Independent Regulation of Rap1 and Mitogen-Activated Protein Kinase by the α Chain of G_o

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Key Words

G proteins • G_o • Mitogen-activated protein kinase • Rap1 • Cell growth • Cell type differentiation

Abstract

Receptors coupled to $G_{i/o}$ proteins stimulate the mitogen-activated protein kinase (MAPK) cascade. The intracellular pathways linking the α chains of these G proteins to MAPK activation are not completely understood. One of the signaling molecules which has been suggested to act downstream of $G_{\alpha_{i/o}}$ is the small G protein Rap1. We investigated the role of Rap1 in MAPK stimulation by G_{α_o} in Chinese hamster ovary (CHO) cells. Our previous results have shown that in this cell system activated G_{α_o} strongly potentiates the MAPK response to the epidermal growth factor (EGF) receptor. Rap1 regulation was examined in cells transfected with Rap1 and wild-type G_{α_o} or the activated mutant G_{α_o} -Q205L. Immunocytochemical analysis detected both Rap1 and the G_{α_o} subunit at the plasma membrane as well as on perinuclear cytoplasmic vesicles. Expression of wild-type G_{α_o} had no significant effect on the levels of activated Rap1. In contrast, G_{α_o} -Q205L virtually abolished the activation of Rap1 induced by EGF. Further experiments showed that MAPK stimulation by EGF was greatly inhibited by expression of activated Rap1, suggesting that Rap1 inhibition could mediate the effect of G_{α_o} on the MAPK cascade. However, G_{α_o} -Q205L efficiently inhibited the activation of Rap1 induced by fibroblast growth factor (FGF). We have previously found that the

ability of FGF to activate MAPK is not modified by G_{α_o} . In addition, expression of the GAP protein RAP1GAPII blocked Rap1 activation without affecting EGF- or FGF-dependent MAPK stimulation. These findings provide evidence for independent regulation of Rap1 and MAPK by the $G_o \alpha$ chain.

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Introduction

Many G-protein-coupled receptors regulate growth and differentiation of selected cell types by activating the mitogen-activated protein kinase (MAPK) cascade [1, 2]. Different G-protein subunits and a variety of intracellular pathways appear to be involved in these responses [1–3]. The ability of the α chains of the pertussis toxin-sensitive $G_{i/o}$ proteins to activate MAPK is well documented. Studies expressing activated mutants have demonstrated that $G_{\alpha_{i2}}$ induces MAPK activation in Rat-1 fibroblasts [4] and G_{α_o} enhances growth factor-dependent stimulation of MAPK in Chinese hamster ovary (CHO) cells [5]. In addition, the use of pertussis toxin-insensitive or dominant negative mutants of G_{α_o} has shown that this subunit mediates the MAPK response to muscarinic M_1 receptors and opioid receptors in CHO and neuroblastoma × glioma NG108-15 cells, respectively [6, 7]. The mechanisms used by $G_{\alpha_{i2}}$ and G_{α_o} to activate MAPK remain only partially understood [2, 5, 6].

There is evidence that $G\alpha_{i/o}$ control the function of the small G protein Rap1 by interacting with the GAP protein Rap1GAPII [8–10]. Two different models of Rap1GAPII regulation by $G\alpha_{i/o}$ have been proposed. Studies investigating the action of $G\alpha_o$ have shown that the inactive form of this subunit binds to Rap1GAPII and prevents the action of the GAP protein on Rap1. Activation of $G\alpha_o$ would then lead to release of Rap1GAPII and termination of the Rap1 signal [8]. Some results indicate that $G\alpha_{i2}$ regulates Rap1GAPII by a similar mechanism [10]. However, other studies have demonstrated that the active, and not the inactive, form of $G\alpha_{i2}$ interacts with Rap1GAPII. This in turn would result in Rap1 inactivation [9]. Recent data suggest that Rap1 regulation by $G_{i/o}$ α chains is probably even more complex because, at least in the case of $G\alpha_o$, interaction with Rap1GAPII also targets the GAP protein for proteasomal degradation [10].

The role of Rap1 in regulation of the MAPK pathway is controversial. There have been several suggestions that Rap1 can inhibit the MAPK cascade by acting as an antagonist of Ras [11–13]. Rap1 has also been shown to stimulate MAPK via activation of the B-Raf kinase in neurons and other cell systems [13–15]. However, other studies have failed to detect any significant effect of Rap1 on MAPK in various cell types [12, 16, 17]. The ability of Rap1 to inhibit the MAPK cascade has suggested that $G_{i/o}$ α chains stimulate MAPK via Rap1GAPII-mediated inactivation of Rap1. Indeed, in 293T cells the MAPK response to the G_i -coupled muscarinic M_2 receptor is enhanced by expression of Rap1GAPII and inhibited by a Rap1GAPII dominant negative mutant [9]. Whether stimulation of the MAPK cascade by $G\alpha_o$ involves regulation of Rap1 has not been investigated yet.

We have previously shown that activated $G\alpha_o$ strongly potentiates the MAPK response to the epidermal growth factor (EGF) receptor in CHO cells [5]. Here we report that in the same cell system, expression of wild-type $G\alpha_o$ does not modify the levels of activated Rap1. In contrast, the constitutively active mutant $G\alpha_o$ -Q205L potently inhibits the activation of Rap1 induced by EGF. Our results demonstrate that Rap1 inhibition does not mediate the stimulatory action of $G\alpha_o$ on MAPK and indicate the existence of Rap1-independent pathway(s) connecting this G-protein subunit to the MAPK cascade.

Materials and Methods

Materials

The $G\alpha_o$ antiserum used for immunoblot analysis was generated by immunization of rabbits with a synthetic peptide corre-

sponding to the C-terminal sequence ANNLRGCGLY. The anti-HA mouse monoclonal antibody 12CA5 was from Boehringer Mannheim (Mannheim, Germany); the anti-phospho-p44/42 MAPK (Thr202/Tyr204) rabbit polyclonal antibody was from New England BioLabs (Beverly, Mass., USA); the mouse monoclonal antibody against Rap1 was from Transduction Laboratories (Lexington, Ky., USA); the anti- $G\alpha_o$ mouse monoclonal antibody used for immunocytochemistry and anti-Rap1 rabbit polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Glutathione-Sepharose 4B and protein A-Sepharose CL-4B were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Isopropyl β -D-1-thiogalactopyranoside and polyornithine were from Sigma (St. Louis, Mo., USA), human EGF was from Boehringer Mannheim and human fibroblast growth factor (FGF) was from Calbiochem (La Jolla, Calif., USA). All other chemicals were reagent grade.

DNA Constructs

The cDNAs encoding wild-type $G\alpha_o$ and the activated mutant $G\alpha_o$ -Q205L, the human EGF receptor and HA-p44 MAPK were as previously described [5]. The plasmid for bacterial expression of a GST fusion protein of the Rap1-binding domain of Ral GDP dissociation stimulator (RalGDS-RBD) and pMT2-HA vectors containing the coding sequences of Rap1GAPII, wild-type Rap1A and the activated mutant Rap1A-G12V were kindly provided by J.L. Bos (University of Utrecht, The Netherlands).

Cell Culture, Transfections and Immunoblot Analysis of Protein Expression

CHO cells were maintained in culture in Dulbecco's modified Eagle's medium-Ham's F12 (1:1) supplemented with 10% FCS. Transient transfections were performed using the Transfectam reagent (Promega, Madison, Wisc., USA) as previously described [5]. The total amount of transfected DNA was kept constant by addition of empty vector. Cells were then incubated for 24 h in 10% FCS medium and serum starved for 18 h in medium containing 0.5% FCS. The expression of HA-tagged proteins was assessed by immunoblot analysis of cell lysates with anti-HA antibodies. Proteins were separated by 12% (for HA-Rap1) or 10% (for HA-p44 MAPK and HA-Rap1GAPII) SDS-PAGE and transferred to nitrocellulose filters (Schleicher & Schuell, Keene, N.H., USA). Filters were blocked for 1 h in Blotto (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20, 0.01% sodium azide, and 5% non-fat dry milk), incubated for 2 h at room temperature in Blotto containing 2.5 μ g/ml anti-HA antibodies, incubated for 2 h in Blotto with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, Calif., USA) and finally processed for enhanced chemiluminescence (Amersham Pharmacia Biotech). For immunoblot analysis of $G\alpha_o$ expression, lysates were subjected to 10% SDS-PAGE and filters were incubated with $G\alpha_o$ antiserum (1:3,000) overnight at 4°C in Blotto. Detection was performed as described above.

Rap1 Activation Assay

Activation of Rap1 was measured using the specific probe RalGDS-RBD [18]. The GST fusion protein of RalGDS-RBD was expressed in *Escherichia coli* by induction with 1 mM isopropyl β -D-1-thiogalactopyranoside for 3 h at 37°C. Bacterial cells were harvested by centrifugation and resuspended in PBS containing 1 mM EDTA, 0.5 mM DTT, 1 mM Na_3VO_4 , 1 mM PMSF, 10 μ g/ml

aprotinin and 10 $\mu\text{g/ml}$ leupeptin. After sonication and incubation with 1% Triton X-100 for 40 min at 4°C, lysates were clarified by centrifugation at 13,000 rpm for 10 min at 4°C. GST-RalGDS-RBD was immobilized by incubating lysates for 1 h at 4°C with glutathione-Sepharose beads pre-equilibrated in PBS containing 1 mM EDTA, 0.5 mM DTT, 1% Triton X-100, 10% glycerol, 0.1 mM Na_3VO_4 , 1 mM PMSF, 1 $\mu\text{g/ml}$ aprotinin and 1 $\mu\text{g/ml}$ leupeptin. Beads were washed three times with the same buffer and three times with Ral buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2.5 mM MgCl_2 , 1 mM DTT, 1% Nonidet P-40, 10% glycerol, 1 mM Na_3VO_4 , 10 mM NaF, 250 μM PMSF, 10 $\mu\text{g/ml}$ aprotinin and 10 $\mu\text{g/ml}$ leupeptin). Immobilized GST-RalGDS-RBD was then used to pull down activated Rap1. Cells in 10-cm dishes were incubated in serum-free medium in the absence or presence of EGF or FGF, washed once with ice-cold Ca^{2+} - and Mg^{2+} -free PBS and lysed in 0.8 ml of Ral buffer. After centrifugation at 13,000 rpm for 10 min at 4°C, the supernatant containing about 1.5 mg of total proteins was immediately incubated with glutathione-Sepharose beads (200 μl of 50% slurry containing about 80 μg of GST-RalGDS-RBD) for 2 h at 4°C. Samples were washed three times with Ral buffer, proteins were separated by 12% SDS-PAGE, and activated Rap1 was detected by immunoblot analysis with anti-HA antibodies as described above.

Analysis of MAPK Phosphorylation

To assess phosphorylation of MAPK, cells in 6-cm dishes were incubated in serum-free medium in the absence or presence of EGF or FGF and lysed in 0.6 ml of buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM DTT, 200 μM Na_3VO_4 , 40 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 mM PMSF and 10 $\mu\text{g/ml}$ chymostatin, leupeptin, antipain and pepstatin. Lysates were cleared by centrifugation at 13,000 rpm for 15 min at 4°C, and 50 μg of proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose filters. Filters were kept for 2 h in BSA blocking solution (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.02% sodium azide, and 5% BSA) and incubated in the same solution with anti-phospho-MAPK antibodies (1:2,000) for 2 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence. In a set of experiments, transfected HA-p44 MAPK was immunoprecipitated from cell lysates by incubation with 2 μg of HA-antibodies for 2 h at 4°C. Immune complexes were collected with protein A-Sepharose (50 μl of 50% slurry) for 1 h at 4°C, centrifuged, and washed three times in lysis buffer. Two equal parts of each immunoprecipitate were then separated by 10% SDS-PAGE and transferred to nitrocellulose filters. Filters were probed with anti-phospho-MAPK antibodies or anti-HA antibodies as described above.

Immunocytochemistry and Confocal Laser Microscopy

For immunocytochemistry studies, CHO cells on polyornithine-coated glass coverslips were washed with PBS, fixed for 1 h with 4% paraformaldehyde and quenched with 100 mM glycine in 120 mM phosphate buffer. After 1 h at 4°C in 120 mM phosphate buffer containing 1% BSA and 0.4% saponin, coverslips were incubated for 3 h at room temperature in the same solution containing primary antibodies (1:200) and extensively washed. Subsequently, coverslips were incubated for 2 h with FITC-, Texas Red- or Cy5-conjugated fluorescent antibodies (Jackson Immuno-Research, West Grove, Pa., USA), extensively washed and mounted on glass slides with FluorSave Reagent (Calbiochem,

Darmstadt, Germany). Samples were observed on a Zeiss Axioskop microscope equipped with a confocal head (LSM 510). Confocal images were scanned using a Plan-Apochromat 63 $\times$ /1.40 DIC objective. Excitation was obtained by Argon/2 (488 nm), helium-neon1 (543 nm) and helium-neon2 (633 nm) lasers. Signals were generated and collected individually on a frame-by-frame basis at 2,048 $\times$ 2,048 resolution. Similar pinhole sizes and amplifier settings were used to obtain double labeling images, and no further manipulation of image files was performed. Laser intensities and amplification settings for each individual channel were selected such that autofluorescence seen with control cells was minimized. Images were exported as JPG files and assembled in panels by Adobe Photoshop.

Results

Activated $\text{G}\alpha_o$ Inhibits EGF Receptor-Dependent Rap1 Activation

Rap1 regulation was investigated in CHO cells transiently transfected with wild-type or mutationally activated $\text{G}\alpha_o$, the EGF receptor and HA-Rap1. As shown in figure 1A, cell exposure to EGF caused a marked accumulation of activated HA-Rap1, with a maximal response at 2 min after the addition of the growth factor. EGF-dependent HA-Rap1 activation was not significantly modified by expression of wild-type $\text{G}\alpha_o$, but was virtually abolished in the presence of $\text{G}\alpha_o$ -Q205L (fig. 1B). Expression of either wild-type or activated $\text{G}\alpha_o$ did not produce detectable changes in the levels of activated HA-Rap1 in unstimulated cells (fig. 1B). Immunoblot analysis with specific antibodies revealed that these results could not be accounted for by variable levels of expression of HA-Rap1 or $\text{G}\alpha_o$ in cells transfected with different cDNA combinations.

We verified the subcellular localization of transfected Rap1 and $\text{G}\alpha_o$ by immunocytochemical analysis. Figure 2A shows the pattern of staining obtained with anti-HA antibodies in cells expressing wild-type HA-Rap1, where a clear signal was detected in the perinuclear region as well as on the plasma membrane. As shown in figure 2B, cells transfected with the mutationally activated mutant Rap1-G12V gave comparable results. A similar distribution of endogenous Rap1 has been previously described [19]. Unfortunately, staining with commercially available anti-Rap1 antibodies was not sensitive enough to detect endogenous Rap1 in CHO cells (data not shown). As expected, both wild-type and mutationally activated $\text{G}\alpha_o$ were found localized at the plasma membrane (fig. 2C, D). $\text{G}\alpha_o$ -transfected cells also displayed labeling of cytoplasmic vesicular structures most abundant in the perinuclear region. As the anti- $\text{G}\alpha_o$ antibod-

Fig. 1. Effects of the EGF receptor and the $G\alpha_o$ subunit on Rap1 activation. CHO cells were transfected in 10-cm dishes, placed for 18 h in 0.5% FCS medium and incubated in the absence or presence of 100 ng/ml EGF. GTP-bound HA-Rap1 was isolated and detected with anti-HA antibodies as described under Materials and Methods. Immunoblot analysis of cell lysates with anti-HA or anti- $G\alpha_o$ antibodies was performed to determine the total amounts of HA-Rap1 and $G\alpha_o$. **A** Time course of HA-Rap1 activation by EGF. Cells were transfected with 4 μ g of HA-Rap1 and 4 μ g of EGF receptor and stimulated with EGF for the indicated periods of time. The data are representative of two independent experiments performed in duplicate. **B** Effect of $G\alpha_o$ on Rap1 activation. Cells were transfected with 4 μ g of HA-Rap1, 4 μ g of EGF receptor and 4 μ g of empty vector, $G\alpha_o$ wild-type or $G\alpha_o$ -Q205L and stimulated with EGF for 2 min. The data are representative of three independent experiments performed in duplicate. Similar results were obtained when cells were exposed to EGF for 5 or 10 min.

ies used in our studies failed to detect the relatively low levels of endogenous protein present in untransfected CHO cells, we do not know whether the same structures also contain the endogenous $G\alpha_o$ subunit. This possibility would be consistent with previous reports that showed association of endogenous G proteins with various intracellular membranes [20, 21]. Analysis of cells cotransfected with $G\alpha_o$ and HA-Rap1 revealed colocalization of the two proteins at the level of both the plasma membrane and the cytoplasmic vesicles (fig. 2E, F).

Expression of Activated Rap1 Inhibits MAPK Stimulation by EGF

To clarify the role of Rap1 inhibition in the stimulatory effect of $G\alpha_o$ on the MAPK cascade, we examined whether Rap1 can somehow regulate MAPK in CHO cells. For these experiments, cells were transfected with the EGF receptor, HA-p44 MAPK and wild-type or mutationally activated Rap1. Immunoblot analysis of cell lysates or anti-HA immunoprecipitates with anti-phospho-

MAPK antibodies revealed that wild-type and, to a much greater extent, activated Rap1 inhibited the stimulation of HA-p44 MAPK induced by EGF (fig. 3). In cell lysates, inhibition of the response of endogenous p42 and p44 MAPK could also be detected. No significant changes in MAPK activation in the presence of either wild-type or activated Rap1 were observed in unstimulated cells. In all these experiments, we noticed that the levels of expression of Rap1-G12V were considerably lower than those observed after transfection of the wild-type protein (fig. 3A).

Inhibition of Rap1 Activation by $G\alpha_o$ or Rap1GAPII Does Not Affect Growth Factor-Induced MAPK Stimulation

The results obtained in cells expressing activated Rap1 appear consistent with the possibility that the action of $G\alpha_o$ on the MAPK pathway is mediated by inhibition of Rap1 activation. We have previously found that expression of $G\alpha_o$ -Q205L in CHO cells does not modify

Fig. 2. Subcellular distribution of transfected Rap1 and $G\alpha_o$. CHO cells were grown on coverslips and transfected with HA-Rap1 wild-type (**A**), HA-Rap1-G12V (**B**), $G\alpha_o$ wild-type (**C**) or $G\alpha_o$ -Q205L (**D**). The cell shown in **E** and **F** was cotransfected with HA-Rap1 wild-type and $G\alpha_o$ -Q205L. Transfections were performed with 0.75 μ g of each construct and cells were serum starved as described in the legend to figure 1. Pictures were obtained by confocal analysis of immunofluorescence labelings with anti-HA (**A**, **B**, **E**) and anti- $G\alpha_o$ (**C**, **D**, **F**) antibodies. Cells coexpressing wild-type HA-Rap1 and wild-type $G\alpha_o$ gave results similar to those obtained after transfection of wild-type HA-Rap1 and activated $G\alpha_o$. Inserts display enlargements of plasma membrane areas indicated by arrows. Bars: 10 μ m.

the stimulation of MAPK induced by endogenous FGF receptors [5]. We therefore investigated the effect of activated $G\alpha_o$ on Rap1 in cells stimulated with FGF. As shown in figure 4A, FGF induced a marked activation of HA-Rap1 with a maximal response at 5 min. The stimulatory effect of the growth factor on HA-Rap1 was blocked by expression of $G\alpha_o$ -Q205L (fig. 4B). The fact that inhibition of FGF-dependent Rap1 activation by $G\alpha_o$ does not result in an amplified MAPK response could be explained by a different sensitivity to Rap1 of the intracellular pathways linking EGF and FGF receptors to MAPK. However, expression of activated Rap1 proved to be able to efficiently inhibit FGF-induced HA-p44 MAPK activation (fig. 4C). A similar, but weaker, effect was detected in the presence of wild-type Rap1. In contrast to the results obtained in cells cotransfected with EGF receptors, we did not observe a significant inhibition of FGF activation of endogenous p42 and p44 MAPK by Rap1. This is most likely explained by the fact

that endogenous FGF receptors stimulate p42 and p44 MAPK in the entire cell population whereas Rap1 inhibition occurs only in the relatively limited fraction of transfected cells [5].

The effect of $G\alpha_o$ on the FGF response suggests that the activation of endogenous Rap1 which is induced by growth factors does not significantly regulate MAPK in CHO cells. To confirm this conclusion, we examined MAPK activation by EGF and FGF in cells expressing HA-Rap1GAPII. As shown in figure 5A, immunocytochemical analysis of HA-Rap1GAPII distribution revealed a diffuse cytosolic staining [9]. Some labeling of the plasma membrane was also observed. In the presence of Rap1GAPII, activated HA-Rap1 was undetectable under basal conditions and failed to accumulate upon stimulation with EGF or FGF (fig. 5B, and data not shown). As previously reported in cells transfected with the GAP protein Rap1GAP [22], Rap1GAPII also caused a significant decrease in the levels of expression of HA-Rap1. Fig-

Fig. 3. Rap1 expression inhibits EGF-induced MAPK activation. CHO cells in 6-cm dishes were transfected with 2 μ g of HA-p44 MAPK, 2 μ g of EGF receptor, and 2 μ g of empty vector, HA-Rap1 wild-type or HA-Rap1-G12V. Cells were then serum starved for 18 h and incubated in the absence or presence of 100 ng/ml EGF for 5 min. Activation of HA-p44 MAPK was assessed by immunoblot analysis of cell lysates (**A**) or anti-HA immunoprecipitates (**B**) with anti-phospho-p42/44 MAPK antibodies. Phosphorylation of endogenous p42 and p44 MAPK was also detected in cell lysates. Immunoblots with anti-HA antibodies were performed to determine total amounts of HA-Rap1 and HA-p44MAPK. Results are representative of two similar experiments in duplicate.

ure 5C shows HA-p44 MAPK activation by EGF and FGF in the absence or presence of Rap1GAPII. In control cells, activation of HA-p44 MAPK by EGF was already clearly detectable at 2 min, further increased at 5 min and was considerably reduced at 15 min. The effect of FGF on HA-p44MAPK was rather weak at 2 min, markedly increased at 5 min and declined at 15 min. Expression of Rap1GAPII did not significantly modify the basal levels of HA-p44 MAPK activation and the magnitude and time course of the responses induced by EGF and FGF. Consistently, no significant effect of Rap1GAPII on endogenous p42 and p44 MAPK was observed in these experiments. It should be noted that immunoblot analysis revealed multiple bands corresponding to HA-Rap1GAPII and that a change in the relative intensities of these bands was reproducibly observed after stimulation with either EGF or FGF. It is conceivable that these observations reflect modifications of the phosphoryla-

tion state of Rap1GAPII induced by the growth factors [23]. Taken together, these results indicate that the stimulatory effect of $G\alpha_o$ on the MAPK cascade observed in CHO cells is not explained by its ability to inhibit Rap1 activation.

Discussion

Various G-protein α subunits appear able to control the function of small G proteins [2, 3, 8–10, 24]. The results reported here demonstrate that activated $G\alpha_o$ can negatively regulate the Rap1 pathway in CHO cells. This conclusion is consistent with a previous report which showed inhibition of Rap1 activation by $G\alpha_o$ -Q205L in PC12 cells [8]. In both PC12 and Neuro-2A cells, expression of wild-type $G\alpha_o$ has been previously found to increase the basal levels of activated Rap1 [8, 10]. This effect

Fig. 4. Effects of $G\alpha_o$ and Rap1 in cells stimulated with FGF. CHO cells transfected in 10-cm (**A**, **B**) or 6-cm (**C**) dishes were serum starved and incubated in the absence or presence of 40 ng/ml FGF. **A** Time course of Rap1 activation by FGF. Cells were transfected with 4 μ g of HA-Rap1 and stimulated with FGF for the indicated periods of times. **B** Inhibition of FGF-dependent Rap1 activation by $G\alpha_o$. Cells were transfected with 4 μ g of HA-Rap1 and 4 μ g of empty vector, $G\alpha_o$ wild-type or $G\alpha_o$ -Q205L and stimulated with FGF for 5 min. **C** Inhibition of FGF-dependent MAPK activation by Rap1. Cells were transfected with 2 μ g of HA-p44 MAPK and 2 μ g of empty vector, HA-Rap1 wild-type or HA-Rap1-G12V and stimulated with FGF for 5 min. Rap1 and MAPK activation were determined as described in the legends to figures 1 and 3, respectively. Data are representative of two to three experiments in duplicate.

Fig. 5. Effects of Rap1GAPII expression on Rap1 and MAPK activation. **A** Subcellular distribution of HA-Rap1GAPII. CHO cells grown on coverslips were transfected with 0.75 μ g of HA-Rap1GAPII, serum starved, labeled with anti-HA antibodies and analyzed as described in the legend to figure 2. The insert displays an enlargement of the plasma membrane area indicated by the arrow. Bar: 10 μ m. **B** Effect of Rap1GAPII on Rap1 activation. CHO cells in 10-cm dishes were transfected with 4 μ g of HA-Rap1, 4 μ g of EGF receptor, and 4 μ g of empty vector or HA-Rap1GAPII. Cells were then serum starved and stimulated with EGF for 2 min. **C** Effect of Rap1GAPII expression on MAPK activation. CHO cells transfected in 6-cm dishes were serum starved and stimulated with 100 ng/ml EGF (upper panels) or 40 ng/ml FGF (lower panels) for the indicated periods of time. For EGF stimulation, cells were transfected with 2 μ g of HA-p44 MAPK, 2 μ g of EGF receptor, and 2 μ g of empty vector or HA-Rap1GAPII. For FGF stimulation, cells were transfected with 2.5 μ g of HA-p44 MAPK and 2.5 μ g of empty vector or HA-Rap1GAPII. Rap1 and MAPK activation were measured as described in the legends to figures 1 and 3, respectively. Expression levels of HA-Rap1GAPII were determined by immunoblot analysis of cell lysates with anti-HA antibodies. Data are representative of two to three experiments in duplicate.

has been explained by the ability of the wild-type subunit to sequester Rap1GAPII away from Rap1 and to promote proteasomal degradation of the GAP protein. Based on the response to prolonged stimulation of the cannabinoid receptors in Neuro-2A cells, it has been proposed that also the active form of $G\alpha_o$, which interacts weakly with Rap1GAPII, causes Rap1 activation by enhancing the degradation of Rap1GAPII [10]. In our experiments, we could not detect any stimulatory effect of $G\alpha_o$ on the levels of activated Rap1. However, we found that wild-type and, to a limited extent, activated $G\alpha_o$ decrease the expression of transfected Rap1GAPII (unpubl. observations). These contradictory results could be explained by differences in the expression of Rap1 and Rap1GAPII or

in the regulation of Rap1 by GAP proteins in the different cell systems investigated. Regardless of the actual mechanism(s) involved, our study suggests that in certain cell types the $G\alpha_o$ subunit exclusively exerts an inhibitory effect on Rap1 function. Clearly, further work is needed to elucidate the regulation of the Rap1 pathway by $G\alpha_o$.

The subcellular localization of Rap1 has recently raised considerable interest. Rap1 is detected at high levels on cytoplasmic vesicles particularly abundant in the perinuclear region of the cell, which have been identified as endosomes or maturing phagosomes [19, 25]. The plasma membrane of different cell types seems also to be enriched in Rap1 [19, 26]. Some uncertainty about the sub-

cellular site of activation of Rap1 by growth factors still remains, with reports suggesting that this event takes place in the perinuclear region [27] or at the plasma membrane [19]. Consistently with the latter possibility, it has been shown that the α chain of G_z inhibits NGF-dependent Rap1 activation by translocating Rap1GAP2 from the cytosol to the plasma membrane [28]. In our experiments, transfected Rap1 and G_{α_o} were found to be present and to colocalize both at the plasma membrane and on cytoplasmic vesicles, suggesting that both scenarios are theoretically possible in CHO cells.

Two lines of evidence demonstrate that inhibition of Rap1 activation does not account for the ability of G_{α_o} to regulate MAPK in CHO cells: (1) G_{α_o} -Q205L inhibits Rap1 activation by both EGF and FGF but only potentiates the MAPK response induced by EGF, and (2) Rap1GAP2 expression does not significantly modify growth factor-dependent activation of MAPK. Previous results have shown that G_{α_o} -dependent MAPK stimulation requires the B-Raf kinase and both PKC and PI3K [5, 6]. The molecular mechanisms involved in the pathway initiated by G_{α_o} remain to be further elucidated. The data reported here also imply that, in CHO cells, Rap1 does not play an important role in the stimulation of the MAPK cascade induced by growth factor receptors. A lack of correlation between Rap1 function and MAPK activation has been previously observed [12, 16, 17, 22]. In our experiments, there is a clear discrepancy between the effects of activated Rap1 and the results obtained in cells expressing Rap1GAP2. The simplest explanation of this discrepancy is that the response to activated Rap1 is due to expression of the protein at high levels and/or for prolonged periods of time. Alternatively, Rap1 activation by receptors might take place only at a specific subcellular location while transfection of Rap1 is followed by a diffuse action within the cell.

There is evidence that Rap1 inhibition mediates stimulation of MAPK by G_{α_i} during activation of the M_2 receptor in 293T cells [9]. Obviously, such a mechanism can only operate in cells in which Rap1 negatively regulates the MAPK pathway. It is therefore possible to imagine that in some systems, such as CHO cells, G_{α_o} activates MAPK independently of Rap1 while in other specific cell types it uses Rap1 inactivation to stimulate the MAPK cascade. It should also be mentioned that the ability of Rap1 to activate MAPK in neurons and other cell types [13–15] implies that G_{α_o} , by inducing Rap1 inactivation, can exert a negative rather than a positive effect on MAPK activity. Indeed, it has been shown that activated G_{α_o} blocks MAPK activation by NGF receptors in PC12 cells and is involved in MAPK inhibition by the dopamine D_2 receptor in pituitary cells [8, 29].

Regulation of Rap1GAP2 and Rap1 can mediate some of the effects of the G_{α_o} subunit on the MAPK cascade. Recent studies have also shown that a Rap1-dependent pathway is involved in stimulation of neurite outgrowth by G_{α_o} in Neuro-2A cells [10]. Continuously increasing evidence indicates that Rap1 plays an important role in the control of many cell functions including synaptic transmission and plasticity, adhesion, motility and phagocytosis [12, 13, 30–32]. It will be of considerable interest to investigate the effects of G_{α_o} on the various responses controlled by Rap1. These studies will clarify the role of Rap1 regulation by G_{α_o} in CHO cells and, most conceivably, other cell types in which the Rap1 and MAPK responses to this G-protein subunit are independent processes.

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