Formation of the Ras Dimer Is Essential for Raf-1 Activation

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Although it is well established that Ras requires membrane localization for activation of its target molecule, Raf-1, the reason for this requirement is not fully understood. In this study, we found that modified Ras, which is purified from SF9 cells, could activate Raf-1 in a cell-free system, when incorporated into liposome. Using a bifunctional cross-linker and a protein-fragmentation complementation assay, we detected dimer formation of Ras in the liposome and in the intact cells, respectively. These results suggest that dimerization of Ras in the lipid membrane is essential for activation of Raf-1. To support this, we found that, when fused to glutathione S-transferase (GST), unprocessed Ras expressed in Escherichia coli could bypass the requirement for liposome. A Ras-dependent Raf-1 activator, which we previously reported (Mizutani, S., Koide, H., and Kaziro, Y. (1998) Oncogene 16, 2781-2786), was still required for Raf-1 activation by GST-Ras. Furthermore, an enforced dimerization of unmodified oncogenic Ras mutant in human embryonic kidney (HEK) 293 cells, using a portion of gyrase B or estrogen receptor, also resulted in activation of Raf-1. From these results, we conclude that membrane localization allows Ras to form a dimer, which is essential, although not sufficient, for Raf-1 activation.

Ras GTPases (Ha-, Ki-, and N-Ras) are the key proteins in eukaryotic signal transduction directed toward cellular proliferation and differentiation (1-3). The biological activity of Ras is controlled by a regulated GDP/GTP cycle. Guanine-nucleotide exchange factors (Ras-GRF1/2, mSos1/2) induce dissociation of GDP from Ras-GDP to form an active, GTP-bound form of Ras. On the other hand, GTPase-activating proteins (p120GAP, NF1) accelerate the intrinsic GTP hydrolytic activity of Ras. On binding of GTP, Ras alters its conformation to promote the formation of an inactive, GDP-bound form of Ras. Upon binding of GTP, Ras alters its conformation to interact with multiple downstream effectors. One of the well characterized effectors is a serine/threonine kinase Raf-1 (4, 5), which induces activation of a dual specificity kinase MEK. 1 Activated MEK in turn activates a serine/threonine kinase ERK, which phosphorylates a variety of proteins including protein kinases, transcription factors, and cytoskeletal proteins (6).

Although it has been demonstrated that Ras binds to Raf-1 directly, the precise mechanism of Raf-1 activation by Ras is not fully understood. According to the current model (5), when Ras is activated, the effector region of Ras interacts with the Ras binding domain of Raf-1, which leads to the binding of cysteine-rich domain of Raf-1 to Ras. These interactions relieve the masking of the C-terminal catalytic domain of Raf-1 by the N-terminal regulatory domain and allow Raf-1 to interact with the downstream kinase, MEK. However, since direct interaction of Ras with Raf-1 is insufficient for Raf-1 activation (5, 7), an additional molecule(s) has been expected to be involved in this activation. In fact, using a cell-free system, we have found a Ras-dependent Raf-1 activator in the cytosolic fraction (8).

Ras is known to undergo a series of post-translational modifications at the CAAX (where A indicates an aliphatic amino acid) motif in its C terminus, such as farnesylation at Cys-186 and palmitoylation of Cys-181 and Cys-184. The modifications are necessary to localize Ras on the plasma membrane (3). Membrane localization is essential for Ras function, because oncogenic Ras mutant proteins lacking Cys-186, including Ras(G12V,C186S), lose their ability to activate Raf-1 (9). On the other hand, it has been reported that Raf-1 can be activated when targeted to the plasma membrane by addition of the CAAX motif of Ras (10, 11). These observations suggest that a certain event(s) necessary for Raf-1 activation occurs in the plasma membrane. The results described in this paper indicate that the dimer formation of Ras is an essential event in the plasma membrane for Raf-1 activation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Anti-Ha-Ras antibody (sc-520) and anti-Ki-Ras antibody (sc-30) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG epitope antibody (M2), ethylene glycolbis(succinimidyldimethylamine) (EGS), and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) were from Eastman Kodak Co., Pierce, and Nakalai Tesque (Kyoto, Japan), respectively.

**Plasmid Construction**—Complementary DNA of Ki-ras (4B), which was generously given by Y. Takai (Osaka University), was subcloned into a baculovirus transfer vector pAcYM1 (12), a kind gift from Y. Matsuura (National Institute of Health, Tokyo). To construct pGEX-Ha-ras and -Ki-ras, Ha-ras (2-189) and Ki-ras were subcloned into pGEX-2T (Amersham Pharmacia Biotech), respectively. Plasmids pBS-gyrA, pCMX-ER, and pWZL-Δω were kindly provided by M. A. Farrar and R. M. Perlmutter (Merck Research Laboratories), M. Nakafuku (University of Tokyo), and H. M. Blau (Stanford University), respectively. With these plasmids, we generated pCMV-FLAG-gyrB-Ha-ras(G12V,C186S), pCMX-ER-Ha-ras(G12V,C186S), and pCMV-Δω-Ha-ras(G12V), pCMV-Δω-Ki-ras(G12V), and pCMV-Δω-Ras(G12V,C186S) by inserting the genes of fusion proteins into either pCMV5 (13) or pCMX (14).

**Protein Preparation**—Modified Ki-Ras was purified from SF9 cells infected with baculovirus encoding Ki-Ras as described in Ref. 15.

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<sup>1</sup> The abbreviations used are: MEK, mitogen-activated protein kinase/nuclear-extracellular signal-regulated kinase kinase; EG5, ethylene glycolbis(succinimidylsuccinate); ER, the hormone binding domain of estrogen receptor; β-gal, β-galactosidase; GST, glutathione S-transferase; Gyrb, the C-terminal domain of the B subunit of gyrase; 4-HT, 4-hydroxytamoxifen; RafFH, Raf-1 with FLAG epitope tag and six histidine residues in its C terminus; X-gal, 5-bromo-4-chloro-3-indoyl-β-D-galactoside; DTT, dithiothreitol; HEK, human embryonic kidney.
Ras Dimerization for Raf-1 Activation

RESULTS

Modified Ras Incorporated into Liposome Can Activate Raf-1—A possible explanation for the requirement of the plasma membrane was that the plasma membrane may contain a protein molecule(s) indispensable for Ras-induced Raf-1 activation. We examined this possibility using a cell-free system, which is based on the activation of Raf-FH (Raf-1 with FLAG epitope tag and six histidine residues in its C terminus) in the cytosolic fraction prepared from HEK293 cells expressing Ras-GFP together with pLNC-raf-1:FH6, a kind gift from M. McMahon (University of California, San Francisco). After 24-h starvation, cells were treated with coumermycin (Sigma) or 4-hydroxytamoxifen (Sigma) at 37 °C. Then cells were harvested and lysed with lysis buffer (10 mM HEPES/NaOH (pH 7.4), 10 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10 mM NaF, 25 mM β-glycerophosphate, 4 μg/ml aprotinin, 10 μg/ml leupeptin, 1% Triton X-100, 10% glycerol). In all experiments, kinase activity of Raf-FH was determined using His-MEK and GST-kdMAPK, kind gifts from E. Nishida (Kyoto University), as described previously (8).

Unmodified Ha-Ras was prepared as described previously (16). GST-fused Ha-Ras and Ki-Ras were produced using pGEX-Ha-ras and pGEX-Ki-ras, respectively, and purified according to the manufacturer’s instructions. To obtain unmodified Ki-Ras, GST-fused Ki-Ras was digested with thrombin, and thrombin and GST were removed using benzamidine- and glutathione-Sepharose (Amersham Pharmacia Biotech). Preparation of the GTP- and GDP-bound forms of Ras was carried out as described previously (17), except that samples were incubated for 30 min instead of 10 min.

Preparation of Liposome—Phosphatidylcholine (Sigma, P-5638) in CHCl3/CH3OH (9:1, v/v) was thoroughly dried under nitrogen gas and resuspended in 20 mM HEPES/NaOH (pH 7.4), 5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 10 mM NaF, 25 mM β-glycerophosphate, by sonication (5 x 3 times). Large vesicles were removed by centrifugation at 9,000 x g for 10 min. Then the supernatant was ultracentrifuged at 100,000 x g for 30 min, and the pellet was resuspended in 20 mM HEPES/NaOH (pH 7.4), 5 mM MgCl2, 1 mM DTT, 250 mM sucrose. To incorporate Ki-Ras into liposome, the supernatant was incubated with purified Ki-Ras for 2 h at 4 °C prior to the ultracentrifugation.

Raf-1 Activation—The cytosolic fraction (S100) was prepared from HEK293 expressing Raf-FH by homogenization and ultracentrifugation. In the experiments with liposome, the cytosolic fraction was used as the Raf fraction and incubated with Ki-Ras-containing liposome at 16 °C for 30 min. For the experiments using GST-Ras, the cytosolic fraction was subjected to a Resource Q column (Amersham Pharmacia Biotech), and purified Ki-Ras for 2 h at 4 °C prior to the ultracentrifugation.

Detection of a Ras Dimer in Lipid Membrane—Since it has been reported that artificial dimerization of Ras results in Raf-1 activation (18, 19), we thought that Ras might require liposome for its dimerization. To test this possibility, we incubated Ki-Ras-GFP-containing liposome with a homobifunctional amine-reactive cross-linker, EGS. In the absence of EGS, we observed only a single band of monomeric Ras (21 kDa) (Fig. 2A). On the other hand, an additional band appeared in the presence of EGS, whose mobility corresponded to the molecular weight of dimerized Ras. This band was not observed when unmodified Ki-Ras-GTP was incubated with EGS in the presence of liposome. These results suggest that modified Ras can dimerize when reconstituted in liposome.

To examine if Ras dimerization occurs also in intact cells, we performed a protein-fragmentation complementation assay using β-galactosidase (β-gal) (20). In this assay, two deletion mutants of β-gal, Δα and Δω, show β-gal activity only when they are forced to interact with each other. We fused the two deletion mutants to the N terminus of Ha-Ras and transiently expressed them in HEK293 cells. When either Δα-Ha-Ras[G12V] or Δω-Ha-Ras[G12V] alone was expressed, no blue-stained (i.e. β-gal-positive) cells were observed (data not shown). However, when both molecules were expressed together, a large number of blue-stained cells appeared (Fig. 2B).

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On the other hand, only a few cells were stained when unfused FLAG-tagged Δα was expressed in place of Δα-Ha-Ras[G12V], although expression levels were approximately the same between Δα-Ha-Ras[G12V] and unfused Δα, as judged from Western blotting analysis (data not shown). These data suggest that Ras forms a dimer in HEK293 cells with association of Δα and Δω fragments to restore the β-gal activity.

Artificial Dimerization of Ras Leads to Raf-1 Activation in a Cell-free System—If Ras dimerization in lipid membrane is an essential process for Raf-1 activation, bacterially produced, unmodified Ras protein may restore its Raf-1 activating ability by synthetic dimerization. To explore this possibility, we produced dimerized Ha-Ras by fusion with glutathione-S-transferase (GST) in Escherichia coli. Dimerization of GST-fused Ha-Ras (GST-Ras) was confirmed by gel filtration (data not shown). When bacterially produced, unmodified Ha-Ras was incubated with the Raf fraction, Ha-Ras could not activate Raf-FH even in the GTP-bound form (Fig. 3A). On the other hand, GST-Ras induced Raf-FH activation in a GTP-dependent manner. The activation was not observed when unfused Ha-Ras and GST were incubated together with the Raf fraction. These results suggest that dimerization of Ras by fusion with GST can bypass incorporation of Ras into liposome for Raf-1 activation.

Since the Raf fraction contains a Ras-dependent Raf-1 activator (8), we examined whether dimerized Ras still requires the activator for Raf-1 activation. After Raf-FH was immunoprecipitated from the Raf fraction by anti-FLAG antibody, the precipitate was washed extensively to remove the activator. When the immunoprecipitated Raf-FH was incubated with GST-Ras, no activation of Raf-FH was observed (Fig. 3B). On
the other hand, RafFH was activated when the activator, which was partially purified from rat brain cytosols (8), was added to the reaction mixture. These results indicate that dimerization of Ras is not sufficient for Raf-1 activation and that the activator is still necessary for this activation.

Unmodified Ras Activates Raf-1 in a Dimerization-dependent Manner in Intact Cells—As described above, dimerization of Ras leads to Raf-1 activation in the cell-free system. To determine whether the same is true in intact cells, we fused the N terminus of Ha-Ras[G12V,C186S] to the N-terminal domain of the B subunit of E. coli gyrase (GyrB) or the hormone binding domain of murine estrogen receptor (ER). Ras[G12V,C186S] has been reported to bind with Raf-1, but not to activate it because of the lack of its farnesylation site (9). GyrB and ER are known to form a homodimer in response to their specific ligands, coumermycin and 4-hydroxytamoxifen (4-HT), respectively (18, 21). When HEK293 cells were transfected with GyrB-Ras[G12V,C186S], treatment with coumermycin efficiently induced RafFH activation (Fig. 4A). In contrast, novo- biocin, a monovalent analogue of coumermycin that cannot induce GyrB dimerization, failed to stimulate RafFH activation (data not shown). ER-Ras[G12V,C186S] also activated RafFH in a 4-HT-dependent manner (Fig. 4B). These results suggest that enforced dimerization of Ras induces Raf-1 activation in intact cells, as well as in the cell-free system.

**DISCUSSION**

Although activation of Raf-1 is initiated by association with Ras-GTP on the cytoplasmic membrane, binding of Ras-GTP to Raf-1 does not induce Raf-1 activation in vitro. Several studies suggested that an additional factor(s) in the plasma membrane is required for Ras-induced activation of Raf-1 (10, 11). One of the candidate molecules is a membrane-bound tyrosine kinase Src, since Ras and Src synergistically activate Raf-1 (22, 23).

![FIG. 2. Detection of Ras dimerization.](image1.png)

**FIG. 2.** Detection of Ras dimerization. A, Ki-Ras-containing liposome or unmodified Ki-Ras plus liposome was incubated with or without 2 mM EG for 1 h at 4 °C. Samples were then subjected to SDS-polyacrylamide gel electrophoresis, followed by Western blotting analysis using anti-Ki-Ras antibody. B, HEK293 cells were transfected with pCMV5-Δα-Ha-ras[G12V] or pCMV5-Δα-FLAG together with pCMV5-Δα-Ha-ras[G12V]. After 48 h, cells were fixed with 0.25% glutaraldehyde for 10 min at 4 °C. Then cells were incubated with X-gal to monitor β-galactosidase activity. In both experiments, the results shown are representative of several experiments.

![FIG. 3. Raf-1 activation by bacterially produced GST-Ras in the cell-free system.](image2.png)

**FIG. 3.** Raf-1 activation by bacterially produced GST-Ras in the cell-free system. A, the Raf fraction was incubated alone or with GST-Ras, unmodified Ha-Ras, or GST plus unmodified Ha-Ras for 2 h at 4 °C. RafFH was then immunoprecipitated, and its kinase activity was measured. B, after extensive washing, immunoprecipitated RafFH was incubated alone or with GST-Ras-GTP in the presence or absence of the activator for 2 h at 4 °C. Then the immunoprecipitate was washed again and RafFH kinase activity was measured. The results shown are representative of three independent experiments.

![FIG. 4. Activation of Raf-1 by dimerization of modification-deficient Ras mutant in intact cells.](image3.png)

**FIG. 4.** Activation of Raf-1 by dimerization of modification-deficient Ras mutant in intact cells. A, HEK293 cells were transfected with the empty vector (open bars) or pCMV5-FLAG-gyrB-Ha-ras[G12V,C186S] (filled bars) together with pLNC-raf-1:FH6. After 24-h starvation, cells were incubated for 15 min at 37 °C with or without 1 μM coumermycin (Cm). B, HEK293 cells were transfected with pCMX-ER-Ha-ras[G12V,C186S] and pLNC-raf-1:FH6. After starvation, cells were treated with or without 1 μM 4-HT for 2 h at 37 °C. In both experiments, after stimulation, cells were lysed and RafFH was immunoprecipitated to measure its kinase activity. Data are presented as the mean ± S.E. of three separate experiments.

However, our present data indicate that purified Ras incorporated into liposome can activate Raf-1 in the absence of other membrane proteins. Thus, it is likely that, although other membrane molecules including Src may augment Ras-dependent Raf-1 activation in vivo, they are dispensable in the presently used cell-free system.

Using a cell-free system, Stokoe et al. (24) have also shown that Ras is the only required protein in the membrane for Raf-1 activation. However, in their system, modified Ras did not require liposome for Raf-1 activation. The reason for the discrepancy between their results and ours is presently unknown.
Since Raf-1 interacts with phosphatidylerine or phosphatidic acid (25, 26), it was also possible that phospholipid in the liposome might enhance the Raf-1 activation. However, this is unlikely since GST-Ras could induce Raf-1 activation even in the absence of liposome (Fig. 3A). The association of phospholipids with Raf-1 may not be absolutely required for Raf-1 activation, although it may facilitate the translocation and/or activation of Raf-1 in vivo.

Experiments using EGS and a β-gal system (Fig. 2) suggest that Ras forms a homodimer when modified and localized on the plasma membrane. However, it remains unclear how Ras dimerizes on the membrane. Since no difference was observed in cross-linking efficiency between Ras-GTP and Ras-GDP (data not shown), it is possible that Ras forms a dimer constitutively. Furthermore, it seems that the affinity of the protein moiety of Ras is not high enough for a dimer formation, because bacterially produced (i.e., unmodified) Ras is eluted as a monomer on gel filtration chromatography (27), and we could neither cross-link unmodified Ras (Fig. 2A) nor immunoprecipitate a Ras dimer from cell lysate (data not shown). Thus, it appears that not protein, but lipid moiety, may be responsible for the dimer formation. An alternate possibility is that, although the affinity between Ras proteins is too low to form a stable dimer, membrane localization increases accessibility of the protein moieties of Ras.

Previously, we found a Ras-dependent Raf-1 activator in the cytosolic fraction (8). The importance of the dimer formation of Ras in Raf-1 activation suggested the possibility that the activator may be an inducer of Ras dimerization. However, GST-Ras failed to activate Raf-1 in the absence of the activator (Fig. 3B). Requirement of the activator was also observed in the experiments using Ras-containing liposome (data not shown). Thus, it is unlikely that the role of the activator in Raf-1 activation is to induce Ras dimerization.

In conclusion, we have demonstrated that Ras requires the lipid membrane for Raf-1 activation and that this requirement can be bypassed by the enforced dimerization of Ras both in vivo and in vitro. Furthermore, we found that Ras dimerization occurs in the membrane. These results strongly suggest that Ras-Ras interaction in the plasma membrane is an essential step for Ras-induced activation of Raf-1. Subsequent association of two Raf-1 molecules on the dimeric Ras may facilitate the increase of Raf-1 kinase activity. The present results raise the intriguing possibility that other low molecular weight GTP-binding proteins may also function as dimers. Such a possibility is currently being investigated in our laboratory.

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