Coassociation of Rap1A and Ha-Ras with Raf-1 N-terminal Region Interferes with Ras-dependent Activation of Raf-1*

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Raf-1 is a major downstream effector of mammalian Ras. Binding of the effector domain of Ras to the Ras-binding domain of Raf-1 is essential for Ras-dependent Raf-1 activation. However, Rap1A, which has an identical effector domain to that of Ras, cannot activate Raf-1 and even antagonizes several Ras functions in vivo. Recently, we identified the cysteine-rich region (CRR) of Rap1A as another Ras-binding domain. Ha-Ras proteins carrying mutations N26G and V45E, which failed to bind to CRR, also failed to activate Raf-1. Since these mutations replace Ras residues with those of Rap1A, we examined if Rap1A lacks the ability to bind to CRR. Contrary to the expectation, Rap1A exhibited a greatly enhanced binding to CRR compared with Ha-Ras. Enhanced CRR binding was also found with Ha-Ras carrying another Rap1A-type mutation E31K. Both Rap1A and Ha-Ras(E31K) mutant failed to activate Raf-1 and interfered with Ha-Ras-dependent activation of Raf-1 in S99 cells. Enhanced binding of Rap1A to CRR led to co-association of Rap1A and Ha-Ras with Raf-1 N-terminal region through binding to CRR and Ras-binding domain, respectively. These results suggest that Rap1A interferes with Ras-dependent Raf-1 activation by inhibiting binding of Ras to Raf-1 CRR.

Ras belongs to a family of small GTP-binding proteins playing essential roles in cell proliferation and differentiation. Mammalian ras genes carrying activating mutations are found in many types of neoplastic tissue and are able to induce morphological transformation in vitro when transfected into fibroblast cell lines. However, the rap1A gene (1), encoding a 21-kDa GTP-binding protein with high homology to Ras, has been shown to induce reversion of the transformed phenotype in Ki-ras-transformed NIH3T3 cells (2). In addition to the overall structural homology, Rap1A shares two important structural features with Ras. One is that Rap1A has an identical effector domain (amino acids 32–40) to that of Ras. The effector domain of Ras is essential for the association with and activation of its effectors (3). The other is that Rap1A undergoes similar post-translational modification to Ras at its C terminus except that Ras is farnesylated and Rap1A is geranylgeranylated (4). This modification is essential for the function of Rap1A as observed for Ras (5, 6).

Rap1, a serine/threonine kinase regulating the mitogen-activated protein kinase cascade, is a major mammalian Ras effector and is thought to play a key role in Ras-induced cellular transformation (7). Although the precise mechanism of Ras-dependent Raf-1 activation remains unclear, it is known that the effector domain of Ras interacts with the N-terminal RBD1 (amino acids 51–131) of Raf-1 and that this interaction is essential for physical association between these proteins as well as for the activation of Raf-1 (7). Rap1A, too, has been shown to associate with Raf-1 N-terminal fragment in vivo (8), and a recent x-ray diffraction study of the crystal of the complex between Rap1A and Raf1 RBD has provided evidence for this association at the atomic level (9). These studies suggest the possibility that the suppression of Ras function by Rap1A is due to the competitive inhibition of Ras-RBD interaction (10), although it is unclear why Rap1A cannot activate Raf-1.

We have recently identified Rap1A CRR (amino acids 152–184) as another Ras-binding domain and demonstrated that interaction of Ras with both BCR and CRR is necessary for the activation of Raf-1 (11). Two mutations, N26G and V45E, were found to abolish the interaction of Ha-Ras with CRR and attenuate the activation of Raf-1 by Ha-Ras. The fact that both of these mutations replaced Ha-Ras residues with corresponding Rap1A residues prompted us to examine the possibility that the inability of Rap1A to activate Raf-1 is due to its failure to interact with Raf1 CRR. Contrary to the expectation, we found that Rap1A exhibited a greatly enhanced ability to bind to CRR.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Rap1A and Ha-Ras Proteins—Rap1A cDNA was amplified from a human lung fibroblast cDNA library by polymerase chain reaction (12) using a pair of primers, 5′-CGGGATCCGATATGCCGGAGCATACAAGCTAG-3′ and 5′-AACTGGACGACCTA-GAGCCAGGACATGATTTCC-3′. After cleavage with BamHI and PstI in the primer sequences, it was cloned into matching cleavage sites of the baculovirus transfer vector pBlueBac III (Invitrogen Inc., San Diego, CA). The cDNA for an activated Rap1A, Rap1AV52F, was prepared by oligonucleotide-directed mutagenesis (13) and cloned into pBlueBac III as for the wild-type cDNA. pV-IEK, another baculovirus transfer vector for expressing proteins as GST fusions, was provided by Dr. D. Midda (University of California, San Francisco, CA) through Dr. A. Kikuchi (Hiroshima University, Hiroshima, Japan) (14). For expression of Ha-Ras fused to GST, Ha-Ras cDNA was amplified by polymerase chain reaction using a pair of primers, 5′-CCGGCTCTAAGATGAGG-GAATTAAGCTGTTG-3′ and 5′-GCCGAATTTCTCAGGAGGACA-1

1 The abbreviations used are: BBD, Ras-binding domain; CRR, cysteine-rich region; MBP, maltose-binding protein; GTPγS, guanosine 5′-O-(3-thiotriphosphate); GST, glutathione S-transferase; MEK, mitogen-activated protein kinase kinase/extracellular signal-regulated kinase kinase; KNERK, a kinase negative mutant of ERK2; PAGE, polyacrylamide gel electrophoresis.
CACCCTTG-3'. After digestion with XbaI and EcoRI in the primer sequences, it was cloned into matching cleavage sites of pV-ICS. Structures of the constructs were confirmed by DNA sequence analysis. Preparation of recombinant baculoviruses expressing Ha-Ras, Rap1A, and their mutants and the purification of the post-translationally modified proteins from infected SF9 cells were carried out as described (15, 16).

Assay for Rap1A and Ha-Ras Binding—MBP fusion proteins of Raf-1 N-terminal fragments were expressed in Escherichia coli and immobilized on amylase resin as described (11). Binding reaction was carried out by incubating 20 μl of the resin carrying various amounts of MBP-Raf-1 proteins with various amounts of GTP-S- or GDP-bound Ha-Ras or Rap1A in a total volume of 100 μl of buffer A (20 mM Tris/HCl, pH 7.4, 40 mM NaCl, 1 mM EDTA, 1 mM diethiothreitol, 5 mM MgCl2, and 0.1% Lubrol PX) as described (11). After incubation at 4 °C for 2 h, the resin was washed, and the bound proteins were eluted with buffer A containing 10 mM maltose and subjected to SDS-PAGE followed by Western immunoblot detection with anti-Ras monoclonal antibody Y13-259 (Oncogene Science Inc., Manhasset, NY) or anti-Rap1A polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California). Both anti-Ha-Ras and anti-Rap1A antibodies exhibited little cross-reactivity to Rap1A and Ha-Ras, respectively (data not shown). The assay for competitive inhibition of Ras binding to the MBP-Raf-1 fusion proteins by Rap1A and Ha-Ras were carried out by including a fixed amount of Ha-Ras and various amounts of Rap1A in the same binding reaction. For the in vivo co-association of Rap1A with GST-Ha-Ras, GST-Ha-Ras in SF9 cell lysate was first immobilized on glutathione-Sepharose and then loaded with GTP-S. The resin was then incubated with GTP-S-bound Rap1A in the absence or the presence of purified MBP-Raf-1(51–131) or MBP-Raf-1(48–206). The binding condition was the same as described above except that the bound proteins were eluted with 10 mM glutathione in buffer A.

Suppression of Ras-dependent Activation of Raf-1 by Rap1A and Ha-Ras Mutant E31K in SF9 Cells—Monolayers of SF9 cells (2 × 106 cells) were triply infected with the recombinant baculoviruses expressing the full-length Raf-1 and Ha-Rasα12, along with that expressing Ha-Rasα12/E31K or Rap1α12 (1 × 106 plaque-forming units each). After 72 h post-infection, the cells were lysed by sonication in 1 ml of buffer B (20 mM Tris/HCl, pH 7.5, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 1 mM sodium vanadate) and centrifuged at 13,000 × g for 30 min. Raf-1 was immunoprecipitated from the supernatant (200 μl) with the anti-Raf-1 antibody C12 (2 μl) (Santa Cruz Biotechnology Inc.) and protein A-agarose. The Raf-1 activity was determined by incubating the immunoprecipitates in the presence of GST-MEK (15 μg) and GST-KNERR2 (1 μg) in 30 μl of kinase reaction mixture (20 mM Tris/HCl, pH 7.5, 10 mM MnCl2, 10 mM MgCl2, 20 mM β-glycerophosphate, and 50 μM γ-[32P]ATP (4,000 cpm/pmol)) for 30 min at 25 °C. After the incubation, proteins in the reaction mixture were fractionated by SDS-PAGE and subjected to autoradiography to detect phosphorylation of GST-KNERR2 as described (11, 14).

RESULTS

Rap1A Has Enhanced Binding Activity to Raf-1 CRR—In the previous study, we have shown that Ha-Ras binds to immobilized MBP-Raf-1(132–206), representing CRR, demonstrating that Rap1A is shown in Fig. 1C. Ha-Ras bound to CRR yielded roughly 10-fold less signal than that to RBD even though the amounts of CRR in the binding reaction were doubled, indicating that the ability of Ha-Ras to bind to CRR was roughly 20-fold less than that to RBD. This estimation by immunoblot was consistent with our kinetic measurement of the affinity of Ha-Ras for RBD and CRR using a competitive inhibition of Ha-Ras-dependent activation of Saccharomyces cerevisiae adenyl cyclase (15, 16).2 In a striking contrast, Rap1A bound to CRR yielded stronger signal than that to RBD under the same condition, indicating that it has the ability to bind equally to RBD and to CRR (see also Fig. 1A). After taking account of the observation that Rap1A yielded twice as much signal as Ha-Ras on the equimolar basis (Fig. 1D), the ability of Rap1A to bind to CRR was roughly 10-fold greater than that of Ha-Ras. In addition, the ability of Rap1A to bind to RBD was about one-half of that of Ha-Ras after the same normalization. These results indicated that Rap1A has greatly enhanced rather than impaired activity to bind to CRR.

In our previous report, we found that the binding of Ha-Ras to CRR required post-translational modification (11). To test if binding of Rap1A to CRR was also dependent on its post-translational modification, we incubated RBD and CRR with a lysate of SF9 cells expressing Rap1A. As shown in Fig. 1E, RBD bound both modified and unmodified Rap1A represented by the faster and slower migrating bands, respectively, on the Western immunoblot as described before (17). In contrast, CRR bound only the modified form of Rap1A. These results indicated

2 C.-D. Hu, K. Kariya, and T. Kataoka, unpublished results.
Mechanism of Suppression of Ras by Rap1A

The induce phosphorylation of GST-KNERK2 in the presence of GST-MEK.

extract by the anti-Raf-1 antibody and examined for its activity to ther, triple expression of either of them along with Ha-Ras V12 RasV12(E31K) indeed could not activate Raf-1 (Fig. 1). The results showed that Rap1AV12 and Ha-Ras fusion protein onto glutathione-Sepharose and incubated it with Rap1A in the absence or the presence of MBP-Raf-1 fusion proteins. As shown in Fig. 3C, no Rap1A was found to be associated with GST-Ha-Ras when they were incubated in the absence of MBP-Raf-1 (lane 1). Incubation in the presence of MBP-Raf-1(50–131) did not result in association of Rap1A with GST-Ha-Ras either, whereas MBP-Raf-1(50–131) was associated with GST-Ha-Ras (lane 2). Remarkably, when incubated in the presence of MBP-Raf-1(48–206), not only MBP-Raf-1(48–206) but also Rap1A was found to be associated with GST-Ha-Ras (lane 3). These results indicated that Rap1A and Ha-Ras co-associate with Raf-1 N-terminal region only when it contains both RBD and CRR. Similar results could be obtained if association of GST-Ha-Ras with MBP-Raf-1(48–206) induced dimerization of MBP-Raf-1(48–206), leading to subsequent binding of Rap1A to the second MBP-Raf-1(48–206) molecule. However, induced dimerization of MBP-Raf-1(48–206) was not the case because incubation of GST-Ha-Ras and untagged Ha-Ras in the presence of MBP-Raf-1(48–206) (the same experiment as Fig. 3C, lane 3, except that Ha-Ras was used instead of Rap1A) did not result in any association between GST-Ha-Ras and Ha-Ras (data not shown).

**DISCUSSION**

The current model of Ras-suppressive action of Rap1A involves competitive inhibition by Rap1A of the interaction between Ras and Raf-1 RBD (10). However, we found in this study that Rap1A possessed greatly enhanced ability to bind to CRR, another Ras-binding domain identified by us (11) and
Mechanism of Suppression of Ras by Rap1A

A study with Ras/Rap1A chimera indicated that transforming potential of Ras requires both of the two regions, residues 21–31 and 45–54. On the other hand, Rap1A anti-oncogenicity requires mainly residues 21–31, although residues 45–54 is also required for full activity to suppress transformation (21).

Disregarding that are changed conservatively, that are variable among the Ras family, and that are not exposed on the protein surface, three residues, 26, 31, and 45, have been postulated to determine whether the protein is oncogenic or anti-oncogenic (3, 21–24). In fact, replacements of residues 26 (or 26 plus 27), 31 (or 30 plus 31), or 45 of activated Ras with those of Rap1A resulted in attenuation of transforming activity (22, 24). However, it remained unclear which of these residues plays the most critical role.

Residues 26–28 (including 26) and 42–49 (including 45) have been proposed to constitute a contiguous domain on the surface of Ras protein (3, 25). The domain, termed “activator domain,” was suggested to play an important role for activation of effectors through some physical interaction with them. In the previous study, we found that Ha-Ras proteins carrying mutations N26G and V45E failed to bind to Raf-1 CRR (11).

Because we have previously shown that binding of Ras to both CRR and RBD is necessary for Raf-1 activation (11), it is very likely that this triple complex formation will result in impairment of Raf-1 activation due to the failure of Ha-Ras to bind to CRR. The previous observations that unmodified Rap1A cannot suppress Ras function (5, 6) is also consistent with this, because we found that modification of Rap1A is essential for its binding to CRR.

We found here that E31K mutation alone enhanced binding to CRR and RBD due to the newly created ionic interaction. Although the solution structure of CRR has been solved (27), further understanding of the mechanism of CRR binding should await the structural analysis of the complex between CCR and Rap1A or Rap1A.

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