Effect of Phosphorylation on Activities of Rap1A to Interact with Raf-1 and to Suppress Ras-dependent Raf-1 Activation*

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Rap1A is phosphorylated by cAMP-dependent protein kinase (PKA), and this phosphorylation has been shown to modulate its interaction with other proteins. However, it is not known whether Rap1A phosphorylation is involved in regulation of its cellular functions, including suppression of Ras-dependent Raf-1 activation. We have previously shown that this suppressive activity of Rap1A is attributable to its greatly enhanced ability to bind to the cysteine-rich region (CRR, residues 152–184) of Raf-1 compared with that of Ras. Here, we show that phosphorylation of Rap1A by PKA abolished its binding activity to CRR. Furthermore, a mutant Rap1A(S180E), whose sole PKA phosphorylation residue, Ser-180, was substituted by an acidic residue, Glu, to mimic its phosphorylated form, failed to suppress Ras-dependent Raf-1 activation in COS-7 cells. These results indicate that the CRR binding activity and the Ras-suppressive function of Rap1A can be modulated through phosphorylation and suggest that Rap1A may function as a PKA-dependent regulator of Raf-1 activation, not merely as a suppressor.

Rap1A belongs to the Ras family of small GTP-binding proteins. Accumulated evidences indicate that Rap1A is phosphorylated at its C-terminal Ser-180 both in vitro and in vivo by PKA1 (for reviews, see Refs. 1 and 2). Phosphorylation of Rap1A induced by cAMP-elevating agents is observed in a number of cell types including Rat-1 cells (3), IL-60 cells (4), PC12 cells (5), and neutrophils (6, 7). The observation in the neutrophils that the association of Rap1A with cytosome b is dramatically reduced by phosphorylation (7) suggests a regulatory role of Rap1A phosphorylation in its interaction with other proteins. However, there has been no report demonstrating the effect of phosphorylation on cellular functions of Rap1A.

Rap1A has a very high homology to Ras and associates with almost all cellular effectors of Ras including Raf-1 (8), B-Raf (5), and RalGDS (9–11) in mammalian cells as well as Caenorhabditis elegans PLC210 (12). Raf-1 is a serine/threonine protein kinase regulating the mitogen-activated protein kinase cascade (for a review, see Ref. 13). Ras activates Raf-1 by physically associating with it at the plasma membrane. This physical association is mediated mainly by the interaction between RBD (residues 51–131) of Raf-1 and the effector region of Ras (residues 32–40 of Ha-Ras) (13). In addition to RBD, we and others have recently found that CRR (residues 152–184) of Raf-1 also interacts with Ras and shown that this novel interaction is essential for activation of Raf-1 (Refs. 14–19; for a review, see Ref. 20).

In contrast to Ras, Rap1A cannot activate Raf-1. Instead, Rap1A inhibits Ras-dependent Raf-1 activation. Because Rap1A has the identical effector region to that of Ras and associates well with RBD (8), we speculated that it would have a defect in association with CRR. However, to our surprise, we found that Rap1A has a greatly enhanced ability to associate with CRR compared with Ras (21). Further, the enhanced CRR binding property of Rap1A resulted in formation of a ternary complex with Ras and Raf-1 in which Rap1A and Ras independently associate with CRR and RBD of Raf-1, respectively. Raf-1 in this complex cannot be activated by Ras, presumably because interaction of CRR with Ras is hampered by Rap1A tightly bound to CRR.

Association with CRR seems to involve the C-terminal region of Rap1A, because it was shown that the association was dependent on the C-terminal posttranslational modification of Rap1A (21). We therefore reasoned that phosphorylation of Rap1A at its C-terminal Ser-180 might affect its ability to associate with CRR. Here we report that the phosphorylation specifically abolished the activity of Rap1A to bind to CRR. Further, a Rap1A mutant mimicking the phosphorylated form was found to have lost the activity to suppress Ras-dependent Raf-1 activation.

EXPERIMENTAL PROCEDURES

Preparation of Various Recombinant Proteins—MBP-Raf-1(51–131), MBP-Raf-1(132–206), and MBP-Raf(1–48–206) are MBP fusion proteins containing the indicated residues of human Raf-1 described before (14). MBP-PLC210 is an MBP fusion protein containing the Ras-associating domain (residues 1570–1670) of PLC210 (12). MBP-RalGDS is an MBP fusion protein containing the Ras-interacting domain (residues 724–852) of rat RalGDSb (9–11). The MBP fusion proteins were expressed in Escherichia coli harboring pMal vectors carrying the corresponding cDNA fragments. The E. coli cells were lysed and centrifuged at 100,000 × g for 30 min. The MBP fusion proteins in the resulting supernatant fractions were immobilized on amyllose resin and used for the in vitro binding assays as described below. cDNAs encoding Rap1AV12(S180E) and Rap1AV12(S180A), carrying a substitution of Glu or Ala, respectively, for Ser at residue 180 in addition to an activating mutation of Gly to Val at residue 12, were prepared by polymerase chain reaction using oligonucleotide primers carrying the correspond-
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ing mutations and the Rap1A V12 cDNA as a template (21, 22). Cloning of the mutant Rap1A cDNAs into a baculovirus transfer vector and preparation of the recombinant baculoviruses expressing them were carried out as described before (23). Procedures for purification of the posttranslationally modified forms of Ras and Rap1A from Sf9 cells infected with baculoviruses expressing the respective proteins were described before (21). Expression of a GST fusion Ha-Ras (GST-Ha-Ras) in Sf9 cells using a baculovirus vector was described before (21).

In Vitro Binding and Complex Formation Assays—The in vitro binding reactions were carried out by incubating 20–30 µl of amylase resin carrying various immobilized MBP fusion proteins with GTP-S or GDP-loaded Rap1A (total volume of 100 µl of 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) as described (14, 21). 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-Rap1A polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California). The ECL system (Amersham Pharmacia Biotech) was used for signal development. For examination of the ternary complex formation, GST-Ha-Ras in Sf9 cell lysate was first immobilized on glutathione-Sepharose resin and then loaded with GTP-S. The resin was subsequently incubated with GTP-S-loaded Rap1A in the presence of MBP-Raf-1 (48–206). The experimental condition was the same as described above, except that the bound proteins were eluted with 10 mM glutathione in buffer B (100 mM Tris/HCl, pH 7.5, 10 mM MnCl2, 1 mM MgCl2, 0.5 mM Na2EDTA, 0.1% Lubrol PX, 20% glycerol, and 5 mM TTYADPIASRTGRBN (21, 22)) and then loaded with GTP and 10 pmol of unphosphorylated Rap1A with the anti-Rap1A antibody. Experiments shown for the Raf-1 kinase activity in COS-7 Cells—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. For the activation of Raf-1 by Ras or Rap1A, cells in 60-mm dishes (50% confluency) were cotransfected with a combination of a Raf-1 expression vector pH5-Raf-1 (25) and either one of pcDNA3.1-Ha-Ras V12, pSR-Rap1A V12 (25), or pSR-Rap1A V12 (25) by using SuperFect Transfection Reagent (Qiagen GmbH, Germany). For examination of suppression of the Ras-dependent Raf-1 activation by Rap1A or its mutants, cells were cotransfected with a combination of pcDNA3.1-Ha-Ras V12, pSR-Raf-1, and either one of pSR-Rap1A V12, pSR-Rap1A V12 (25) by using SuperFect Transfection Reagent (Qiagen GmbH, Germany). For examination of the effect of phosphorylation of Rap1A on its binding activities toward RBD and CRR in the same in vitro binding assay. First, we incubated Rap1A with a recombinant catalytic subunit of PKA and confirmed its quantitative phosphorylation by observing its mobility shift on SDS-PAGE as reported (4) (Fig. 1A). After incubation of PKA by the protein kinase inhibitor peptide, Rap1A was loaded with GTP-S and incubated with 10 pmol of GTP and 20 pmol of MBP-Raf-1 (48–206). As shown in Fig. 1B, the phosphorylated Rap1A exhibited no detectable activity to bind to CRR in a sharp contrast to the unphosphorylated Rap1A. The same result was obtained with the phosphorylated Rap1A loaded with GDP (data not shown). In contrast, when tested with MBP-Raf-1 (51–131), the unphosphorylated and the phosphorylated Rap1A did not exhibit any significant difference in their RBD binding activities (Fig. 1C). GTP dependence of the RBD binding was unaffected also. These results indicate that the phosphorylation of Rap1A specifically impaired the Rap1A-CRR association.

In addition to Raf-1, we examined the effect of Rap1A phosphorylation on interaction with other Ras effectors, RaLGDS, and PLC210 (12). As shown in Fig. 1, D and E, we observed GTP-dependent binding of Rap1A to both the Ras-interacting domain of rat RaLGDS and the Ras-associated domain of C. elegans PLC210, which were produced as MBP fusions. However, we did not observe any difference of binding between the phosphorylated and the unphosphorylated forms of Rap1A (Fig. 1, D and E). These results further indicate that the effect of Rap1A phosphorylation is specific to Raf-1 CRR. Phosphorylated Rap1A Lacks Its Ability to Form a Ternary Complex with Ras and Raf—We next examined the effect of Rap1A phosphorylation on its ability to form a ternary complex.
with Ras and Raf-1. In this complex, Ras and Rap1A were independently associated with RBD and CRR, respectively. When Rap1A in the unphosphorylated form was incubated with GST-Ha-Ras immobilized on glutathione-Sepharose resin in the presence of MBP-Raf-1(48–206) containing both RBD and CRR, all three proteins were trapped on the resin and thereby co-eluted with 10 mM glutathione (Fig. 1F, lane 1). In contrast, when the phosphorylated Rap1A was used in the same reaction in place of the unphosphorylated form, no Rap1A was detectable in the eluate even though a similar amount of MBP-Raf-1(48–206) was co-eluted with GST-Ha-Ras (Fig. 1F, lane 2). These results indicate that the phosphorylation of Rap1A abrogates its ability to form a ternary complex with Ha-Ras and Raf-1.

Phosphorylation at Ser-180 of Rap1A Is Responsible for the Effect on CRR Binding—Accumulated evidences indicate that Ser-180 is a sole phosphorylation site of Rap1A by PKA examined both in vivo and in vitro (1, 2). To prove that phosphorylation at Ser-180 was responsible for the observed effect of PKA treatment of Rap1A, we examined the effect of substitution of Ala for Ser-180. Rap1AV12(S180A) exhibited an activity to associate with Raf-1 CRR comparable with wild-type Rap1AV12 (Fig. 2A). In contrast to the case with wild-type Rap1AV12, no visible mobility shift was observed after treatment of this Rap1A mutant by PKA, suggesting that it was not phosphorylated by PKA (Fig. 2B). Furthermore, the PKA treatment of Rap1AV12(S180A) did not affect its activity to bind to CRR (Fig. 2C). This result indicates that Ser-180 is indeed the sole phosphorylation site responsible for the loss of the CRR binding activity of Rap1A.

Rap1A(S180E) Mutant Mimics the Phosphorylated Rap1A and Fails to Suppress Raf-1 Activation by Ras—Because we previously found that the tight association of Rap1A with CRR is responsible for the Ras-suppressive activity of Rap1A, the above findings prompted us to test the effect of Rap1A phosphorylation on its cellular function, in particular suppression of Ras-dependent Raf-1 activation (1, 2). For this purpose, we could not employ the conventional approach of stimulating cells with cAMP-elevating agents or overexpressing PKA catalytic subunit, because it has been shown that Raf-1 is phosphorylated by PKA and thereby rendered insensitive to activation by Ras (26, 27). To circumvent this problem, we turned to test the feasibility of using a mutant Rap1AV12(S180E) in which the phosphorylation residue Ser-180 was replaced by an acidic residue Glu. This mutant is similar to the reported mutant Rap1A(S180D), which was shown to mimic the phosphorylated Rap1A (5). Rap1AV12(S180E) was expressed in S9 cells, purified, and examined for its activity to associate with CRR. As shown in Fig. 2A, the mutant exhibited no binding activity toward CRR, which is similar to the case with the phosphory-
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Rap1A has been reported to associate with almost all Ras effectors and other proteins in cells. Although it is assumed that phosphorylation of Rap1A may be a physiological event, regulating its interaction with other proteins based on the observation that phosphorylated Rap1A does not associate with cytochrome b (7), there has been no report that phosphorylation also affects the association of Rap1A with Ras effectors. In the present study, we have shown that the phosphorylated Rap1A lacks its ability to bind to Rap1A effector. This represents the second direct evidence that phosphorylation of Rap1A affects its interaction with other proteins. Consistent with our previous finding that CRR binding requires the C-terminal lipid modification of Rap1A (21), this result provides further support to the idea that the C-terminal region of Rap1A is involved in CRR binding. Significantly, we have shown that the C80E mutant, mimicking the phosphorylated Rap1A, can no longer suppress Ras-dependent Rap1A activation. This result not only agrees well with our hypothesis that the enhanced CRR binding is involved in suppression of Ras-dependent Rap1A activation by Rap1A but also, to our knowledge, represents the first clear demonstration that the cellular function of Rap1A is controlled by phosphorylation.

The first indication of Ras-antagonizing activity of Rap1A came from its ability to induce reversion of Ras-transformed fibroblasts (28). Subsequently, this antagonizing activity of Rap1A was also observed in nontransformed cells (1, 2). Although these studies employed expression of a large amount of Rap1A, it has recently been shown that expression of Rap1AV12 at levels similar to the endogenous wild-type protein also efficiently inhibits activation of mitogen-activated protein kinase by growth factors (29). Because this mitogen-activated protein kinase activation was found dependent on Ras, the observation suggested that a physiological amount of endogenous Rap1A may be sufficient for its antagonistic effect on Ras during normal cell growth (29). Based on the present results that the antagonizing activity of Rap1A can be controlled by phosphorylation, we propose further that Rap1A may function as a PKA-dependent regulator of Rap1A activation, not merely as a suppressor.

In addition to Rap1A, PKA phosphorylates many cellular proteins including Rap1 (26, 27). Phosphorylation of Rap1 by PKA renders it incapable of associating with Ras, thereby precluding its activation by Ras. Taken together with our present finding, one can hypothesize that PKA may exert both positive and negative regulatory effects on Ras-dependent Rap1A activation. Although these ambivalent effects appear puzzling, they may be required for fine-tuning the signal output from Rap1 in different cell-type or developmental contexts. It should also be pointed out that there exists a possibility that a protein kinase other than PKA may specifically phosphorylate Rap1A, but not Rap1, under control of a certain extracellular signal. Identification of such a protein kinase might provide a further insight into the regulatory mechanism of Rap1A activity by Rap1A.

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