Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 8683–8686, September 1993
Biochemistry

GTP-dependent association of Raf-1 with Ha-Ras: Identification of Raf as a target downstream of Ras in mammalian cells

(signal transduction/GTP-binding proteins/serine/threonine kinases)

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Communicated by Arthur Kornberg, June 21, 1993

ABSTRACT **Ras is involved in signal transduction of various factors for growth, differentiation, and oncogenesis. Recent studies have revealed several proteins that function upstream and downstream of the Ras signaling pathway. However, its immediate downstream target molecule has not yet been identified. In an effort to identify the Ras-associated downstream proteins, we added recombinant Ha-Ras in a GTP-bound form to cell-free lysates and used several antibodies against Ras to immunoprecipitate Ras complexes. We found that a serine/threonine kinase, Raf-1, was coimmunoprecipitated with Ha-Ras by two anti-Ras antibodies (LA069 and Y13-238), whereas a neutralizing antibody against Ras (Y13-259) could not precipitate Raf-1. The coimmunoprecipitation was observed with a complex of Ras and guanosine 5′-[γ-thio]triphosphate but not with a complex of Ras and guanosine 5′-[β-thio]diphosphate. The GTP-dependent association of Ha-Ras with Raf-1 was observed with lysates of various types of cultured cells, including NIH 3T3, pheochromocytoma (PC) 12, Ba/F3, and Jurkat T cells, and also with crude extracts from rat brain. Furthermore, Raf-1 was precipitated with a transforming Ha-Ras mutant ([Val^{12}]Ras) and wild-type Ha-Ras but not with an effector-region mutant ([Leu^{35},Arg^{37}]Ras) that lacks transforming activity. These results indicate that Ras·GTP physically associates with Raf either directly or through other component(s) and strongly suggest that Raf functions in close downstream proximity to Ras in mammalian cells.**

Ras is a member of a family of low molecular weight GTP-binding proteins and plays an important role in various signaling pathways for cellular growth, differentiation, and transformation (1, 2). Like other GTP-binding proteins (3), Ras cycles between an active GTP-bound form (Ras·GTP) and an inactive GDP-bound form (Ras·GDP) and thus functions as a molecular switch in signal transduction (1, 2). To understand further details of the mechanism of signal transduction mediated by Ras, it is important to clarify its upstream and downstream components. Recent studies have revealed various signals that activate Ras through tyrosine kinases (4), adaptor proteins (5, 6), and modulator proteins such as exchange stimulators (7–12) and GTPase-activating proteins (13).

Analysis using several Ras mutants has shown that several proteins function downstream of Ras in mammalian cells. A dominant inhibitory Ras mutant was reported to block growth-factor-induced activation of Raf-1 kinase, mitogen-activated protein (MAP) kinase, and ribosomal S6 kinase (14–17, 51). On the other hand, an activated Ras mutant was found to stimulate MAP kinase both *in vivo* and *in vitro* (18–22). These observations suggest that Raf-1, MAP kinase kinase, MAP kinase, and ribosomal S6 kinase act downstream of Ras. However, to our knowledge, the direct target of Ras has not yet been identified.

The *raf-1* gene was originally identified as an oncogene of a murine sarcoma virus 3611, which encodes a 72- to 76-kDa cytoplasmic protein with serine/threonine kinase activity (23). The kinase domain of Raf-1 is located in the C-terminal portion and its N-terminal portion is thought to be a regulatory domain (23). In *Drosophila melanogaster* (24) and *Caenorhabditis elegans* (25), Raf-1 has been genetically assigned as a downstream component in Ras-mediated signaling pathways. Specification of R7 cells in eye development of *Drosophila* requires activation of sevenless receptor tyrosine kinase, whose signal comes down to Ras1 through adaptor protein Drk (26, 27) and modulator proteins Sos and Gap1 (28–30) and then down to Draf (or Draf-1) (24). Similarly, the signal for induction of vulval differentiation in *C. elegans* is transduced from tyrosine kinase let-23 to let-60 (Ras) (31) through adaptor protein sem-5 (32) and leads to lin-45 (Raf) (25). In mammalian cells, Raf-1 has also been shown to function downstream of Ras. It has been demonstrated that expression of a dominant inhibitory mutant or an antisense mRNA of Raf-1 inhibits v-*ras*-induced transformation of NIH 3T3 cells (33), while v-*raf*-transformed NIH 3T3 cells are unaffected by injection of an anti-Ras antibody (34). Since Raf-1 can induce activation of MAP kinase kinase (35–37), Raf-1 appears to be located closer to Ras than any other known downstream molecules.

In the present study, we examined a possible association between Ha-Ras and Raf-1 using several anti-Ras antibodies. LA069, an antibody against the C-terminal portion of Ha-Ras, and Y13-238, a nonneutralizing antibody against Ras, coimmunoprecipitated Raf-1. This was observed with the GTP-bound form but not with the GDP-bound form of Ras. A Ha-Ras mutant that has mutations at an "effector" region (38, 39) did not associate with Raf-1, indicating that the effector region of Ras is probably involved in this association. Our results indicate that Ras·GTP and Raf associate to form a complex, although the possibility has not been ruled out that unknown molecule(s) may link Ras to Raf. Recently, Moodie *et al.* (40) have independently demonstrated that Raf-1 and MAP kinase kinase associate with the GTP-bound form of immobilized Ras.

MATERIALS AND METHODS

Antibodies and Peptides. An anti-Raf-1 antibody (Sp63) and an anti-Ras antibody (Has 6) were kind gifts from U. Rapp

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Abbreviations: MAP, mitogen-activated protein; GTP[γS], guanosine 5′-[γ-thio]triphosphate; GDP[βS], guanosine 5′-[β-thio]diphosphate.

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(NCI) and T. Tanaka (Hiroshima University, Japan), respectively. An anti-Ras antibody (LA069) and peptides 146 and 147 were purchased from Quality Biotech (Camden, NJ). An anti-mouse IgG antibody and an anti-rat IgG antibody were purchased from Cappel.

Cell Culture. Mouse NIH 3T3 fibroblast cells, rat pheochromocytoma (PC) 12 cells, mouse Ba/F3 pro-B cells, and human Jurkat T cells were cultured, respectively, in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal bovine serum; DMEM containing 10% fetal bovine serum and 5% (vol/vol) horse serum; RPMI 1640 medium containing mouse interleukin 3 (10 ng/ml), 50 μM 2-mercaptoethanol, and 10% fetal bovine serum; and RPMI 1640 medium containing 10% fetal bovine serum. All cells were grown at 37°C in an atmosphere of 5% CO_2/95% air.

Preparation of the GTP- or GDP-Bound Form of Ha-Ras. Recombinant wild-type Ha-Ras and Ha-[Val^{12}]Ras proteins were produced in *Escherichia coli* and purified as described (41). A mutant Ha-Ras ([Leu^{35}, Arg^{37}]Ras) protein was kindly given by D. Lowy (NCI). To exchange the Ras-bound guanine nucleotide, the recombinant Ras was mixed with a 20-fold excess of guanosine 5'-[γ-thio]triphosphate (GTP[γS]) or guanosine 5'-[β-thio]diphosphate (GDP[βS]) and incubated for 10 min at 30°C in 50 mM Tris·HCl (pH 7.5) containing 5 mM EDTA, 2 mM $MgCl_2$, 150 mM NH_4Cl, and bovine serum albumin (0.5 mg/ml). After the exchange reaction was completed, $MgCl_2$ was added to a final concentration of 7 mM.

Immunoprecipitation Experiments. Cells were lysed with 10 mM Hepes (pH 7.3) containing 150 mM NaCl, 10 mM $MgCl_2$, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, phosphatase inhibitors (10 mM NaF/25 mM β-glycerophosphate/1 mM sodium orthovanadate), and protease inhibitors [aprotinin (20 μg/ml)/leupeptin/pepstatin A (10 μg/ml)]. In the case of rat brain, brain tissue from a Wistar rat (Simonsen Laboratories, Gilroy, CA) was homogenized with the lysis buffer described above except that the buffer contained 0.25 M sucrose instead of 150 mM NaCl. To the lysate (50 μl) was added an antibody, which was premixed with protein G-Sepharose 4 Fast Flow (Pharmacia) and with either anti-mouse or anti-rat IgG antibody. The mixture was incubated at 4°C for 2 hr with 1 μg of the wild-type or mutant Ha-Ras complexed with either GTP[γS] or GDP[βS]. In the experiment using peptides, 1 μg of peptide was added to the lysate at this step. After incubation, the Sepharose was washed, and bound proteins were eluted from the Sepharose by boiling with the sample buffer for SDS/polyacrylamide gel electrophoresis (SDS/PAGE). The eluted samples were fractionated by SDS/PAGE. Immunoblots were probed with either Sp63 or Has6 antibody and detected using the ECL immunodetection system (Amersham).

RESULTS

Coimmunoprecipitation of Raf-1 with the GTP-Bound Form of Ha-Ras. We prepared a cell-free lysate from the mouse pro-B cell line, Ba/F3, and performed immunoprecipitation by mixing the lysate with recombinant Ha-Ras and LA069, an antibody against the C-terminal portion of Ha-Ras. Immunoprecipitates were dissolved and separated on a polyacrylamide gel. After Western blot analysis, Raf-1 was detected in the immunoprecipitates only when Ha-Ras was added as Ras·GTP[γS] (Fig. 1*A*). A faint band observed with Ras·GDP[βS] might be due to the presence of endogenous Ras·GTP, since the intensity of the band was similar to the sample without added Ha-Ras (Fig. 1*A*). By immunoblot analysis with an anti-Ras antibody (Has6), we found practically no difference between the amount of immunoprecipitated Ras·GTP[γS] and Ras·GDP[βS] (data not shown). Thus, it was concluded that Raf-1 was immunoprecipitated with Ras·GTP[γS] but not with Ras·GDP[βS]. To confirm that this observation was not due to nonspecific immunoprecipitation, an epitope peptide for LA069 (peptide 146, residues 157–181 of Ha-Ras) was added to the lysate as a competitor of immunoprecipitation. The peptide inhibited the coimmunoprecipitation of Raf-1 (Fig. 1*B*) and precipitation of Ras (data not shown), whereas a different peptide (peptide 147) whose sequence corresponds to the same region (residues 157–180) of Ki-Ras failed to inhibit precipitation of either Raf-1 (Fig. 1*B*) or Ras (data not shown).

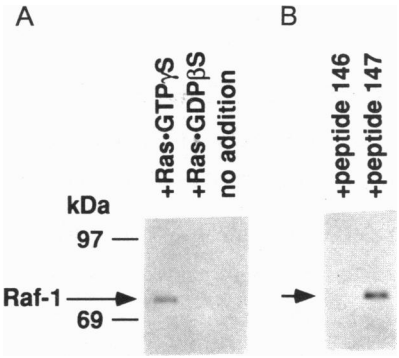

FIG. 1. GTP-dependent coimmunoprecipitation of Raf-1 with Ha-Ras. A lysate from Ba/F3 cells was incubated with LA069 in the presence of Ha-Ras·GTP[γS] or Ha-Ras·GDP[βS] or in the absence of Ha-Ras (*A*). The epitope peptide for LA069 (peptide 146) or another peptide (peptide 147) was then added in the presence of Ha-Ras·GTP[γS] (*B*). After a 2-hr incubation, samples were separated by SDS/PAGE (7.5% gels), and Western blot analysis was carried out with Sp63. Arrows indicate the Raf-1 band.

The coprecipitation of Ha-Ras and Raf-1 was observed in three other kinds of cells—i.e., NIH 3T3, PC12, and Jurkat cells (Fig. 2). In all cases, Ras·GTP[γS] carried down Raf-1 in the immunoprecipitate, whereas the amounts of Raf-1 coprecipitated with Ras·GDP[βS] were very small. In addition to the cultured cell lines, coprecipitation was also observed with crude extracts from rat brain tissue (Fig. 3). In this case also, the coimmunoprecipitation of Ha-Ras with Raf-1 was dependent on GTP[γS].

Difference Among Antibodies. Other antibodies against Ras, Y13-238 and Y13-259, were tested for their ability to coprecipitate Raf-1. Raf-1 was detected in the immune complex with Ras·GTP[γS] using the Y13-238 antibody (Fig. 4*B*), which does not inhibit Ras function. In contrast to LA069 and Y13-238, Y13-259, the neutralizing antibody, failed to coprecipitate Raf-1 in the presence of either GTP[γS] or GDP[βS] (Fig. 4*C*), although the amount of Ras in immunoprecipitates was comparable to that obtained with LA069 or Y13-238 (data not shown).

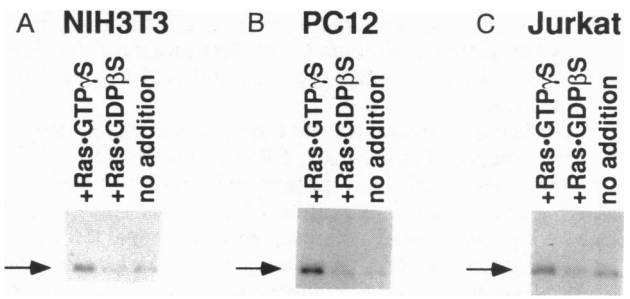

FIG. 2. Coimmunoprecipitation of Raf-1 with Ha-Ras from lysates of NIH 3T3 (*A*), PC12 (*B*), and Jurkat (*C*) cells. The lysates from the three cell lines were incubated with LA069 in the presence or absence of Ha-Ras·GTP[γS] or Ha-Ras·GDP[βS]. Immunoprecipitates were resolved by SDS/PAGE (7.5% gel). Arrows indicate the Raf-1 band.

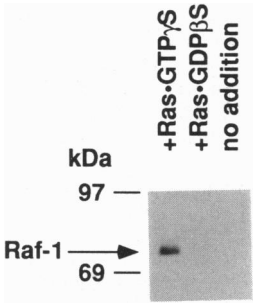

FIG. 3. Coimmunoprecipitation of Raf-1 with Ha-Ras from a crude rat brain extract. Crude extract was mixed with LA069 and Ha-Ras and incubated at 4°C. After a 2-hr incubation, samples were subjected to SDS/PAGE (7.5% gel) and Western blot analysis with Sp63. An arrow indicates the Raf-1 band.

Coimmunoprecipitation of Raf-1 with Ha-Ras Mutants. In addition to the wild-type Ha-Ras, we examined two types of mutants, an oncogenic mutant ([Val^{12}]Ras) and an effector-region mutant ([Leu^{35},Arg^{37}]Ras), for their ability to form a complex with Raf-1. As shown in Fig. 5, Raf-1 was observed in the immune complex with both the wild-type Ras and the oncogenic mutant Ras in the GTP[γS]-bound form. On the other hand, we could not detect any coprecipitation of Raf-1 with the effector-region mutant that is not active in transforming NIH 3T3 cells (39).

DISCUSSION

In the present study, we attempted to demonstrate the physical association of Raf with Ras by using several anti-Ras antibodies. Anti-Ras antibody Y13-259 has been used widely for precipitation and immunoblot analysis of Ras. The epitope of this antibody (approximately residues 63–73) (38, 39) is located within the switch II region (approximately residues 61–77) (42, 43), which is structurally close (44, 45) to the effector region (approximately residues 30–40). Since both regions are thought to be important for the interaction of Ras with its effector, Y13-259 antibody may compete with an effector molecule. In fact, this antibody inhibits Ras-mediated signals (46–48) and also blocks the interaction between Ras and GTPase-activating protein (49), which is thought to bind to the effector region (49, 50). Therefore, we considered that Y13-259 may not be suitable for detection of the association of Ras and a downstream target molecule.

On the other hand, monoclonal antibody LA069 appeared to be suitable for this purpose. This antibody is raised against the epitope at the C-terminal sequences of Ha-Ras and, therefore, would not be expected to interfere with the association of the effector molecule with Ras. As expected,

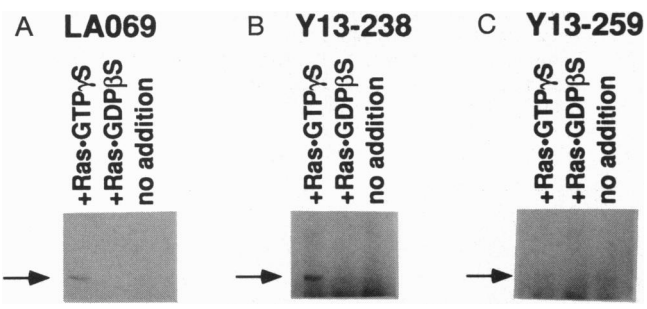

FIG. 4. Immunoprecipitation of Raf-1 using several anti-Ras antibodies. Immunoprecipitation was performed with the lysate from Ba/F3 cells using LA069 (*A*), Y13-238 (*B*), or Y13-259 (*C*). SDS/PAGE was carried out with 10–20% gradient gel. Arrows indicate the Raf-1 band.

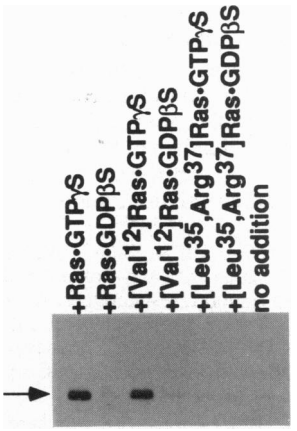

FIG. 5. Coimmunoprecipitation of Raf-1 with the wild-type Ras, [Val^{12}]Ras, and [Leu^{35},Arg^{37}]Ras. The lysate from Ba/F3 cells was incubated with the wild-type or mutant Ha-Ras for 2 hr at 4°C and subjected to SDS/PAGE (10–20% gel). Western blot analysis was performed with Sp63. An arrow indicates the Raf-1 band.

LA069 but not Y13-259 carried down Raf-1 in the immunoprecipitate with Ras. Another monoclonal antibody Y13-238, recognizing a region apart from the effector region and the switch II region (39), was also able to carry down Raf-1 with Ras. These observations suggest that the Raf-1 itself, or the molecule(s) that may be required for the association of Raf-1 with Ras, probably interacts with the effector region of Ras.

Since Raf acts downstream of Ras function, the association of Ras and Raf should take place with the wild-type and an oncogenic mutant Ras complexed with GTP[γS] but not with a mutant Ras·GTP[γS] that lacks signal-transducing ability. As shown in Fig. 5, the wild-type Ras and an oncogenic mutant ([Val^{12}]Ras) coprecipitated Raf-1, but a Ha-Ras mutant ([Leu^{35},Arg^{37}]Ras) that has mutations at the effector region and lacks transforming activity (39) did not bind with Raf-1. This result suggests that the complex formation between Ha-Ras and Raf-1, which we demonstrated in this study, requires the effector region and is important for signal transduction, though we have not yet examined whether the association by itself induces the increase in Raf-1 kinase activity.

Both Ras and Raf have three subtypes—Ha-Ras, Ki-Ras, and N-Ras (1, 2) and A-Raf, B-Raf, and Raf-1 (23), respectively. In the present experiment, we have added recombinant Ha-Ras protein to the lysates of cells and precipitated Ras with anti-Ras antibody LA069, which is specific to Ha-Ras. The immunoprecipitates were analyzed for Raf using an anti-Raf antibody Sp63, which is specific to Raf-1. Therefore, we do not know whether the other subtypes of Raf may exist in the Ha-Ras precipitates, nor we know whether different Ras subtypes may have different specificities toward each subtype of Raf.

The association of Ha-Ras·GTP and Raf-1 was observed in a wide range of cultured cells including fibroblast cells (NIH 3T3), neuronal cells (PC12), B cells (Ba/F3), and T cells (Jurkat). Moreover, this coimmunoprecipitation also occurred in crude brain extracts. Therefore, a similar signal-transducing mechanism between Ha-Ras and Raf-1 may generally exist in all kinds of mammalian cells.

Since we cannot rule out the possibility of the presence of other molecule(s) linking Ha-Ras and Raf-1 in the present study, the direct interaction of recombinant Raf-1 and recombinant Ha-Ras molecules should be tested. If the association is direct, Raf-1 kinase activity may be stimulated through conformational change induced by interaction with Ras·GTP. On the other hand, if intermediate molecules are utilized, the general existence of the Ras–Raf association

should enable us to purify and characterize the linking molecule(s) and the other components in the precipitated complex. These intermediate proteins, in addition to Ras, may be required for stimulation of Raf-1 activity. It is expected that the studies along this line will shed more light on the molecular mechanism of Ras-mediated signaling pathways and lead to the understanding of the processes of cellular growth, differentiation, and oncogenesis.

We are grateful to Drs. Ulf R. Rapp, Takeo Tanaka, and Douglas R. Lowy for providing us with anti-Raf and anti-Ras antibodies and the recombinant Ras mutant, respectively. We also thank Dr. Martin McMahon and Mr. Michael L. Samuels for valuable discussion and Dr. Dovie Wylie for reading the manuscript. DNAX Research Institute is supported by Schering-Plough Corporation.

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