Regulation of Interaction of ras p21 with RalGDS and Raf-1 by Cyclic AMP-dependent Protein Kinase*

(Received for publication, June 7, 1995, and in revised form, October 2, 1995)

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RalGDS is a GDP/GTP exchange protein for ras p21, a member of small GTP-binding protein superfamily. We have recently shown that RalGDS interacts directly with the GTP-bound active form of ras p21 through the effector loop of ras p21 in vitro, in insect cells and in the yeast two-hybrid system. These results suggest that RalGDS functions as an effector protein of ras p21. Here, we report that RalGDS interacts with ras p21 in mammalian cells in response to an extracellular signal. Epidermal growth factor (EGF) induced the interaction of c-ras p21 and RalGDS in COS cells expressing both proteins, but not in the cells expressing RalGDS and c-ras p211,2,3,5, which is an effector loop mutant of ras p21. We also found that cyclic AMP-dependent protein kinase (protein kinase A) regulated the selectivity of ras p21-binding to either RalGDS or Raf-1. Protein kinase A phosphorylated RalGDS as well as (1–149)Raf (amino acid residues 1–149). Although the phosphorylated (1–149)Raf had a lower affinity for ras p21 than the unphosphorylated (1–149)Raf, both the phosphorylated and unphosphorylated RalGDS had the similar affinities for ras p21. The phosphorylation of RalGDS did not affect its activity to stimulate the GDP/GTP exchange of ras p21. Pretreatment of COS cells with forskolin further stimulated the interaction of ras p21 and RalGDS induced by EGF under the conditions that EGF-dependent Raf-1 activity was inhibited. These results indicate that ras p21 distinguishes between RalGDS and Raf-1 by their phosphorylation by protein kinase A.

ras p21 is a member of the small GTP-binding protein superfamily and plays a pivotal role in cell growth and differentiation (1, 2). ras p21 has GDP/GTP-binding and GTPase activity and cycles between the GDP-bound inactive and GTP-bound active forms. Recent studies have shown that growth factor receptors that have tyrosine kinase activity regulate the GDP/GTP exchange reaction and modulate the activity of ras p21 (3, 4). Growth factors such as EGF1 and platelet-derived growth factor induce autophosphorylation of their receptors and create specific binding sites for Src homology 2-containing proteins such as Grb2, phospholipase C-γ, and the p85 subunit of PI 3-kinase (3, 5). Grb2 is in a complex with Sos, a GDP/GTP exchange protein for ras p21, in cytosol in the absence of growth factors (6–8). After a growth factor induces the autophosphorylation of its receptor, Grb2-Sos complex translocates from cytosol and associates with the receptor in membranes, thereby placing it in the vicinity of ras p21. Sos stimulates the conversion of the GDP-bound inactive form of ras p21 to the GTP-bound active form. The GTP-bound active form of ras p21 then transduces a signal(s) to downstream effector protein(s).

One identified effector protein is Raf-1, a serine/threonine kinase (9–16). ras p21 interacts directly with Raf-1 and activates Raf-1, although the detailed mechanism of activation is not known. Then Raf-1 activates mitogen-activated protein kinase (9–16), which in turn activates extracellular signal-regulated kinase, and Raf-1 exerts its function through this protein kinase cascade (9, 10, 17–19). These data are consistent with previous observations that Raf-1 acts downstream of ras p21 in signaling pathways that mediate both the growth and differentiation responses to receptor tyrosine kinases (20–22). However, it is possible that ras p21 has effector proteins other than Raf-1, since ras p21 exerts multiple functions (1, 2). The first possible effector protein of ras p21 was GAP (23). GAP interacts with only the GTP-bound form of ras p21 and fails to interact with the effector loop mutant of ras p21. Although it has been reported that GAP has an influence downstream of ras p21 in various signaling pathways (23–25), it is not clear whether GAP is a real effector protein because GAP acts as a negative regulator of ras p21 by stimulating the GTPase activity of ras p21 (23). Another possible effector protein of ras p21 is PI 3-kinase (26). PI 3-kinase consists of two subunits, p85 and p110. p110 associates with the GTP-bound form of ras p21. v-ras p21 elevates phosphorylated phosphoinositide levels, products of PI 3-kinase, in COS cells, and a dominant negative mutant of ras p21 inhibits nerve growth factor-induced phosphorylated phosphoinositide production in PC12 cells. However, we have reported that platelet-derived growth factor receptor mutant, which lacks the ability to bind to PI 3-kinase, is not able to stimulate GDP/GTP exchange of ras p21 in Chinese hamster ovary cells and epithelial murine mammary gland cells and that a constitutively active form of PI 3-kinase stimulates the GDP/GTP exchange of ras p21 in Xenopus oocytes (27, 28). These results suggest that PI 3-kinase acts upstream of ras p21. Therefore, whether GAP and PI 3-kinase are effector proteins of ras p21 might be dependent on cell types.

We have recently shown that RalGDS is an effective effector protein of ras p21 (29). RalGDS has been originally isolated by...
polymerase chain reaction using regions conserved between CDC25 and ras6 genes, two GDP/GTP exchange proteins known to regulate ras p21 in Saccharomyces cerevisiae and Saccharomyces pombe, respectively (30). RalGDS is a 115-kDa protein that shares a high homology with the region of CDC25, which is important to stimulate the GDP/GTP exchange of ras p21. However, RalGDS does not affect the GDP/GTP exchange of ras p21. Among 13 different small G proteins, RalGDS stimulates the GDP/GTP exchange only of ras p24 and rasB p24. ras p24 has been originally isolated by probing with an oligonucleotide corresponding to one of the GTP-binding domain of ras p21 (31). Although the function of ras p24 has not yet been understood, RalGDS has been implicated in the regulation of the GTP state of ras p24 (30). We have found that RalGDS interacts with the GTP-bound active form of ras p21 but not with the GDP-bound inactive form, that the interaction of ras p21 and RalGDS requires the effector loop of ras p21, and that RalGDS inhibits the interaction of ras p21 with Raf-1 and GAP (29). Thus, RalGDS fulfills the criteria expected of ras p21-effector protein interactions. Two other groups have reported similar results (32, 33).

However, we have not yet demonstrated the interaction of ras p21 and RalGDS in intact mammalian cells in response to an extracellular signal. It has not been clarified how ras p21 distinguishes these possible effector proteins. Here we demonstrate that when COS cells are treated with EGF, RalGDS can be immunoprecipitated with ras p21. Furthermore, we show that protein kinase A regulates the selectivity of ras p21-binding to either RalGDS or Raf-1.

**EXPERIMENTAL PROCEDURES**

Materials and Chemicals—The RalGDS and ras p21 cDNAs and the anti-RalGDS antibody were provided by Drs. B. W. Giddings, C. F. Albright, and R. A. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA) (30). The c-Ha-ras p21 cDNA, dominant-negative ras p21 cDNA (ras p21(N17) (a form of ras p21 mutant in which Ser-17 is changed to Asn)), and the hybridoma cells producing anti-ras p21 antibody (Y13-259) were provided by Dr. J. Downward (Imperial Cancer Research Institute, United Kingdom). Mammalian expression vectors, pBJ-1 and pCGN, and the mouse anti-influenza virus NS2 (rev) monoclonal antibody 12CA5 (34) were provided by Dr. Q. Hu (University of California, San Francisco, CA). pCGN was designed to express a 16-amino acid epitope from influenza virus HA fused to protein. The rabbit anti-GST polyclonal antibody was provided by Dr. Kenamer (University of California, San Francisco, CA). COS-7 cells were obtained from Type Culture Collection (University of California, San Francisco, CA). The anti-RalGDS antibody were provided by Drs. B. W. Giddings, C. F. Albright, and R. A. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA). Mammalian expression vectors, pBJ-1 and pCGN, and the mouse anti-influenza virus NS2 (rev) monoclonal antibody 12CA5 (34) were provided by Dr. Q. Hu (University of California, San Francisco, CA). pCGN was designed to express a 16-amino acid epitope from influenza virus HA fused to protein. The rabbit anti-GST polyclonal antibody was provided by Dr. Kenamer (University of California, San Francisco, CA). COS-7 cells were obtained from Type Culture Collection (University of California, San Francisco, CA). The anti-RalGDS antibody were provided by Drs. B. W. Giddings, C. F. Albright, and R. A. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA). The anti-RalGDS antibody were provided by Drs. B. W. Giddings, C. F. Albright, and R. A. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA). The anti-RalGDS antibody were provided by Drs. B. W. Giddings, C. F. Albright, and R. A. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA). The anti-RalGDS antibody were provided by Drs. B. W. Giddings, C. F. Albright, and R. A. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA).

**Interaction Assay of ras p21 and RalGDS in Vitro**—To make the GTP-γ-S-bound form of ras p21, c-ras p21 (20 pmol) was incubated for 10 min at 30°C in 40 μl of reaction mixture (20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 5 mM MgCl2, 1 mM DTT, and 25 μM GTP-S). After the incubation, 600 μM MgCl2 was added at a final concentration of 15 mM. The GTP-γ-S-bound form of ras p21 was incubated for 30 min at 4°C in 200 μl of reaction mixture (20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM MgCl2, 0.5 mM DTT, and 25 μM GTP-S) in the presence or absence of GST-1-(149)Raf. Then the anti-ras p21 antibody (Y13-238) was added to this mixture, followed by immunoprecipitation. The precipitate was subjected to SDS-PAGE, transferred to nitrocellulose filters, and probed with the anti-RalGDS antibody.

**Interaction Assay of ras p21 and Raf-1 in Vitro**—The GTP-γ-S-bound form of ras p21 was made as described above and incubated for 30 min at 4°C with GST-1-(149)Raf (20 pmol) in 100 μl of reaction mixture (20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM MgCl2, 0.5 mM DTT, and 25 μM GTP-S). Then, the anti-ras p21 antibody (Y13-238) was added to this mixture, followed by immunoprecipitation. The precipitate was subjected to SDS-PAGE, transferred to nitrocellulose filters, and probed with the anti-RalGDS antibody.
mGlu₉, 1 mM DTT, 100 mM NaCl, and 50 μM ATP. To detect phosphorylated bands by autoradiography, 50 μM [γ-³²P]ATP (2,000–4,000 cpm/pmol) was used instead of 50 μM ATP.

RalGDS Assay—To make [³H]GDP-bound form of ras p24, GST-ras p24 (5 pmol) was preincubated for 10 min at 30 °C in 5 μl of reaction mixture (25 mM Tris-HCl (pH 7.5), 10 mM EDTA, 5 mM MgCl₂, 1 mM DTT, and 5 μM [³H]GDP, 3,000–4,000 cpm/pmol). To this preincubation mixture, 45 μl of reaction mixture (55 mM Tris-HCl (pH 7.5), 11 mM MgCl₂, 1.1 mg/ml bovine serum albumin, and 110 μM GTP) containing the indicated amounts of RalGDS was added, and the mixture was further incubated for 10 min at 30 °C. Assays were quantified by rapid filtration on nitrocellulose filters (30).

Raf-1 Activity Assay in COS Cells—COS-7 cells stimulated with EGF were lysed in 0.5 ml of lysis buffer, and 0.2 ml of lysates (160 μg of protein) were immunoprecipitated with the anti-Raf-1 antibody. The Raf-1 kinase activity was determined by incubating the Raf-1 immunoprecipitates with GST-mitogen-activated protein kinase kinase and KNERK in 30 μl of kinase reaction mixture (20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM MnCl₂, 20 mM β-glycerophosphate, and 50 μM [γ-³²P]ATP (1,000–2,000 cpm/pmol)) for 30 min at 25 °C (16). After the incubation, the reaction was stopped by the addition of Laemmli’s loading buffer (37), the samples were subjected to SDS-PAGE (10% polyacrylamide gel), and the phosphoproteins were visualized by autoradiography. The radioactivity incorporated in KNERK was determined using scintillation counting.

Other Assays—Protein concentrations were determined with bovine serum albumin as a standard (38).

RESULTS

Interaction of ras p21 and RalGDS in COS-7 cells—To examine whether ras p21 interacts with RalGDS in mammalian cells, we coexpressed v-ras p21 and RalGDS in COS-7 cells. RasGDS was tagged with a 16-amino acid epitope from influenza virus HA, which was recognized by the monoclonal antibody 12CA5. The expression level of transfected RasGDS in COS cells expressing RasGDS alone was similar to that in the cells coexpressing v-ras p21 and RasGDS, as assessed by immunoblotting using the anti-HA antibody (Fig. 1A, lanes 1-3). When the lysates coexpressing v-ras p21 and RasGDS were immunoprecipitated with the anti-ras p21 antibody, both ras p21 and RasGDS were immunoprecipitated with the anti-HA antibody (Fig. 1A, lane 1).

Interaction of ras p21 and RalGDS in COS cells. COS cells expressing RasGDS alone (lane 1), v-ras p21 alone (lane 2), both v-ras p21 and RasGDS (lanes 3 and 5), and both ras p21S¹⁷⁴ and RasGDS (lane 4) were lysed, and the proteins of the lysates were immunoprecipitated with the anti-ras p21 antibody (Y13-238) (lanes 1-4) or nonimmune rat immunoglobulin (lane 5). The precipitates were probed with the anti-HA and ras p21 antibodies. C, inability of Y13-259 to immunoprecipitate a ras p21-RasGDS complex. COS cells expressing both v-ras p21 and RasGDS were lysed, and the proteins of the lysates were immunoprecipitated with Y13-238 (lane 1) or Y13-259 (lane 2). The precipitates were probed with the anti-HA and ras p21 antibodies. An arrowhead and an arrow indicate the positions of RasGDS and ras p21, respectively. IP, immunoprecipitation; Ig, immunoglobulin. The results shown are representative of three independent experiments.
p21 and RalGDS were detected in the ras p21 immune complex (Fig. 1B, lane 3). When the lysates expressing RalGDS alone or v-ras p21 alone were immunoprecipitated with the anti-ras p21 antibody, RalGDS was not detected (Fig. 1B, lanes 1 and 2). Neither RalGDS nor ras p21 was immunoprecipitated with nonimmune immunoglobulin from the lysates expressing both proteins (Fig. 1B, lane 5).

To characterize the interaction of ras p21 and RalGDS further, we examined the ability of RalGDS to interact with a ras p21 mutant, ras p21<sup>S17N</sup>, ras p21<sup>S17N</sup> is well known as a dominant negative mutant, which has a higher affinity for GDP than GTP and strongly interacts with upstream molecules but not with downstream molecules (1, 2, 39). The expression level of ras p21<sup>S17N</sup> was similar to that of v-ras p21 (Fig. 1A, lanes 3 and 4). When the lysates coexpressing ras p21<sup>S17N</sup> and RalGDS were immunoprecipitated with the anti-ras p21 antibody, RalGDS was not coprecipitated with ras p21<sup>S17N</sup> under the same conditions that were used to coprecipitate v-ras p21 and RalGDS (Fig. 1B, lanes 3 and 4). We used Y13-238 as the anti-ras p21 antibody to immunoprecipitate ras p21 for these experiments. Another antibody, Y13-259, was tested for its ability to immunoprecipitate a ras p21-RalGDS complex. Y13-259 is known to be the neutralizing antibody (1, 40). In contrast to Y13-238, Y13-259 could not immunoprecipitate the ras p21-RalGDS complex from the lysate coexpressing v-ras p21 and RalGDS (Fig. 1C, lanes 1 and 2). Y13-238 and Y13-259 immunoprecipitated the similar amounts of ras p21 from the lysates (Fig. 1C, lanes 1 and 2). These results indicate that RalGDS makes a complex with v-ras p21 but not with ras p21<sup>S17N</sup> in COS cells, and the interaction of ras p21 and RalGDS requires the effector loop of ras p21. These results in COS cells are consistent with our previous observations in Sf9 cells (29).

Interaction of ras p21 and RalGDS in Response to an Extracellular Signal—To examine whether ras p21 interacts with RalGDS in mammalian cells in response to an extracellular signal, we coexpressed c-ras p21 and RalGDS in COS cells (Fig. 2A, lane 2). Then, the cells were stimulated with EGF. EGF is known to activate ras p21 (2, 4). RalGDS was coprecipitated with ras p21 in a dose-dependent manner of EGF (Fig. 2B). We also coexpressed RalGDS and ras p21<sup>T35A</sup> in COS cells (Fig. 2A, lane 3). The expression level of ras p21<sup>T35A</sup> was similar with that of c-ras p21, ras p21<sup>T35A</sup> is an effector loop mutant of ras p21, which fails to interact with effector proteins (1, 2, 12–14, 23). RalGDS was not coprecipitated with ras p21<sup>T35A</sup> by stim-
utilization with EGF (Fig. 2B). The interaction of ras p21 and RalGDS was observed within 2 min after stimulation with EGF, reached to the maximal level at 20 min, and then gradually decreased (Fig. 2C). This time course was almost the same as that of EGF-dependent Raf-1 activity to activate mitogen-activated protein kinase kinase (data not shown). In contrast, ras p21\textsuperscript{72SA} did not make a complex with RalGDS within 60 min. These results demonstrate that ras p21 interacts with RalGDS in mammalian cells in response to an extracellular signal and that the effector loop of ras p21 is important for the interaction of ras p21 and RalGDS.

Phosphorylation of RalGDS by Protein Kinase A—The results described above taken together with our previous observations (29) strongly suggest that RalGDS is an effector protein of ras p21. Since Raf-1 is an effector protein of ras p21, we next asked how ras p21 distinguishes between RalGDS and Raf-1. It has been reported that Raf-1 is phosphorylated by protein kinase A, resulting in the inhibition of Raf-1 binding to ras p21 and Raf-1 activity (35, 41–43). Therefore, we examined the effect of protein kinase A on the interaction of ras p21, RalGDS, and Raf-1. ras p21, GST-(1–149)Raf, and RalGDS were purified to the near homogeneity (Fig. 3A, lanes 1–3). GST-(1–149)Raf contains the ras p21-binding domain and protein kinase A-phosphorylation site (35). ras p21 was faintly phosphorylated by the catalytic subunit of protein kinase A (Fig. 3B, lanes 1 and 2). About 0.1 mol of phosphate was incorporated into 1 mol of ras p21, as consistent with the previous observations (44, 45). RalGDS was phosphorylated by protein kinase A as well as GST-(1–149)Raf (Fig. 3B, lanes 3–6). The phosphorylation of RalGDS by protein kinase A was a dose-dependent manner and a time-dependent manner (data not shown). About 0.7 mol of phosphate was incorporated into 1 mol of RalGDS. As consistent with the previous observations (35), the phosphorylation of GST-(1–149)Raf reduced the ability of GST-(1–149)Raf to complex with ras p21 (Fig. 3C). However, the phosphorylation of RalGDS did not affect the interaction of RalGDS and ras p21 (Fig. 3D). RalGDS was found to stimulate the dissociation of GDP from ras p24 (30). The phosphorylation of RalGDS did not affect this activity of RalGDS (Fig. 4).

Effect of the Phosphorylation of Raf-1 on Its Inhibitory Action for the Interaction of ras p21 and RalGDS in Vitro—We examined the effect of the phosphorylation of Raf-1 by protein kinase A on its inhibitory action for the interaction of ras p21 and RalGDS. GST-(1–149)Raf made a complex with ras p21 in a dose-dependent manner of GST-(1–149)Raf, and the phosphorylated form of GST-(1–149)Raf had a lower affinity for ras p21 than the unphosphorylated form (Fig. 5A). When GST(1–149)Raf was incubated with the indicated concentrations of forskolin for 15 min and then stimulated with 100 ng/ml EGF for 10 min. The complex formation of ras p21 and RalGDS (●) was assayed as described in legend to Fig. 2. The amount of RalGDS bound to ras p21 was expressed as percentage of that in the cells stimulated with EGF in the absence of forskolin. Raf-1 activity (■) was measured using GST-mitogen-activated protein kinase kinase and KNERK as substrates. The results shown are representative of three independent experiments.
cells. After COS cells overexpressing both ras p21 and RalGDS were pretreated with forskolin, the cells were stimulated with EGF, and EGF-dependent Raf-1 activity and the interaction of ras p21 with RalGDS induced by EGF were examined. Consistent with the previous observations (42, 43), EGF-dependent Raf-1 activity was inhibited by forskolin (Fig. 6). From the results of Fig. 5, we expected that forskolin treatment would promote the EGF-dependent interaction of ras p21 and RalGDS by decreasing the competition by Raf-1. However, forskolin did not affect the interaction of ras p21 and RalGDS induced by EGF in COS cells overexpressing both ras p21 and RalGDS (Fig. 6). We thought that one of the reasons for the failure of forskolin to stimulate the interaction of these proteins might be due to overexpression of ras p21, which was enough to bind to both RalGDS and Raf-1. Therefore, we expressed RalGDS alone in COS cells and examined the interaction of endogenous ras p21 and overexpressed RalGDS. In these cells, forskolin treatment further stimulated the interaction of ras p21 and RalGDS induced by EGF (Fig. 7).

DISCUSSION

We have demonstrated here that the interaction of ras p21 and RalGDS occurs in intact mammalian cells in response to an extracellular signal. RalGDS makes a complex with v-ras p21 but not with ras p21<sup>S17N</sup> in COS cells. It is believed that v-ras p21 is a GTP-bound form and that ras p21<sup>S17N</sup> is a GDP-bound form in intact cells (1, 2, 39). One ras p21 antibody, Y13-238, precipitates the ras p21-RalGDS complex, but another antibody, Y13-259, does not. It is known that Y13-259 is a neutralizing antibody and that this antibody does not recognize the ras p21-effector complex (1, 2, 14, 29, 40). Furthermore, EGF induces the complex formation of RalGDS with c-ras p21, but not with ras p21<sup>T35A</sup>. ras p21<sup>T35A</sup> is an effector loop mutant of ras p21 and fails to interact with Raf-1 and GAP (11–14). These observations clearly show that RalGDS interacts with the GTP-bound form of ras p21 through the effector loop of ras p21 by stimulation with EGF in COS cells. Therefore, it is likely that RalGDS is an effector protein of ras p21 in mammalian cells.

Our results suggest that RalGDS provides a potential link between ras p21 and Raf-1. The results showing that one small G protein act downstream of other small G proteins has been reported (46, 47). Genetic analysis of yeast have demonstrated thatcdc42sp, a member of small G protein of S. pombe, lies downstream of ras1 in S. pombe and that CDC42, a member of small G protein of S. cerevisiae, acts downstream of RSR1, another member of small G protein of S. cerevisiae (46). It has been also shown that rac p21 is involved in the action of rho p21 to regulate the cytoskeleton (47). Although the function of rap24 has not yet been understood, it is possible that rap24 acts downstream of ras p21 and that rap24 modulates some ras p21-dependent processes.

It has been reported that RalGDS is phosphorylated in COS cells and that phosphoserine, but not phosphotyrosine, is detected in the phosphorylated RalGDS (30). Our results show that protein kinase A phosphorylates RalGDS. But, the phosphorylation of RalGDS affects neither its interaction with ras p21 nor its GTP activity for rap24. The physiological significance of the phosphorylation of RalGDS by protein kinase A remains to be clarified. Among many small G proteins, rap1 is known to be phosphorylated by protein kinase A (45). The GDP/GTP exchange reaction of rap1 is regulated by Smg GDP dissociation stimulator and the phosphorylation of rap1 enhanced the Smg GDP dissociation stimulator action (48). Although we do not known whether rap24 is phosphorylated by protein kinase A, rap24 has consensus sequences of phosphorylation by protein kinase A. It is intriguing to speculate that the phosphorylation of rap24 makes it sensitive to the action of RalGDS to stimulate the GDP/GTP exchange reaction.

Evidence has accumulated that there are several effector proteins of ras p21 (9, 11–16, 23, 26, 29, 31, 32). However, it has not yet been clarified how ras p21 distinguishes these effector proteins. Our results provide one possible model. It is known that Raf-1 is phosphorylated by protein kinase A and that phosphorylation of Raf-1 reduces its affinity for ras p21 (35, 41–43). The change of the characteristics of Raf-1 by phosphorylation could be one of the mechanisms by which protein kinase A inhibits ras p21-dependent Raf-1 activation. Our results show that the phosphorylation of RalGDS by protein kinase A does not affect its binding to ras p21 under the conditions that the phosphorylation of Raf-1 by protein kinase A inhibits its binding to ras p21 in vitro. Our results also show that when Raf-1 is phosphorylated by protein kinase A, the inhibitory action of Raf-1 for the interaction of RalGDS and ras p21 is attenuated in vitro. Furthermore, our results demonstrate that protein kinase A stimulates the interaction of ras p21 and RalGDS induced by EGF under the condition that EGF-dependent Raf-1 activity is inhibited in COS cells. Taken together with the previous observations (35, 41–43), these results indicate that protein kinase A inhibits the signal from ras p21 to Raf-1 but not to RalGDS. Therefore, it is likely that RalGDS and Raf-1 plays a role in cross-talk between the protein kinase A system and the tyrosine kinase-ras p21 system. Further studies are necessary to clarify the definitive function of RalGDS in signal transduction.

Acknowledgments—We thank Drs. B. W. Giddings, C. F. Albright, and R. A. Weinberg for the RalGDS cDNAs and the RalGDS antibody and Dr. Q. Hu for the mammalian expression vectors and the anti-HA antibody.

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