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Binding of the H-ras p21 GTPase Activating Protein by the Activated Epidermal Growth Factor Receptor Leads to Inhibition of the p21 GTPase Activity in Vitro†

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ABSTRACT: There is strong, albeit indirect, evidence for a mitogenic signal transduction pathway comprising growth factors, growth factor receptors, the GTPase activating protein (p120-GAP), and $p21^{ras}$. To demonstrate a direct physical association between these proteins in the absence of other cell constituents, their interaction was studied in vitro. Our results obtained with homogeneous protein preparations show that the activated epidermal growth factor (EGF) receptor phosphorylates p120-GAP at one site. Phosphorylated p120-GAP remains firmly bound to the receptor at physiological salt concentration; this leads to product inhibition of the receptor kinase activity as shown by diminished autophosphorylation activity and lack of turnover in p120-GAP phosphorylation. Phosphorylated p120-GAP is as active in stimulating the $p21^{ras}$·GTPase as unphosphorylated GAP. p120-GAP, however, when bound to the EGF receptor is by a factor of 2 less active in stimulating the $p21^{ras}$·GTPase than free p120-GAP. This effect might contribute to regulate the steady-state level of p21·GTP.

$p21^{ras}$ is a guanine nucleotide protein which plays a central role in growth control. It is active in the GTP form and inactive in the GDP form [for review, cf. Barbacid (1987) and Grand and Owen (1991)]. $p21^{ras}$ has a weak intrinsic GTPase activity which can be strongly stimulated by GTPase activating proteins p120-GAP and NF1-GAP (Trahey & McCormick, 1987; Xu et al., 1990; Martin et al., 1990). The steady-state level of $p21^{ras}$·GTP is considered to be a determinant for a proliferative response [for review, cf. Hall (1990), McCormick (1990), Parsons (1990), Marshall (1990), and Lowy et al. (1991)]. Transforming mutants of $p21^{ras}$ are more likely to be found in the GTP form (Feig & Cooper, 1986) because, in general, they have a much lower GTPase activity. This activity cannot be accelerated by p120-GAP and NF1-GAP, although both interact with the oncogenic p21 mutants (Vogel, 1988; Bollag & McCormick, 1991). Mitogenic stimulation of various cells, e.g., of T-cells by phytohemagglutinin (Downward et al., 1990) and Swiss 3T3 or NIH 3T3 cells by platelet-derived growth factor (PDGF) as well as EGF (Satoh et al., 1990a,b; Gibbs et al., 1990), results in the accumulation of $p21^{ras}$·GTP. Also, transformation of cells with the erbB-2 oncogene, which encodes an activated growth factor receptor, leads to an increase of $p21^{ras}$·GTP (Satoh et al., 1990b). Similar observations were made with cells transformed with v-src and v-abl (Gibbs et al., 1990). On the other hand, it was shown that stimulation of various cells by growth factors, e.g., of fibroblasts by PDGF (Molloy et al., 1989; Kaplan et al., 1990; Kazlauskas et al., 1990; Anderson et al., 1990) or EGF (Anderson et al., 1990; Ellis et al., 1990; Margolis et al., 1990; Bouton et al., 1991) and macrophages by colony stimulating factor (Reedijk et al., 1990), leads to the phosphorylation of p120-GAP and, in the case of PDGF and EGF, to its association with the respective growth factor receptor. These results were obtained by immunoprecipitation experiments with whole cell extracts analyzed by SDS–PAGE. The interaction between p120-GAP and EGF receptor requires the SH2 domain of p120-GAP [Moran et al., 1990; Anderson et al., 1990; for review, cf. Koch et al. (1991) and Heldin (1991)] and the phosphorylated carboxy terminus of the EGF receptor (Margolis et al., 1990). A possible mechanism for the regu-

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lation of the p21^{ras}·GTP level in response to the activation of a growth factor receptor is suggested by these results: binding of a growth factor to its receptor causes autophosphorylation as well as p120-GAP phosphorylation and, thereby, inhibits its p21^{ras}·GTPase stimulatory activity. The molecular details of this inhibition have not yet been resolved: it could be that phosphorylated p120-GAP is unable to stimulate the p21^{ras}·GTPase or, alternatively, that phosphorylated p120-GAP remains firmly bound to the receptor and, thereby, is sequestered from interacting with p21^{ras}. To distinguish these possibilities, we have investigated the interaction between the purified individual components of this signal transduction pathway: EGF receptor with full enzymatic activity (Weber et al., 1984; Weber & Gill, 1987), human p120-GAP from recombinant baculovirus infected Sf9 cells (Halenbeck et al., 1990), and human p21^{ras} from genetically engineered *Escherichia coli* cells (Tucker et al., 1986).

Experimental Procedures

Proteins. Three proteins were purified to homogeneity, as shown by SDS–PAGE:

(I) Ha-ras p21 was overexpressed in and isolated from *E. coli* as described (Tucker et al., 1986).

(II) Human p120-GAP was prepared from Sf9 cells infected with recombinant baculovirus (Halenbeck et al., 1990) and stored at 4 °C. Depending on the particular batch, the p120-GAP preparation contained in addition to full-length p120-GAP truncated GAP species of $M_r \geq 95000$ which retain full enzymatic activity, presumably because they have an intact C-terminal domain (Halenbeck et al., 1990; Serth et al., 1991).

(III) Human EGF receptor was purified from A341 cells by immunoaffinity chromatography as described (Weber et al., 1984). The preparation contains an approximately 100-fold excess of EGF which was used to elute the EGF receptor from the immunoaffinity column.

Phosphorylation Assay. Autophosphorylation of the activated EGF receptor and p120-GAP phosphorylation were carried out in a reaction mixture containing 20 mM Hepes, pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, and 10 μM [γ-^{32}P]ATP at the temperatures indicated. The phosphorylation reaction was stopped by addition of SDS–PAGE loading buffer and the reaction mixture analyzed by SDS–PAGE on 8% gels. Bands representing phosphorylated EGF receptor and p120-GAP, respectively, were visualized by autoradiography. Individual bands were cut out of the gel, and the radioactivity was determined by scintillation counting.

p21 GTPase Assay. The GTPase activity of p21^{ras} was determined by the charcoal method (Leupold et al., 1983). p21^{ras}·[γ-^{32}P]GTP was freshly prepared by incubating p21^{ras}·GDP with excess [γ-^{32}P]GTP in 20 mM Hepes, pH 7.5, and 10 mM EDTA for 30 min at 25 °C. Unbound nucleotides and EDTA were removed from the preparation by gel filtration (MAP-5, Pharmacia).

Coelectrophoresis Experiments. Eight-microliter aliquots of the phosphorylation mixtures containing phosphorylated p120-GAP and EGF receptor were added to 2 μL of loading solution (1% Ficoll, 0.025% bromophenol blue, 0.025% xylene cyanol) and loaded onto a 3% PAGE gel. The electrophoretic run was performed at 0 °C in 50 mM Tris–borate, pH 8.0, and 0.05% (w/v) Triton X-100. Subsequently, the gel was subjected to autoradiography. Individual bands were cut out of the gel, and their protein composition after elution was analyzed by SDS–PAGE followed by autoradiography.

Results

The autophosphorylation of the EGF receptor and the phosphorylation of p120-GAP by the activated EGF receptor were investigated as described under Experimental Procedures. Kinetic experiments, carried out at 0 °C to slow the reaction, demonstrate that in the presence of ATP and Mn^{2+} ions EGF receptor and p120-GAP become phosphorylated when EGF receptor is activated by EGF and that the autophosphorylation [or rather transphosphorylation (Lammers et al., 1990)] is much faster than the phosphorylation of p120-GAP (Figure 1). Subsequent studies were performed at 25 or 37 °C with varying concentrations of p120-GAP and EGF receptor in order to find out how many phosphoryl groups are incorporated into p120-GAP and EGF receptor by the EGF receptor kinase activity. Figure 2A shows that approximately three phosphoryl groups are incorporated into the EGF receptor. This level is diminished to two in the presence of p120-GAP, indicating that p120-GAP interacts with the receptor and thereby prevents phosphorylation at one of three autophosphorylation sites of the EGF receptor, presumably site P2 or P3 as concluded from the phosphorylation kinetics (Downward et al., 1984; Margolis et al., 1989). The phosphorylation sites are currently being mapped by phosphopeptide analysis. The degree of inhibition of the EGF receptor autophosphorylation is dependent on p120-GAP concentration (data not shown). Figure 2B shows that a maximum of one phosphoryl residue can be incorporated into p120-GAP by the EGF receptor kinase, in agreement with results obtained previously by Liu and Pawson (1991), who showed that the EGF receptor phosphorylates p120-GAP at Tyr-460, adjacent to the SH2 domain. However, this is only the case when EGF receptor is in excess over p120-GAP. The normalized incorporation yield (GAP phosphorylated/total GAP) decreases as the p120-GAP concentration increases. A straightforward explanation for this finding is that after phosphorylation p120-GAP remains firmly bound to the receptor and inhibits the EGF receptor kinase from further enzymatic turnover. Figure 2C shows that at a high excess of p120-GAP over EGF receptor between one and two p120-GAP molecules are phosphorylated per EGF receptor. Given the uncertainty of our concentration determination of active p120-GAP and EGF receptor, this could be interpreted to mean that one phosphorylated p120-GAP remains bound to the receptor and prevents phosphorylation of other p120-GAP molecules.

It must be emphasized that the inhibition of the EGF receptor kinase turnover activity by phosphorylated GAP is

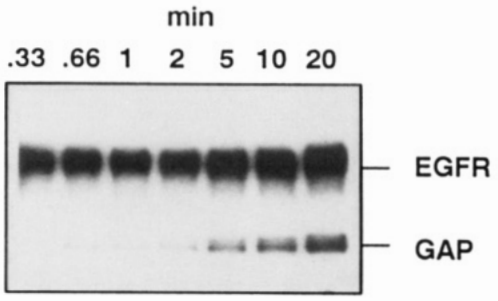

FIGURE 1: Autophosphorylation of activated EGF receptor and phosphorylation of p120-GAP by activated EGF receptor. A mixture of 30 nM EGF receptor saturated with excess EGF and 40 nM p120-GAP was incubated in 150 mM KCl, 20 mM Hepes, pH 7.5, 5 mM $MgCl_2$, and 1 mM $MnCl_2$ with 10 μM [γ-^{32}P]ATP at 0 °C. After 0.33, 0.66, 1, 2, 5, 10, and 20 min the reaction was stopped by addition of SDS–PAGE loading buffer. The reaction mixtures were subjected to SDS–PAGE on 8% gels. Bands representing phosphorylated EGF receptor and p120-GAP were visualized by autoradiography. The band representing p120-GAP is split into a doublet, because the p120-GAP preparation used contained in addition to intact p120-GAP (M_r = 120000) a slightly shortened GAP (M_r = 110000) which is fully active in stimulating the p21^{ras}·GTPase.

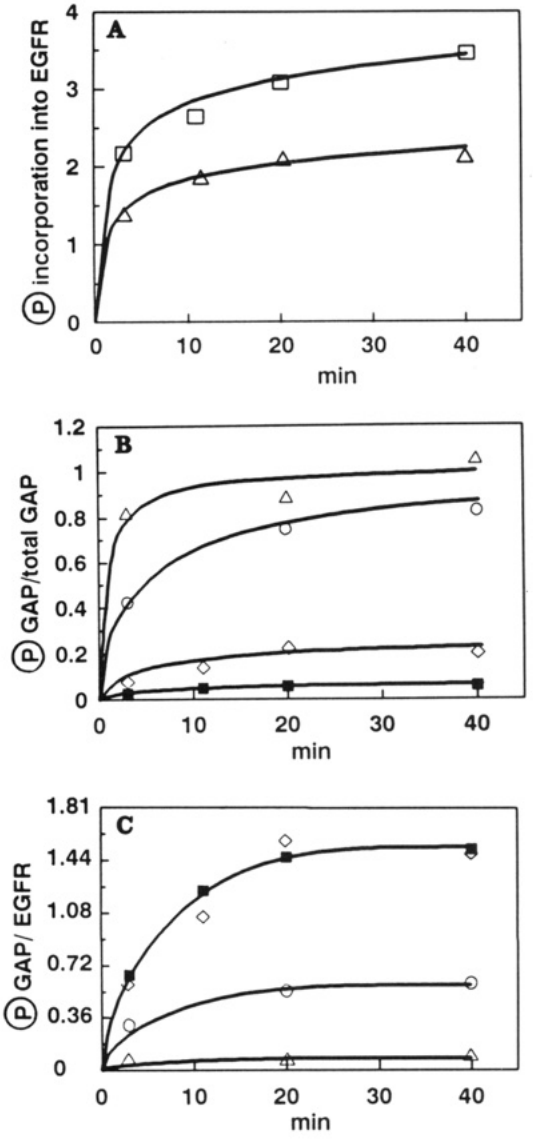

FIGURE 2: Kinetic analysis of the autophosphorylation of EGF receptor and phosphorylation of p120-GAP by the activated EGF receptor. A mixture of 45 nM EGF receptor saturated with excess EGF and 3, 30, 300, and 1050 nM p120-GAP was incubated in 150 mM KCl, 20 mM Hepes, pH 7.5, 5 mM $MgCl_2$, and 1 mM $MnCl_2$ with 10 μM [γ-^{32}P]ATP at 25 °C. Aliquots were withdrawn after 3, 11, 20, and 40 min, the reaction was stopped by addition of SDS–PAGE loading buffer, and the reaction mixture was analyzed by SDS–PAGE on 8% gels. Individual bands were cut out of the gel, and the radioactivity was determined by scintillation counting. The analyses of these data are shown in panels A–C. In (A) the autophosphorylation kinetics for EGF receptor are presented in the absence (□) and presence (△) of 300 nM p120-GAP. In (B) and (C) the phosphorylation kinetics for p120-GAP are represented for 3 (△), 30 (○), 300 (◇), and 1050 (■) nM p120-GAP normalized as phosphorylated p120-GAP/total p120-GAP (B) or phosphorylated p120-GAP/EGF receptor (C). It is important to note that with excess receptor over p120-GAP one phosphoryl residue can be incorporated into p120-GAP (B) and that with excess p120-GAP over receptor not more than 1.5 mol of p120-GAP/EGF receptor becomes phosphorylated (C).

observed at physiological salt concentration (150 mM KCl) and decreases upon lowering the salt concentration. Figure 3 shows the result of phosphorylation experiments carried out at various salt concentrations. In the absence of added salt the EGF receptor kinase shows turnover: phosphorylated GAP accumulates with time. p120-GAP phosphorylated in the absence of added salt was analyzed for its ability to stimulate the p21^{ras}·GTPase. It was found that p120-GAP, whether phosphorylated or not, shows the same p21^{ras}·GTPase stimulating activity (data not shown).

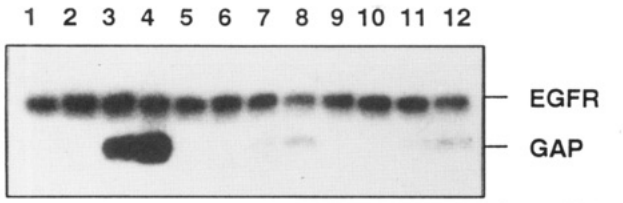

FIGURE 3: Ionic strength dependence of phosphorylation of p120-GAP by activated EGF receptor. EGF receptor (50 nM) saturated with EGF was incubated without (lanes 1, 2, 5, 6, 9, and 10) and with 500 nM p120-GAP (lanes 3, 4, 7, 8, 11, and 12) for 5 and 20 min, respectively, in 20 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 1 mM $MnCl_2$, and 10 μM [γ-^{32}P]ATP. The experiment was carried out in the presence of no (lanes 1–4), 100 mM (lanes 5–8), and 200 mM (lanes 9–12) added salt.

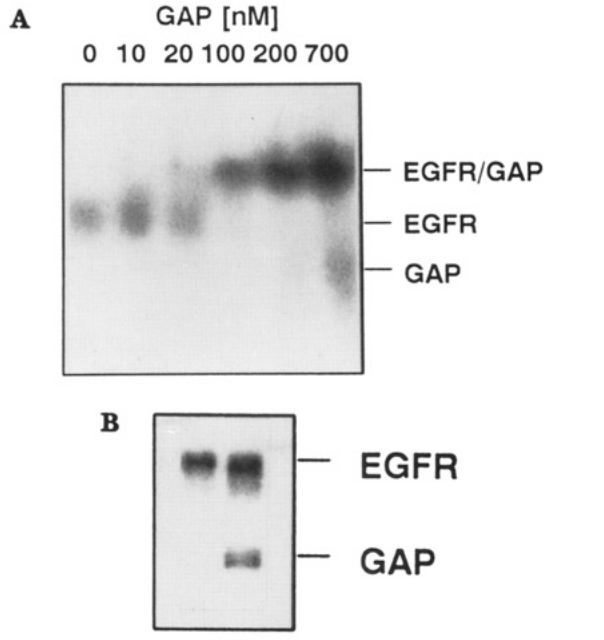

FIGURE 4: Analysis of complex formation between p120-GAP and the activated EGF receptor by coelectrophoresis. EGF receptor (30 nM) saturated with excess EGF was incubated with 0, 10, 20, 100, 200, and 700 nM p120-GAP in the presence of [γ-^{32}P]ATP for 15 min at 25 °C. Then 8 μL of the incubation mixture was loaded onto a 3% PAGE gel. The electrophoretic run was performed at 0 °C in 50 mM Tris–borate, pH 8.0, and 0.05% (w/v) Triton X-100. The gel was subsequently subjected to autoradiography, shown in (A). The analysis of the protein composition of the bands in lane 1 (30 nM EGF receptor) and in lane 5 (30 nM EGF receptor and 200 nM GAP) was done by SDS–PAGE. For this purpose the bands were excised from the native gel and the proteins eluted. After SDS–PAGE the gel was subjected to autoradiography, shown in (B). The lane on the left shows EGF receptor, and the lane on the right shows EGF receptor and GAP.

If the EGF receptor·p120-GAP product complex is as stable at 150 mM KCl as it must be in order to prevent the enzyme from enzymatic turnover, it should be possible to isolate such a complex by coelectrophoresis or cochromatography. Figure 4A shows a coelectrophoresis experiment under nondenaturing conditions. The band representing free EGF receptor disappears with increasing concentration of p120-GAP, and a new band appears which we assign to the complex, because the SDS–PAGE analysis of material eluted from this band shows that it contains EGF receptor and p120-GAP (Figure 4B). It must be emphasized that in this experiment association between EGF receptor and p120-GAP is demonstrated by autoradiography of phosphorylated proteins. Hence, only complexes containing phosphorylated receptor and/or GAP are seen.

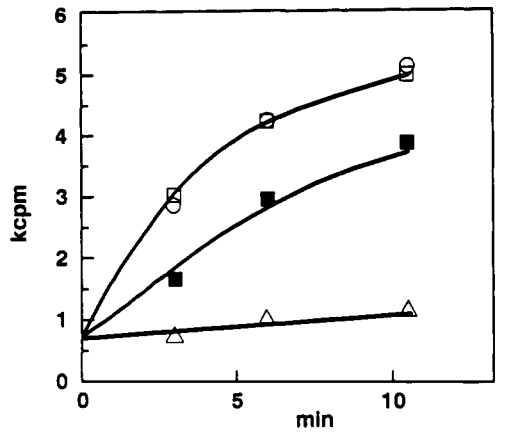

FIGURE 5: Analysis of the effect of p120-GAP binding to activated EGF receptor on the p120-GAP-stimulated $p21^{ras}$·GTPase activity. A mixture of 100 nM EGF receptor saturated with excess EGF and 40 nM p120-GAP was preincubated for 10 min at 37 °C in 150 mM KCl and 20 mM Hepes, pH 7.5, with (■) and without (□) 5 μM ATP. Subsequently, 250 nM $p21^{ras}$·[γ-^{32}P]GTP was added and the incubation continued. After defined time intervals the amount of GTP hydrolyzed was determined by measuring the phosphate release. (△) and (○) denote the control experiments, for which $p21^{ras}$·GTP was incubated in the absence (△) and presence (○) of p120-GAP, both without EGF and EGF receptor.

In order to find out whether the sequestration of phosphorylated p120-GAP by the activated EGF receptor interferes with the function of the p120-GAP-stimulated $p21^{ras}$·GTPase, we have measured the p120-GAP stimulated p21 GTPase in the absence and presence of activated receptor. Figure 5 shows the effect of incubating $p21^{ras}$·GTP with p120-GAP, EGF, and EGF receptor on GTP hydrolysis. In the presence of ATP, i.e. the cosubstrate for the receptor kinase, $p21^{ras}$·GTP is hydrolyzed with a slower rate than observed in the absence of ATP. We conclude that autophosphorylation of the receptor and/or phosphorylation of p120-GAP is (are) responsible for the inhibition of the p120-GAP-stimulated $p21^{ras}$·GTPase activity. The inhibitory effect depends on EGF receptor concentration (data not shown) and reaches 50% at maximum.

Discussion

Our results demonstrate the direct physical association of the activated EGF receptor and p120-GAP. They show that in response to this association, without additional factors, p120-GAP is phosphorylated and remains firmly bound to the receptor at physiological salt concentration. The p120-GAP·EGF receptor complex cannot efficiently carry out the functions associated with its isolated components, viz. activation of the $p21^{ras}$·GTPase by p120-GAP and autophosphorylation of the EGF receptor to the level observed in the absence of p120-GAP. As phosphorylated p120-GAP stimulates the $p21^{ras}$·GTPase to the same extent as nonphosphorylated p120-GAP (this paper; Moran et al., 1991), it must be the sequestration of phosphorylated p120-GAP by the activated EGF receptor rather than the p120-GAP phosphorylation itself which is responsible for the increased lifetime of $p21^{ras}$·GTP. This conclusion concerns the in vitro situation in which the isolated components interact in solution.

Our approach makes use of purified components to reconstitute part of a signal transduction chain and allows us to analyze molecular events important for the processing of proliferative signals under defined conditions. It must be considered, however, that these components interact with many others [for review, cf. Cantley et al. (1991)]. First, the membrane association of the EGF receptor, of $p21^{ras}$, and, under certain conditions, also of p120-GAP via interaction with lipids (Tsai et al., 1989a,b; Serth et al., 1991) could modulate the interactions analyzed here, in particular because the formation of the EGF·EGF receptor complex is followed by rapid internalization and degradation (Carpenter & Cohen, 1990). It might be that the localization of GAP at the plasma membrane in proximity to $p21^{ras}$ may compensate for the EGF receptor induced reduction in GTPase activity. Second, p120-GAP is only one of a variety of other proteins, notably phospholipase C-γ1, phosphoinositide-3-kinase and several nonreceptor protein kinases, that interacts with the EGF receptor [Ullrich & Schlessinger, 1990; Carpenter & Cohen, 1990; for review, cf. Hall (1990) and Parsons (1990)]. The effect of these interactions on complex formation with p120-GAP and the EGF receptor remains to be analyzed. Third, transformation of cells by v-src, v-fps, or v-abl or stimulation with EGF induces the stable association of two phosphorylated proteins, p62–64 and p190, with p120-GAP (Ellis et al., 1990; Bouton et al., 1991; Moran et al., 1990). It seems that p62–64 and p190 compete with the EGF receptor for binding of p120-GAP, because in EGF-stimulated cells p120-GAP is found associated only with p62 and p190 (Moran et al., 1991). The complexity of this network of interactions makes it impossible, at present, to decide how much of p120-GAP is available to interact with $p21^{ras}$ in a given metabolic situation in vivo. The finding that only 10% of the total soluble p120-GAP in PDGF-stimulated cells can be recovered as a complex with the PDGF receptor (Kazlauskas et al., 1990) may reflect that only a subset of the total p120-GAP population is free to interact with a receptor. Furthermore, it is not quite clear at present how much the $p21^{ras}$·GTP concentration must increase in order to induce proliferation. While Downward et al. (1990) have observed that the amount of GTP bound to $p21^{ras}$ rises from 5% to 50% upon stimulation of T-cells, Satoh et al. (1990a,b) and Gibbs et al. (1990) report an approximately 2-fold increase in $p21^{ras}$·GTP when fibroblasts were stimulated by PDGF or EGF. Such an increase in the $p21^{ras}$·GTP concentration may well be achieved by a sequestration of a part of the p120-GAP population, as shown here. On the other hand, it must be considered that GTP hydrolysis of $p21^{ras}$·GTP is stimulated not only by p120-GAP but also by NF1-GAP, both of which may be present in the same cell (Bollag & McCormick, 1991). NF1-GAP, therefore, may compensate in part for the sequestration of p120-GAP. Finally, the steady-state level of $p21^{ras}$·GTP in vivo is determined not only by the stimulation of the $p21^{ras}$·GTPase activity by p120-GAP and possibly NF1-GAP but also by the rate of nucleotide exchange which is catalyzed by guanine nucleotide exchange proteins (Crêchet et al., 1990; Wolfman & Macara, 1990; West et al., 1990). A high intracellular level of p21·GTP can, therefore, be obtained by receptor-mediated inhibition of a GTPase activating protein or receptor-mediated stimulation of a guanine nucleotide exchange factor, as demonstrated for the *Drosophila* Ras1 protein (Simon et al., 1991; Fortini et al., 1992; Bonfini et al., 1992). It is quite possible that both of these mechanisms can be used in concert to regulate $p21^{ras}$·GTP levels.

In conclusion, it is a recurrent theme in several recent reports that phosphorylation and binding of p120-GAP to activated receptor kinases may be responsible for the regulation of the activated state of $p21^{ras}$. Our finding that the sequestration of phosphorylated p120-GAP by the EGF receptor directly leads to an increase of the concentration of $p21^{ras}$·GTP supports this hypothesis. To what extent the binding of p120-GAP to the activated EGF receptor and the resulting inhibition of their activities contribute to the signal transduction of mitogenic

stimuli in vivo cannot be decided by our experiments. Our data clearly show, however, that the interaction between EGF receptor and p120-GAP may contribute to the control of proliferation.

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