Activation of R-Ras by Ras-Guanine Nucleotide-releasing Factor*

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Ras-GRF/CDC25Mm, mSos, and C3G have been identified as guanine nucleotide-releasing factors for Ras family proteins. We investigated in this study the guanine nucleotide-releasing activities of Ras-GRF, mSos, and C3G toward R-Ras, which shows high sequence similarity to Ras. Ras-GRF markedly stimulated the dissociation of GDP from R-Ras, and C3G also promoted the release of R-Ras-bound GDP. Under the same conditions, mSos little affected the reaction. When Ras-GRF and R-Ras were coexpressed in COS7 cells, the remarkable accumulation of the active GTP-bound form of R-Ras was observed. C3G also increased active R-Ras in COS7 cells, while mSos did not give any effect. These results indicated that Ras-GRF and C3G could activate R-Ras. Furthermore, the activation of R-Ras by Ras-GRF was enhanced when cells were treated with ionomycin, which is known to increase the intracellular calcium concentration. The examination of tissue distribution of R-Ras, Ras-GRF, and mSos by the reverse transcription-polymerase chain reaction revealed that Ras-GRF was expressed only in brain and testis, whereas R-Ras, C3G, and mSos were expressed rather ubiquitously. These findings raise the possibility that R-Ras is activated by Ras-GRF in brain and testis, and by C3G in other tissues, respectively.

R-Ras was isolated by low stringency hybridization with the v-Ha-ras gene as a probe (1). The amino acid sequence of R-Ras shows a 55% sequence identity with that of Ras and has an amino-terminal extension of 26 residues. The GTP-binding domains and the effector domain are well conserved between R-Ras and Ras. The function of Ras has been intensively investigated, which revealed that Ras transduces signals from receptor-type tyrosine kinases to downstream effectors and thereby controls the proliferation and the differentiation of various types of cells (2). Despite the close resemblance in structure to Ras, the biological function of R-Ras has not been characterized extensively.

Ras recycles between the active GTP-bound and the inactive GDP-bound forms (2). The GDP-bound form is converted to the active form by guanine nucleotide-releasing factors (GRFs), Ras-GRF/CDC25Mm, mSos, Vav, or SengDGS (3), and interacts with downstream effector molecules (4). The GTP-bound form is converted to the inactive form by the intrinsic GTPase activity, which is accelerated by GTPase-activating proteins (GAPs), p120GAP, neurofibromin, and Gap1™ (5, 6).

In contrast to activated Ras, the activated form of R-Ras does not transform Rat1 fibroblastic cells (1). Rey et al. (7) stated that R-Ras did not induce DNA synthesis in Swiss3T3 cells or the differentiation of PC12 cells. However, two further reports demonstrated that activated R-Ras transforms NIH3T3 cells and the transformant forms tumors in athymic nude mice (8, 9). Since the putative effector domain of R-Ras is highly homologous to that of Ras, R-Ras binds to c-Raf-1 kinase and activates the mitogen-activated protein kinase pathway leading to the induction of e-fos (7, 8, 10). Besides the roles in the stimulation of the proliferation of cells, R-Ras may play roles in some cell biological processes other than those of Ras. It was described that the carboxyl-terminal domain of R-Ras interacts with Bcl-2, which is a key molecule in the control of apoptosis, in a GTP-independent manner (11). The activated form of R-Ras promotes apoptotic cell death induced by growth factor deprivation through a Bcl-2 suppressible mechanism (12). Zhang et al. (13) stated that the active form of R-Ras stimulates cell adhesion through the activation of integrins.

Following the identification of Ras-GRF and mSos as mammalian GRFs for Ras, we isolated C3G as a Crk SH3-binding GRF (14). C3G has a sequence similarity to the catalytic domains of Ras-GRF and mSos. The fact that C3G complemented a temperature-sensitive mutation of Saccharomyces cerevisiae CDC25 gene indicates that C3G can activate RAS in budding yeast cells (14). However, additional biochemical and cell biological analyses revealed that C3G activates Rap1 much more efficiently than does Ras (15). A GRF for Ral was also described (16); however, a GRF specific to R-Ras has not yet been identified.

Considering the high sequence similarity between Ras and R-Ras, we examined the effects of the known GRFs for the Ras family proteins, Ras-GRF, mSos, and C3G on the guanine nucleotide release from R-Ras. The results described in this study indicate that Ras-GRF and C3G can stimulate the nucleotide exchange of R-Ras and raise the possibility that these factors may be activators for R-Ras.

EXPERIMENTAL PROCEDURES

Low Molecular Weight GTP-binding Proteins—Ha-Ras and R-Ras expression plasmids (17), generous gifts of Dr. K. Kaibuchi, directed the expression of Ha-Ras and R-Ras as fusion proteins with glutathione

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S-tranferase (GST). GST fusion proteins were purified by using glutathione-Sepharose (Pharmacia Biotech Inc.) and digested with thrombin (Sigma). Rap1B protein produced in Sf9 cells using a baculovirus vector (18) and Escherichia coli producing the full-length N-Ras protein were kind gifts from Drs. Y. Takai and A. Wittinghofer, respectively.

pEBG-R-Ras, a mammalian GST fusion R-Ras expression vector derived from pEBG (19), was constructed as follows. An entire coding region of R-Ras was amplified by polymerase chain reaction (PCR), and the product was inserted into the BamHI site of pEBG. The Ras expression plasmid SR-Ras was described previously (20).

GRFs—The full-length cDNA of Ras-GRF was kindly provided by Dr. L. Feig. The SoloClaf fragment of Ras-GRF covering the entire coding region was inserted into Xhol site of an expression vector, pCAGGS (21), which yielded pCAGGS-Ras-GRF. The membrane targeting farnesylation signal, CAAX box of the c-Ki-Ras2 gene, was amplified by PCR with primers Ki-RT (5'-GGCAAGCTTGTGTCATCAGAT-3') and Ras-GRF (5'-GCCCTCTAGATGTGGGGAGTTTTGG-3'), corresponding to amino acid residues 559–565 with an artificial CAAX-I site. The amplified product was inserted into the Caax-I site, which is derived from pEBG (19), to yield pEBG-mSos. To obtain a mSos protein, 1 μg of a reaction buffer containing 2.0 pmol of each low molecular weight GTP-binding protein-[3H]GDP complex, an appropriate amount of GRF, and an excess amount of nonradioactive GDP were incubated at 30 °C for 20 min. The activities of exchange factors were measured as a decrease in the amount of GTP-binding protein-[3H]GDP complex, which was quantitated by the nitrocellulose filter method. [3H]GDP bindings to Ras family proteins were 0.5–0.6 pmol/pmol of protein.

Analysis of Guanine Nucleotides Bound to R-Ras and R-Ras in Intact Cells—COS7 cells (1.5 × 10^6 per 100-mm diameter dish) were transfected with the various combinations of expression vectors for Ha-Ras, R-Ras, and GRF (0.3 μg) (15). After 48 h, guanine nucleotides bound to Ha-Ras or R-Ras were analyzed as described previously (15, 20). Briefly, cells were labeled with 0.05 mCi/ml [32P]orthophosphate (Amersham Corp.) in a serum-free medium for 4 h, then the cells were lysed in a lysis buffer. Ras was immunoprecititated by Y13-259 anti-Ras monoclonal antibody, and GST-R-Ras was recovered by using glutathione-Sepharose. Guanine nucleotides bound to Ras and R-Ras were separated by polyethyleneimine thin layer chromatography and quantitated by the BAS 2000 system (Fuji Film, Tokyo). The treatment of cells with ionomycin was carried out as described by Farnsworth et al. (24). Thirty-two hours after transfection of the plasmids, cells were incubated in a serum-free medium for 12 h and then labeled with [32P]orthophosphate for 4 h. Cells were treated with 10 μM ionomycin for 5 min, then Ras-R-Ras-bound nucleotides were analyzed.

RESULTS

Ras-GRF Stimulates GDP Release from R-Ras—We measured the substrate specificity of Ras-GRF by using various Ras family proteins. As expected Ras-GRF stimulated the dissociation of GDP from Ha-Ras and N-Ras in a dose-dependent manner (Fig. 1, left). Ras-GRF also stimulated the dissociation of GDP from R-Ras. We previously reported that Ras-GRF did not stimulate the release of GDP from RalA, Rab3A, or RhoA (15).

We next examined the substrate specificity of mSos, another GRF for Ras. Under the same conditions, mSos stimulated the
Ras-GRF kept on ice was taken as 100%, and the values are expressed relative to this. The value obtained with the sample without the presence of Ras-GRF was about 30%, the value of which may be lower than that expected for the activated R-Ras. Therefore, it may be difficult to determine by these rather indirect methods whether the activators of Ras also activate R-Ras.

**DISCUSSION**

Genetic and biochemical studies established that CDC25 and Sos, and their counterparts in mammalian cells, are GRFs for Ras proteins (reviewed in Ref. 3). For Rap1 and Rap1a, RalGDS (16) and C3G (15) have been identified as GRFs, respectively. However, a GRF for Ras has not yet been described. In the present study, we demonstrated that both Ras-GRF and C3G can activate R-Ras.

While Ras-GRF is expressed only in brain and testis, R-Ras and C3G are widely expressed in all the tissues examined (Fig. 6). Recently, GRF2, the amino acid sequence of which is 80% identical to that of Ras-GRF, was identified (27). GRF2 is widely expressed in various tissues and has GRF activity for Ras (27). Considering the high sequence similarity between Ras-GRF and GRF2, it is highly likely that GRF2 also acts on R-Ras as a GRF. Thus it is conceivable that R-Ras may be activated by Ras-GRF in brain and testis, and by C3G and GRF2 in various tissues. However, this does not exclude the presence of R-Ras-specific GRFs yet to be discovered.

Huff et al. (28) described that the coexpression of known Ras activators with R-Ras did not stimulate the transforming and the transactivating activities of R-Ras. Based on these results they argued that mSos and Ras-GRF are not the activators of R-Ras. However, these activities of R-Ras, even activated by a point mutation analogous to Rasval12, are much weaker than those of activated Ras (8). When the wild type R-Ras and Ras-GRF are coexpressed, the relative amount of GST-bound form of R-Ras was about 30% (Fig. 3), the value of which may be lower than that expected for the activated R-Ras. Therefore, it may be difficult to determine by these rather indirect methods whether the activators of Ras also activate R-Ras. In contrast we demonstrated the activation of R-Ras by directly measuring the ratio of GST-bound form.

The amino acid sequences of the catalytic domains of mSos, Ras-GRF, and C3G show about 30% identity with one another (14). However, there is a marked difference in their substrate specificities. Both mSos and Ras-GRF activated Ras efficiently. However, while Ras-GRF activated R-Ras as thoroughly as Ras, mSos did not activate R-Ras at all. C3G activated most efficiently Rap1, moderately R-Ras, and rather weakly Ras (this study) (14, 15).

Residues of Ha-Ras and RAS2 that are critical for the interaction with GRFs have been identified using yeast (29–32) and mammalian (33–35) systems. They are residues 35, 38 (29), 62, 63, 67, 69 (31, 35), 73–76 (30), 75, 76, 79 (34), 69, 73, 102–105 (32), and 130–140 (33) (codon numbers are of Ha-Ras or the Ha-Ras equivalents). Although they are mostly in the switch II region (61–77) (36) whose conformation dramatically changes
depending on the bound guanine nucleotides, another portion of Ras is obviously necessary for the interaction. Among 17 residues in the switch II region, 12 residues are identical between Ras and R-Ras, whereas 8 residues are conserved between R-Ras and Rap1. This may be the reason why Ras-GRF activates both Ras and R-Ras but not Rap1. Quilliam et al. (35) demonstrated that mutations in this region impair the sensitivities of the mutated Ras proteins to both Ras-GRF and mSos to similar extents, suggesting that Ras-GRF and mSos interact with the similar structure of Ras. However, Ras-GRF but not mSos activates R-Ras, demonstrating that there may be still some difference in the structures recognized by the two factors. Nine residues are identical between switch II regions of Ras and Rap1. Although critical residues of Rap1, R-Ras, and Ras for the interaction with C3G are yet to be identified, C3G showed the broadest substrate specificity. Ras-GRF with or without a membrane-targeting signal exhibited similar activities in the stimulation of both Ha-Ras and R-Ras, whereas mSos with the targeting signal showed higher activity than that without the signal in the activation of Ha-
Ras (Fig. 4). The difference in the effects of the membrane targeting signal may reflect the difference in the cellular localizations of Ras-GRF and mSos. It has been shown that mSos translocates from the cytosol to the plasma membrane by the adapter proteins, Grb2 and Shc, which causes the activation of Ras-GRF and mSos. It has been demonstrated that mSos activates the GTPase activities of both Ras and R-Ras (7, 18606).

The products were analyzed on 1.2% agarose gels, and the gels were stained with SYBER Green I (Molecular Probes).

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FIG. 5. Enhancement of Ras-GRF activity to R-Ras by calcium in COS7 cells. An expression vector for R-Ras was transfected into COS7 cells with either a control vector (pCAGGS) or an expression vector for Ras-GRF or Ras-GRF-F. After 48 h, cells were treated with or without 10 μM ionomycin for 5 min, then guanine nucleotides bound to R-Ras were analyzed as in the legend to Fig. 3. Mean values obtained from three independent experiments were shown with standard deviations.

FIG. 6. The tissue specificities of R-Ras, Ras-GRF, and mSos mRNA form various organs were reverse transcribed and then amplified by PCR as described under “Experimental Procedures.” The products were analyzed on 1.2% agarose gels, and the gels were stained with SYBER Green I (Molecular Probes).

Activated Rap1 also inhibits other Ras GAP molecules and R-Ras GAP, the activation of Rap1 by C3G would favor the formation of a GTP-bound form of R-Ras. Since the in vitro activation of R-Ras by C3G is rather modest compared with the activation of R-Ras in COS7 cells, this indirect mechanism might work in the cells.

It is very intriguing that Ras-GRF and C3G activate R-Ras and Rap1 and R-Ras, respectively, since a single stimulus may activate two different signaling pathways. R-Ras promotes apoptotic cell death when interleukin-3 is withdrawn (12), whereas the expression of activated form of Ras prevents apoptotic cell death caused by interleukin-3 deprivation (41). In this case Ras and R-Ras cause opposite cell biological effects. In contrast, the activated forms of both R-Ras and Ras induce transformation of a particular fibroblast cell line (8, 9). Hence the biological meaning of co-activation of R-Ras and Ras should await further experiments. Similarly, C3G activates Rap1 and R-Ras. Although Rap1 acts antagonistically against Ras function (42), it is not well investigated whether Rap1 and R-Ras interfere with each other in some biological processes.

Recently, it has become a consensus that the activation of low molecular weight GTP-binding proteins is achieved through the functional activation of GRFs rather than the inhibition of GTPase-activating proteins. For Ras, it has been demonstrated that the signals from receptor-type tyrosine kinases and cytosolic tyrosine kinases are transduced to Ras through the translocation of Sos (3). The activity of Ras-GRF is up-regulated by serum or lysophosphatidic acid (38, 39) and by the increase in the intracellular calcium concentration (24). It was also demonstrated that a signal from the βγ subunit of trimeric G proteins activates Ras-GRF in a phosphorylation-dependent manner (43). Activation of Rap1 may be under the control of tyrosine kinases, since its GRF, C3G, is a Crk-binding protein. Although not yet proven, these signals may also activate R-Ras.

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