Cysteine-rich Region of Raf-1 Interacts with Activator Domain of Post-translationally Modified Ha-Ras*

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The interaction between “switch I/effector domain” of Ha-Ras and the Ras-binding domain (RBD, amino acid 51–131) of Raf-1 is essential for signal transduction. However, the importance of the “activator domain” (approximately corresponding to amino acids 26–28 and 40–49) of Ha-Ras and of the “cysteine-rich region” (CRR, amino acids 152–184) of Raf-1 have also been proposed. Here, we found that Raf-1 CRR interacts directly with Ha-Ras independently of RBD and that participation of CRR is necessary for efficient Ras-Raf binding. Furthermore, Ha-Ras carrying mutations (N26G and V45E) in the activator domain failed to bind CRR, whereas they bound RBD normally. On the contrary, Ha-Ras carrying mutations in the switch I/effector domain exhibited severely reduced ability to bind RBD, whereas their ability to bind CRR was unaffected. Mutants that bound to either RBD or CRR alone failed to activate Raf-1. Ha-Ras without post-translational modifications, which lacks the ability to activate Raf-1, selectively lost the ability to bind CRR. These results suggest that the activator domain of Ha-Ras participates in activation of Raf-1 through interaction with CRR and that post-translational modifications of Ha-Ras are required for this interaction.

Raf-1, a 74-kDa cytoplasmic serine-threonine protein kinase regulating the mitogenic-activated protein kinase cascade, is one of the major effectors of Ha-Ras (for a review, see Ref. 13). Raf-1 shares three regions of conservation, termed CR1, CR2, and CR3, with other Raf isoforms and homologs (Fig. 1A) (13). CR1 and CR2 are located in the N-terminal half of Raf-1, and CR3 corresponds to the C-terminal kinase domain. Activation of Raf-1 by N-terminal truncations indicates that the N-terminal portion plays an important regulatory role (14). The minimal region of Raf-1 responsible for the interaction with Ha-Ras has been mapped into 81 amino acids in CR1, RBD (amino acids 51–131) (15), and mutational analyses have suggested that RBD interacts directly with the switch I of Ha-Ras (16–19). However, a mutation (C168S) located in another region of CR1, RBD (amino acids 152–184), has been shown to impair physical interaction of Raf-1 with Ha-Ras and to render Raf-1 unresponsive to Ha-Ras (20, 21). These observations indicate that not only RBD but also CR is critical for Ras-dependent activation of Raf-1. However, the exact mechanism of involvement of Raf-1 CRR in Ras-dependent activation remains to be determined. Here we report that CRR interacts with Ha-Ras independently of RBD and that the activator domain of Ha-Ras is critical not only for this interaction but also for the Ha-Ras-dependent activation of Raf-1. These results suggest that CRR participates in Raf-1 activation through interaction with the activator domain of Ha-Ras. Evidence is also presented that post-translational modifications of Ha-Ras C terminus are necessary for this interaction.

EXPERIMENTAL PROCEDURES

Construction and Expression of Raf-1 Mutants—For Raf-1 deletion mutants (Fig. 1B), various subfragments of the human c-raf-1 cDNA (22) were amplified by polymerase chain reactions (23) using suitable primers and designated Raf-1-x-y, in which x-y represents the range of...
Raf-1 proteins—The recombinant baculovirus expressing wild-type human Ha-Ras protein was described previously (24). Amino acid substitution mutations were introduced into Raf-1(50–131) and Raf-1(132–206), respectively, by oligonucleotide-directed mutagenesis (25). R89L and C168S substitution mutations were introduced into pMal vectors for expression as fusions with MBP in encoded polypeptides in amino acid positions. They were cloned into two subregions surrounding the effector domain.

The activator domain is split into two subregions surrounding the effector domain, B. Shown are the structure of the full-length Raf-1 protein (a) and an enlargement of its N-terminal region (b), on which three conserved regions (CR1, CR2, and CR3) and the two subregions (RBD and CRR) in CR1 are indicated. See the text for the definition of these regions and subregions. The structures of various Raf-1 mutants used in this study are also depicted (c–i). Numbers on the bars represent amino acid positions in both A and B. encoded polypeptides in amino acid positions. They were cloned into pMal vectors for expression as fusions with MBP in Escherichia coli (24). R89L and C168S substitution mutations were introduced into Raf-1(50–131) and Raf-1(132–206), respectively, by oligonucleotide-directed mutagenesis (25) using mutagenic oligonucleotides 5′-GGTGAAGGCGCCAACAGCTATCAAGACA-GAACG-3′ and 5′-TTGTAGCCAGAAGTCT-GCGGGATCCGATATGAC-3′. The resulting DNA fragments Raf-1(50–131, R89L) and Raf-1(132–206, C168S) were cloned into pMal for expression as MBP fusions in E. coli.

Expression and Purification of Wild-type and Mutant Ha-Ras Proteins—The recombinant baculovirus expressing wild-type human Ha-Ras protein was described previously (24). Amino acid substitution mutations were introduced into the synthetic human Ha-Ras cDNA encoding the Ha-Ras protein bearing the oncogenic valine for glycine substitution mutation at position 12 (Ha-RasV12) as described previously (6, 9, 11). The mutant genes were amplified by polymerase chain reactions (23) with a pair of primers 5′-CCGGATCCGATATGACCGAATACAACACCGTACG-3′ and 5′-AACTGCAGCTATCAAGACCGAACGCATTGTG-3′, and, after cleavage with BamHI and PstI in the primer sequences, cloned into matching cleavage sites of the baculovirus transfer vector pBlueBac III (Invitrogen Inc., California). Accuracy of the recombinant expression was confirmed by nucleotide sequencing. The posttranslationally modified form of wild-type or mutant Ha-Ras proteins and unmodified form of wild-type Ha-Ras protein were purified from membrane and cytosolic fractions, respectively, of Sf9 cells infected with recombinant baculoviruses expressing the respective proteins as described (24, 26). The unmodified Ha-Ras is neither farnesylated nor further processed as shown previously (26, 27). Ha-Ras protein was quantitated by GTP-γ-S binding assay.

Assay for Ha-Ras Binding—MBP-Raf-1 fusion proteins in cell lysates were immobilized on amylose resin by incubation at 4 °C for 2 h, followed by extensive washing with buffer A (20 mM Tris/HCl, pH 7.4, 40 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5 mM MgCl2, and 0.1% Lubrol PX). Binding reactions were carried out by incubating GTP-γ-S or GDP-bound Ha-Ras protein with 20 μl of the resin carrying immobilized MBP-Raf-1 proteins in a total volume of 100 μl of buffer A. After incubation at room temperature for 30 min and at 4 °C for 2 h with continuous agitation, the resin was washed with 400 μl of buffer A. The bound proteins were subsequently eluted from the resin with 200 μl of buffer A containing 10 mM maltose and subjected to SDS-polyacrylamide gel electrophoresis, followed by Western immunoblot detection with an anti-Raf-1 monoclonal antibody Y13-259 (Oncogene Science Inc., New York).

Assay for Ras-dependent Activation of Raf-1—The assays were carried out similarly as described (28). E. coli expressing GST fusion protein of MEK1 (GST-MEK) or that of KNRK (GST-KNRK), and a recombinant baculovirus expressing the full-length human Raf-1 were obtained from Dr. A. Kikuchi (Hiroshima University School of Medicine, Hiroshima, J. Japan). Monolayers of Sf9 cells (2 x 107 cells) were coinfected with the recombinant baculovirus expressing the full-length Raf-1 and that expressing either Ha-RasV12 or one of its mutants (1 x 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 x 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 kinase activity was determined by incubating the immunoprecipitates in the absence or presence of GST-MEK (13 μg) and GST-KNRK (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 (4000 cpm)) for 30 min at 25 °C. After the incubation, proteins in the reaction mixture were fractionated by SDS-polyacrylamide gel electrophoresis and subjected to autoradiography to detect phosphoproteins.

RESULTS

CRR of Raf-1 Acts as Another Ras-binding Domain Independently of RBD—First, we attempted to elucidate the role of CRR in the Ras-Raf interaction. We reasoned that if CRR is really important for the physical interaction between Ha-Ras and Raf-1, deletion of CRR should result in reduced Ras-Raf binding. To confirm this, we constructed serial C-terminal deletion mutants of Raf-1 CR1 (Fig. 1B), expressed them in E. coli as fusions with MBP, and performed an in vitro Ha-Ras-binding assay. Consistent with the idea, the amount of Ha-Ras bound to the immobilized MBP-Raf-1 mutants was considerably decreased with the deletion of CRR (Fig. 2A). Contribution of CRR to efficient Ras-Raf binding could be accounted for by either one of the two mechanisms: conformational stabilization of RBD by CRR or a physical interaction between CRR and Ha-Ras. Therefore, we examined the potential of CRR to interact with Ha-Ras independently of RBD. For this purpose, we prepared MBP-Raf-1(132–206), which included 20 amino acids each upstream and downstream of CRR for preservation of the functional CRR conformation, and MBP-Raf-1(50–130), which contained the extra amino acid at the N terminus of RBD. As shown in Fig. 2B, immobilized MBP-Raf-1(132–206) bound Ha-Ras-GTP, although the binding was weaker compared with MBP-Raf-1(50–130). However, with increasing concentration of Ha-Ras, the amount of Ha-Ras bound to MBP-Raf-1(132–206) increased and finally reached to a level comparable to that bound to MBP-Raf-1(50–130). This suggested that the observed weaker binding was ascribable to the lower affinity of CRR for Ha-Ras than that of RBD. Unexpectedly, MBP-Raf-1(132–206) also bound Ha-Ras-GDP almost equally. Parallel bindings of Ha-Ras-GTP and Ha-Ras-GDP were consistently observed with various amounts of Ha-Ras (Fig. 2B) or MBP-Raf-1(132–206) (data not shown). These results indicated that CRR could act as another Ras-binding domain in a GTP-independent manner. To test the specificity of the interactions of RBD and CRR with Ha-Ras, we introduced mutations R89L and C168S into RBD and CRR, respectively, since these mutations have been shown to cause impaired interaction of Raf-1 with Ha-Ras both in vitro and in vivo (17, 20, 21). We expressed these mutant proteins as fusions with MBP, and performed the in vitro binding assay (Fig. 2C). As expected, both MBP-Raf-1(50–130, R89L) and MBP-Raf-1(132–206, C168S) exhibited greatly reduced abilities to bind Ha-Ras. Based on these obser-
The amounts of Ha-Ras proteins bound to various MBP-Raf-1 fusion proteins immobilized on amylase resin in vitro were measured by Western immunoblotting with the anti-Ras antibody Y13-259. A, 10 pmol of GTPγS-bound Ha-Ras (T) or GDP-bound Ha-Ras (D) were incubated with 25 pmol of MBP-Raf-1 fusion proteins or MBP alone. The numbers on the top indicate the range of Raf-1, in amino acid position, expressed as fusions with MBP. B, various amounts of GTPγS-bound Ha-Ras (T) or GDP-bound Ha-Ras (D) were incubated with either 100 pmol of MBP-Raf-1(132–206) (CRR), 50 pmol of MBP-Raf-1(50–131) (RBD), or 100 pmol of MBP alone (MBP). The numbers indicate the amounts of Ras proteins used for the assay. C, 10 pmol of GTPγS-bound Ha-Ras were incubated with 50 pmol of MBP-Raf-1(50–131) (RBD) or its mutant MBP-Raf-1(50–131, R89L) (RBD), and with 100 pmol of MBP-Raf-1(132–206) (CRR) or its mutant MBP-Raf-1(132–206, C168S) (CRR). D, 10 pmol each of GTPγS-bound Ha-Ras WT (WT) or its mutants (N26G, V45E, A59E, and D38N) were incubated with 25 pmol of MBP-Raf-1(50–131) (RBD), 100 pmol of MBP-Raf-1(132–206) (CRR), or 100 pmol of MBP alone (MBP). The mobilities of the Ha-Ras mutants differ from one another as reported previously (4). E, 20 pmol of GTPγS-bound post-translationally unmodified Ha-Ras were incubated with 25 pmol of MBP-Raf-1(50–131) (RBD) or 100 pmol of MBP-Raf-1(132–206) (CRR). Experiments were repeated three times giving equivalent results.

In the present study, we have shown that CRR interacts with Ha-Ras independently of RBD. During the preparation of this manuscript, Brtva et al. also reported that CRR (GST-Raf-1(139–186)) alone can interact with Ha-Ras in a GTP-dependent manner using an ELISA-based assay (29). However, this
result is contradictory to our finding that CRR binds to Ha-Ras in a GTP-independent manner. Considering the GTP-dependent binding of all other Raf-1 mutants to Ha-Ras (Fig. 2, A and B), it seems that the condition of our assay is appropriate to detect GTP-dependent binding. Furthermore, the observations that CRR did not interact with Ha-Ras mutants N26G and V45E, and that the C1685 mutation introduced into CRR could abolish its interaction with wild-type Ha-Ras, strongly support the specificity of the interaction between Ha-Ras and Raf-1 CRR. Finally, our results agree with the fact that the conformation of the activator domain is not affected by GDP/GTP exchange (10, 11). Although the reason for the discrepancy regarding GTP dependence is unknown, it must be pointed out that the source of Ha-Ras protein in our assay is different from that in their assay. We used post-translationally modified Ha-Ras protein purified from infected Sf9 cells, while they used bacterially expressed Ha-Ras protein which was not modified. In our hands, unmodified Ha-Ras protein purified from Sf9 cells did not bind CRR at all (Fig. 2E).

Post-translational modifications of Ha-Ras at its C terminus have been shown to be necessary for its membrane-anchoring as well as for Raf-1 activation (12, 28). Modifications of Ha-Ras may have an indirect role in Raf-1 activation; modified and membrane-anchored Ha-Ras may simply recruit Raf-1 to the plasma membrane, where it is subsequently activated by an unknown membrane-bound factor(s) (30, 31). However, it is possible that modifications may have a direct role in this process; they may be required for establishing a specific mode of interaction essential for Raf-1 activation. Previous in vivo studies using an Ha-Ras C-terminal mutant deficient in modifications could not examine the two possible roles of modifications in Raf-1 activation separately (28). Furthermore, in vitro binding studies using a Raf-1 fragment containing both RBD and CRR did not reveal any significant defect of unmodified Ha-Ras in interaction with Raf-1 (21). In the present study, by separately examining RBD and CRR, we have shown that unmodified Ha-Ras lacks the ability to interact with CRR. This represents a novel molecular defect of unmodified Ha-Ras, which is unrelated to the membrane-anchoring defect. Recently, we have established an in vitro membrane-free system in which modified Ha-Ras can activate B-Raf but unmodified Ha-Ras cannot.2 In another membrane-free system, modifications are also required for yeast Ras2 to activate its effectors, yeast adenylyl cyclase (32). These observations employing the membrane-free systems also indicate that the defect of unmodified Ras proteins in activating the effectors is unrelated to membrane anchoring.

Previous reports examined effects of mutations in RBD or CRR to study roles of these domains in Ras-dependent activation (17, 20, 21). However, these manipulations may either alter conformation of Raf-1 or modulate its basal activity (17, 20). Therefore, use of Ha-Ras mutants that possess the ability to interact differentially with the two Ras-binding domains may provide further insights. In the present study, we actually found these mutants and used them to provide evidence that CRR is involved in Ras-dependent activation of Raf-1. The analysis also showed that residues belonging to the activator domain of Ha-Ras are critical for interaction with CRR, providing a molecular basis for the role of this domain in activation of Raf-1. The binding affinity of CRR for the activator domain is lower than that of RBD for the switch I/effector domain and may not be sufficient for stable association of Raf-1 with GDP-bound Ha-Ras in vivo. However, after establishment of the interaction between RBD and switch I/effector domain of GDP-bound Ha-Ras, the interaction between CRR and the activator domain may take place efficiently and induce further conformational change of Raf-1 to be activated. Post-translational modifications of Ha-Ras C terminus are involved in the latter interaction, although the mechanism of their action remains to be clarified. Further understanding of these interactions will require x-ray crystallographic study of the complex between post-translationally modified Ha-Ras and Raf-1 including both RBD and CRR.

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