-encoded protein by NGF in these cells, this activity is likely to be similar to that characterized previously in brain extracts, which is not specific for *ras*-encoded protein but acts on all members of the *ras*-encoded protein superfamily tested (13). We were not able to determine the specificity of the exchange activity that we have measured in permeabilized fibroblasts for different members of the *ras*-encoded protein superfamily, since highly efficient antibodies are required to perform these assays. It is clear, however, that the effects measured are not nonspecific ones that act on all GTP-binding proteins.

Detailed kinetic analysis of guanine nucleotide exchange on $p21^{ras}$ in whole or permeabilized cells is not possible,

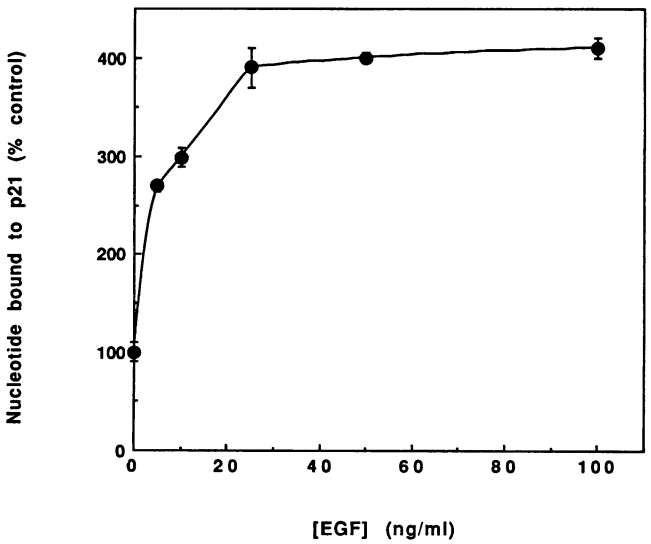

FIG. 3. Dependence of *ras*-encoded protein activation in permeabilized Rat-1 cells on EGF concentration. Permeabilized Rat-1 cells were treated with EGF at the indicated concentrations, and labelled GTP was added for 10 min. The amount of nucleotide bound to *ras*-encoded protein at the end of this period is represented as a percentage of the amount bound in unstimulated cells.

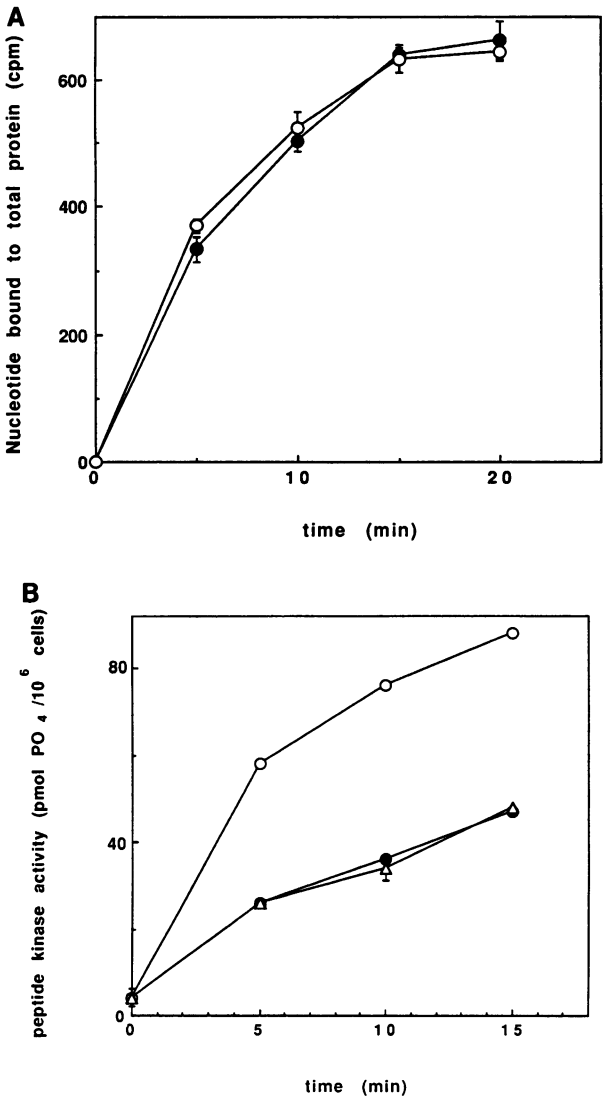

FIG. 4. EGF does not affect nucleotide binding to non-*ras*-encoded proteins. (A) Total nucleotide bound to Triton X-100-soluble protein from permeabilized Rat-1 cells treated with EGF. Protein was immobilized on nitrocellulose filters. ●, EGF treatment; ○, control. (B) Phosphorylation of histone H1-derived peptide substrate for protein kinase C in permeabilized Rat-1 cells treated with 100 ng of phorbol dibutyrate per ml (○) or 100 ng of EGF per ml (●). △, control.

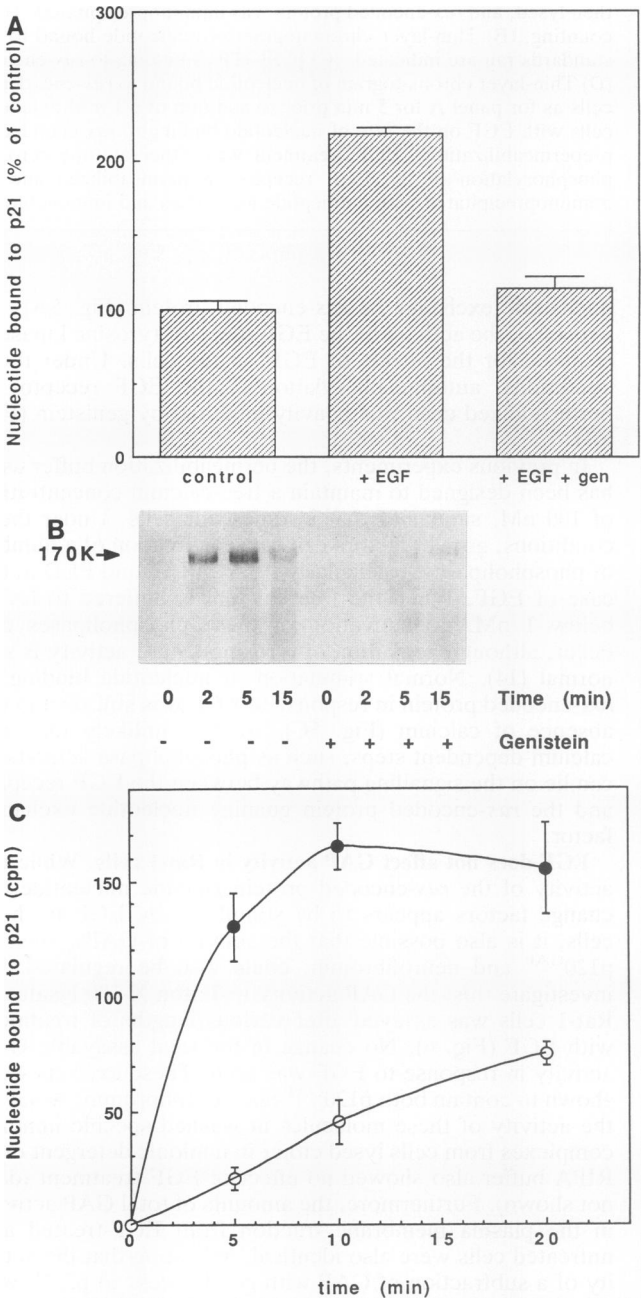

FIG. 5. Role of tyrosine kinase activity and calcium in regulation of *ras*-encoded protein exchange. (A) Effect of the tyrosine kinase inhibitor genistein (gen, 40 μg/ml, added at the same time as streptolysin O) on rate of guanine nucleotide binding to *ras*-encoded protein in EGF-treated, permeabilized Rat-1 cells at the 10-min time point. (B) Effect of genistein on autophosphorylation of the EGF receptor in EGF-treated, permeabilized Rat-1 cells. The EGF receptor was immunoprecipitated from lysates of permeabilized cells and then immunoblotted with antiphosphotyrosine antibodies. 170K, molecular weight of 170,000. (C) EGF stimulation of nucleotide binding to *ras*-encoded protein in Rat-1 cells permeabilized in zero-calcium buffer (same buffer as previously described but with additional 10 mM EGTA). All points are averages of duplicates. ●, EGF treatment; ○, control.

owing to the large number of unknowns involved. It seems likely, however, that to maintain a 10-fold excess of *ras*-encoded protein–GDP over *ras*-encoded protein–GTP, as found in unstimulated cells, the hydrolysis reaction must have a rate of 10 times the GDP-for-GTP exchange reaction. A 10-fold stimulation of the exchange reaction (roughly that seen in the permeabilized-cell system on treatment with EGF) would result in equal rates of hydrolysis and exchange and, hence, equal amounts or *ras*-encoded protein in the GTP- and GDP-bound states (as seen after maximal stimulation with EGF). A mutant *ras*-encoded protein, His116, with a 10-fold-increased nucleotide exchange rate in vitro, shows a level of activation comparable to that of normal *ras*-encoded protein in NIH 3T3 cells (31). Binding of the

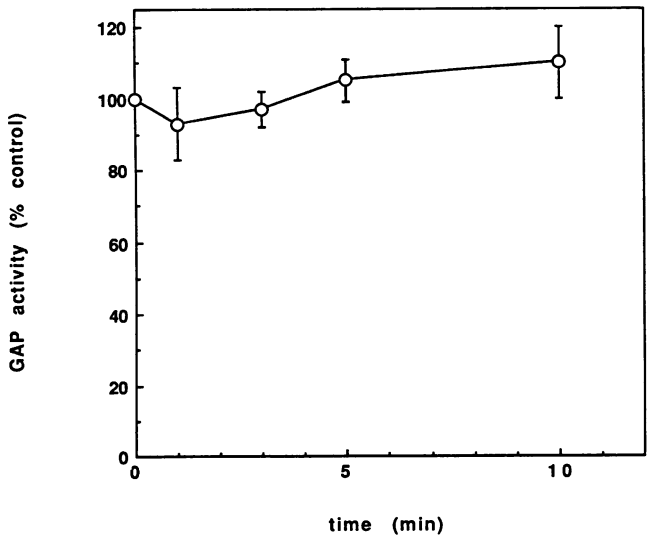

FIG. 6. Effect of EGF on GAP activity in Rat-1 cell lysates. Cells were treated with 100 ng of EGF per ml for the indicated times prior to lysis in Triton X-100 buffer. Total GAP activity in the lysate was measured at various dilutions and expressed as the dilution required to stimulate GTP hydrolysis on *ras*-encoded protein twofold. GAP activity is plotted as a percentage of the activity in untreated cells against time of EGF treatment. Average values of two experiments are shown.

guanine nucleotide to $p21^{ras}$ in EGF-stimulated, permeabilized cells was half maximal in just over 2 min. Taken together, these data are compatible with the notion that the effects on EGF stimulation of exchange on *ras*-encoded protein seen in permeabilized cells are a true reflection of the situation in the whole cell, with changes in nucleotide exchange rate alone being capable of accounting for *ras*-encoded protein regulation. Similar conclusions have recently been reached more indirectly by studying the behavior of exchange and effector mutants of *ras*-encoded protein upon PDGF treatment of whole NIH 3T3 cells (31).

A notable feature of the activation of *ras*-encoded protein in intact Rat-1 fibroblasts is that it is rapid and transient. After 10 min, the level of GTP bound to p21 has declined considerably. However, in permeabilized cells the rate of nucleotide exchange on p21 remains stimulated for about 10 min (Fig. 2E). The slightly slower autophosphorylation of the EGF receptor in permeabilized cells might indicate that this signalling system performs more slowly in permeabilized cells than in intact cells. Cells that have been treated with EGF intact for 5 min before permeabilization show a much smaller stimulation of nucleotide exchange on *ras*-encoded protein than do those which have been treated simultaneously with growth factor and streptolysin O, implying that in whole cells the activation of exchange is transient. Attenuation of the increased exchange activity could therefore be responsible, at least in part, for the rapid return of *ras*-encoded protein to an inactive state following EGF stimulation. The mechanism responsible for returning the exchange activity to basal levels is not known.

The difference between lymphocytes and fibroblasts in the regulation of *ras*-encoded protein is initially somewhat surprising. In permeabilized lymphocytes, there exists a very high basal level of exchange activity which does not alter with cell stimulation (7); this is about 10-fold faster than in quiescent fibroblasts. Extracellular stimuli lead to a decrease of GAP activity in T-cell extracts and accumulation of GTP at the expense of GDP on *ras*-encoded protein in permeabilized T cells without affecting the overall binding rate. By contrast, in fibroblasts GAP activity is not affected by EGF but the rate of guanine nucleotide binding to *ras*-encoded protein increases severalfold without, however, any measurable accumulation of GTP, only that of GDP. The failure to accumulate GTP on *ras*-encoded protein in EGF-stimulated permeabilized fibroblasts probably reflects the high level of total GTPase activity in these cells: only GDP appears to be available to bind to *ras*-encoded protein, presumably owing to the very rapid hydrolysis of GTP by GTPases other than *ras*-encoded protein upon entry into permeabilized cells. We speculate that in intact cells the GTP levels on *ras*-encoded protein increase in response to EGF since higher levels of GTP are maintained in the cytosol.

The mechanism by which the activated EGF receptor stimulates the activity of *ras*-encoded protein exchange factors is not known but is clearly likely to involve tyrosine phosphorylation, either of an exchange factor itself or of an intermediate protein. Tyrosine phosphatase inhibitors have been reported to stabilize *ras*-encoded protein exchange activity purified from rat brain (30). We detected no evidence of involvement of phospholipases in this regulation. The nature of the exchange factor involved is unknown: likely candidates are the mammalian CDC25 homologs (16, 25, 28) or those more closely related to *Drosophila* sos (2, 3, 26), but it is also possible that smg p21 GDS, which acts on K-*ras*-, *rap*-, and *rho*-encoded proteins (17), could be stimulated by EGF.

The considerable differences in the regulation of *ras*-encoded proteins seen in different cell types could reflect the large number of roles that *ras*-encoded protein can play, including stimulation of cell growth, inhibition of cell growth, induction of differentiation, and inhibition of differentiation. In addition, many different types of stimuli are capable of activating *ras*-encoded protein, some only in certain cell types. The manner in which *ras*-encoded protein is regulated in a given cell type may well be determined by the guanine nucleotide exchange factors present, since these appear to be expressed in a tissue-specific manner (25), unlike GAPs. Rapid regulation of *ras*-encoded protein via modulation of GAP activity can be achieved only in cells in which there is a high basal level of guanine nucleotide exchange activity. The *ras*-encoded protein signalling system is clearly very versatile: the existence of multiple regulatory mechanisms may allow fine adjustment to the needs of a particular cell type. The data presented here indicate that in fibroblasts the principal mechanism by which *ras*-encoded protein is controlled in response to growth factors involves modulation of guanine nucleotide exchange factor activity.

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