Physical Association with Ras Enhances Activation of Membrane-bound Raf (RafCAAX)*

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The transforming activity of artificially membrane-targeted Raf1 suggests that Ras-mediated recruitment of Raf1 to the plasma membrane is an important step in Raf1 activation. Cellular Ras is concentrated in the caveolae, a microdomain of the plasma membrane that is highly enriched in caveolin, glycosylphosphatidylinositol-anchored proteins, and signal transduction molecules. Growth factor stimulation recruits Raf1 to this membrane domain. Whether Ras simply promotes Raf association with caveolae membranes or also modulates subsequent activation events is presently unclear. We have identified a ras variant, ras12V,37G, that does not interact with Raf1 but does interact with a mutant raf1(257L). To examine the role of Ras in the activation of membrane-bound Raf1, raf1CAAX, and raf1(257L)/CAAX, membrane-targeted variants of Raf1 and raf1(257L), respectively, were expressed in fibroblasts with or without coexpression of ras12V,37G. Cell fractionation localized both raf1CAAX and raf1(257L)/CAAX to caveolae membranes independent of ras12V,37G expression; however, coexpression of ras12V,37G enhanced the activation of raf1(257L)/CAAX, but not raf1CAAX, as monitored by induction of cellular transformation, increased Raf kinase activity, and induction of activated MAP kinase. These results suggest that the Ras/Raf1 interaction plays a role in Raf1 activation that is distinct from membrane recruitment.

Activation of the Raf-MAP kinase (mitogen-activated protein kinase) kinase cascade is a critical step in cellular transformation induced by oncogenic ras (1, 2). Upon interaction with ras-GTP, the Raf serine/threonine protein kinase is activated and in turn activates MAP kinase through the direct activation of MEK (mitogen-activated or extracellular signal-regulated kinase kinase) (3–6). Activated MAP kinase phosphorylates a number of cytoplasmic and nuclear targets that participate in the transformation response (7, 8).

The mechanism by which Ras interaction mediates Raf1 activation is unclear. Cytoplasmic, inactive, Raf1 translocates to the plasma membrane and is activated in a Ras-GTP-dependent fashion (9–12). Plasma membrane association allows a number of activation events to occur on Raf that are most likely mediated by tyrosine and serine/threonine protein kinases (13–16) and may involve other protein (17) and lipid cofactors (18). Raf1 proteins with carboxyl-terminal fusions to the Ki-ras membrane localization signals (rafCAAX) are targeted to the plasma membrane when expressed in cells and are enzymatically active independently of Ras activation (10, 19). These results suggest that the primary role of Ras in Raf1 activation is to mediate translocation of Raf1 to the plasma membrane (20). However, a number of observations suggest that the regulation of Raf1 by activated Ras is more complex. Mutant forms of Ras have been identified that effectively activate MAP kinase from the cytosol (21); purified farnysylated Ras activates a 14-3-3-B-Raf complex in vitro (22); and the amino-terminal regulatory portion of Raf1 contains two RAS-hiding domains (23), both of which are critical for Ras-mediated Raf1 activation in vivo (24–26).

To determine if Ras contributes to Raf1 activation subsequent to membrane recruitment, we have utilized membrane-targeted versions of raf1 in conjunction with a ras variant, ras12V,37G, that is defective in Raf1 interaction, but that can interact with the compensatory raf variant, raf1(257L) (27). Using stably transfected cell lines, we show that ras12V,37G facilitates the activation of membrane-targeted raf1(257L), but not membrane-targeted Raf1. The membrane localization sequences from Ki-ras, used to create the membrane-targeted raf1 variants, are sufficient to promote localization of the raf1 variants to a specific plasma membrane microdomain independently of ras12V,37G interaction. These results demonstrate that the association of Ras with Raf1 is important not only for plasma membrane recruitment of Raf1 but also for subsequent activation events on Raf1.

EXPERIMENTAL PROCEDURES

Molecular Constructs—Raf1CAAX and raf1(257L)/CAAX were expressed from pcDNA3 Institute for Molecular Biology and Genetics (Spring Harbor Laboratory, Cold Spring Harbor, NY). ras12V,37G was expressed from pSRa-ras12V,37G described previously (27). A polynucleotide encoding the carboxyl-terminal 5 amino acids of Raf1 fused to the carboxyl-terminal 17 amino acids of Ki-ras was ligated onto the AcoI restriction site in raf1(257L)/CAAX, generating a cDNA encoding the complete raf1(257L) protein fused to the Ki-Ras membrane localization sequences. This cDNA was inserted as an EcoRI/XhoI fragment into the EcoRI/XhoI sites of pcDNA3 (Institute for Molecular Biology and Genetics). To make pcDNA3-raf1CAAX, pcDNA3-raf1CAAX is identical to pcDNA3-raf1(257L)/CAAX except that it encodes an entirely wild-type Raf1 protein fused to the Ki-ras membrane targeting signal (provided by J. Stolarov and M. Wiger Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). ras12V,37G was expressed from pSRa-ras12V,37G described previously (27).

Mammalian Cell Culture and Transfection Assays—Stable transfections of NIH 3T3 cells were performed by calcium phosphate precipitation as described (28). 24 h post-transfection, cells were trypsinized and one-half of each transfection was plated into Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 5% calf serum (Life Technologies, Inc.) for focus assays and the other half into Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and 0.5 mg/ml G418 sulfate (Life Technologies, Inc.) to select for stable transfectants. The number of colonies from transfected cells was scored under magnification after 14 days of incubation in 5% serum. G418-resistant colonies were counted after 10 days of...

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growth in selective media. Stably transfected cells lines were isolated by cloning and expanding individual G418 resistant colonies and by pooling mass populations of G418-resistant colonies.

Cell Fractionation—Transfected cells were serum-starved for 24 h. Plasma membrane, low density, and high density plasma membrane fractions were prepared using a detergent-free method as described previously (29).

Antibodies and Protein Expression—Protein samples were trichloroacetic acid precipitated and separated on 12.5% SDS-polyacrylamide gel. Separated proteins were transferred to polyvinylidene difluoride nylon membrane and subjected to Western blotting as described elsewhere (29) using monoclonal anti-Raf1 (Transduction Laboratories), monoclonal anti-Ras (Transduction Laboratories), polyclonal anti-active MAP kinase (Promega Corp.), or polyclonal anti-ERK1 (C-16) (Santa Cruz Biotechnology, Inc.) antibodies.

Raf1 Kinase Assays—Raf1 kinase activity in whole cell lysates was measured as described previously (30).

FIG.1. ras12V,37G cooperates with membrane-targeted raf1(257L), but not with membrane-targeted Raf1, to transform cells. NIH 3T3 cells were transfected with plasmids expressing the indicated proteins. The raf1 variants were introduced (500 ng/transfection) in pSRα. ras12V,37G was introduced (500 ng/transfection) in pSRα. Empty vector was included where necessary to maintain equivalent DNA concentrations and to allow for selection in G418 containing media. A, focus assays. Transfected cells were cultured in 5% calf serum for 14 days, fixed in 10% formaldehyde, and stained with Giemsa to visualize foci of growth transformed cells. B, focus formation frequency for each transfection was determined as the number of foci per number of G418-resistant colonies. Values represent results from two independent transfections (solid bars and stripped bars).

FIG.2. ras12V,37G enhances the enzymatic activity of raf1(257L)CAAX but not raf1CAAX. Whole cell lysates from cell lines selected for equivalent expression of raf and ras variants (see Fig. 3A) were assayed for levels of Raf1 kinase activity and levels of activated MAP kinase. A, Raf1 kinase activity. Raf1 was immunoprecipitated from 300 μg of whole cell lysate from serum-starved cells transfected with vector alone (lane 1), raf1CAAX (lane 2), raf1(257L)CAAX (lane 3), raf1CAAX + ras12V,37G (lane 4), and raf1(257L)CAAX + ras12V,37G (lane 5). The immunoprecipitated Raf protein was incubated with recombinant kinase-inactive MEK1 protein in an in vitro kinase assay in the presence of [γ-32P]ATP. Samples were separated by SDS-PAGE and visualized on a PhosphorImager. Images were quantitated by densitometry from three separate experiments, and values were normalized to the activity measured in vector transformed cells. Error bars represent standard deviation from the mean. A representative experiment is shown below the graph. Similar results were obtained with independently isolated cell lines and with pooled G418-resistant colonies from independent transfections (data not shown). B, levels of activated cellular MAP kinase in 35 μg of cell extract were visualized by Western blotting using the anti-active MAP kinase antibody which selectively recognizes the activated phosphorylated forms of ERK1 and ERK2. Blots were stripped and reprobed with anti-ERK1 that recognizes both active and inactive forms of ERK1 and ERK2. The lane designations are the same as in A. Similar results were obtained with independently isolated cell lines and with pooled G418-resistant colonies from independent transfections (data not shown).

RESULTS

Activity of Plasma Membrane-targeted raf Is Enhanced by ras Interaction—Substitution of glycine for glutamic acid at position 37 of oncogenic ras (rasV12,G37) prevents association with full-length Raf1. A compensatory change in Raf1 was identified, raf1(257L), that rescues productive interaction with ras12V,37G (27). To determine if Ras participates in Raf1 activation via mechanisms distinct from membrane recruitment, we tested the ability of ras12V,37G to enhance the activity of membrane-targeted variants of Raf1 (raf1CAAX) and raf1(257L) (raf1(257L)CAAX) in vivo. If the association of Ras and Raf1 is important for Raf1 activation events subsequent to membrane recruitment, then ras12V,37G should enhance the activation of raf1(257L)CAAX but not raf1CAAX.

Raf1CAAX and raf1(257L)CAAX are full-length Raf1 proteins with carboxyl-terminal fusions containing the membrane localization sequences of Ki-ras. Raf1CAAX has been shown to be sufficient to induce cellular transformation when expressed in some fibroblast cell lines (31). NIH 3T3 cells were transfected with the raf variants alone and in combination with
ras12V,37G. Both raf1CAAX and raf1(257L)CAAX were weakly transforming as determined by induction of foci of growth and morphologically transformed cells. ras12V,37G did not induce foci when expressed alone and did not cooperate with raf1CAAX. Coexpression of ras12V,37G with raf1(257L)CAAX, however, resulted in an 8-fold increase in focus formation frequency above the level induced by raf1(257L)CAAX alone (Fig. 1).

To test if the synergy in cellular transformation observed upon expression of ras12V,37G with raf1(257L)CAAX correlated with enhanced activation of raf1(257L)CAAX enzymatic activity, stably transfected cells were assayed for levels of Raf kinase activity and for levels of activated MAP kinase.

Stable cell lines selected for equivalent expression of raf1CAAX or raf1(257L)CAAX were derived from each of the above transfections (see Fig. 3A). Raf1 variants were immunoprecipitated from lysates of serum-starved cells, and kinase activity was assayed by in vitro phosphorylation of kinase-inactive MEK1. A 3-fold elevation of Raf kinase activity, relative to control cells, was observed in immunoprecipitates from cells coexpressing ras12V,37G and raf1(257L)CAAX. Surprisingly, no significant Raf kinase activity above background was observed in any other cell line despite significant expression of raf1CAAX or raf1(257L)CAAX (Fig. 2A). Transient expression of Raf1CAAX has been shown to result in significant levels of Raf kinase activity (10, 19).

Levels of activated MAP kinase were visualized by Western blot, using an antibody that selectively recognizes the phosphorylated activated forms of the Raf-regulated MAP kinases ERK1 and ERK2. Although little Raf kinase activity was detected in lysates from cells expressing raf1CAAX or raf1(257L)CAAX alone, these cells contained a population of activated ERK1 and ERK2 that was greater than that observed in cells transfected with empty vector (Fig. 2B). The bulk of activated Raf1 that is below the threshold of detection in our kinase assays. Alternatively, some persistent MAP kinase activation in these stable cell lines may no longer be dependent on the presence of activated Raf1. Importantly, cells expressing both raf1(257L)CAAX and ras12V,37G contained a substantially greater population of active ERK1 and ERK2 than cells expressing theraf variants alone or cells expressing both raf1CAAX and ras12V,37G (Fig. 2B).

Raf1CAAX and raf1(257L)CAAX Are Localized to Caveolae Independently of ras Interaction—Targeting of Raf1 to the plasma membrane is a critical step in Raf1 activation (10, 19). The observed increase in raf1(257L)CAAX activity upon coexpression with ras12V,37G suggests that Ras can participate in Raf1 activation events distinct from membrane recruitment. However, the interaction of raf1(257L)CAAX with ras12V,37G could potentially result in enhanced recruitment of raf1(257L)CAAX to the plasma membrane due to targeting signals present on ras12V,37G. To test this, we examined the subcellular localization of the raf1CAAX variants with and without expression of ras12V,37G.

Following serum starvation, detergent-free extracts prepared from the stable cell lines described above were separated into plasma membrane and cytosol fractions on Percoll gradients. Consistent with previous observations (12, 19), very little Raf1 was detected in the membrane fraction of serum-starved, vector-transfected cells (Fig. 3B, lane 1). In contrast, the majority of detectable Raf1 was observed in the plasma membrane fraction of cells expressing raf1CAAX or raf1(257L)CAAX (Fig. 3, lanes 2–5). All cell lines had similar amounts of Raf1 in the cytosol, suggesting that virtually all expressed raf1CAAX and raf1(257L)CAAX is membrane-bound (Fig. 3C). The bulk plasma membranes were further separated into high and low density membrane fractions using density gradient centrifugation. Previous work has demonstrated that cellular Ras is found exclusively in the low density membrane fraction and that cellular Raf1 is recruited to this fraction upon Ras activation (12). The low density membrane fraction is highly enriched in the caveolae marker protein caveolin and is believed to be composed primarily of caveolae membrane microdomains (29). Virtually all of the raf1CAAX and raf1(257L)CAAX in the plasma membrane copurified with the low density membrane fraction independently of ras12V,37G expression (Fig. 3, D and E, compare lanes 2 and 3 to lanes 4 and 5). These results demonstrate that the targeting signals conferred by the amino terminus of Ki-ras are sufficient to efficiently promote localization of theraf1 chimeras to the caveolae membrane fraction.

DISCUSSION

Using mutants of ras and raf1 that affect physical association, we have shown that activated ras will stimulate the kinase activity of membrane-targeted raf1 only when these molecules can physically interact. ras12V,37G interacts with raf1(257L) but not Raf1 (27). Membrane-targeted variants of raf1(257L) and Raf1 (raf1(257L)CAAX and raf1CAAX, respectively) were efficiently localized to the caveolae fraction of the plasma membrane independently of ras12V,37G interaction. Consistent with other published studies (10, 19), these raf1 variants induced a low level of cellular transformation and MAP kinase activation. However, coexpression of ras12V,37G resulted in the elevation of raf1(257L)CAAX kinase activity and enhanced induction of cellular transformation. ras12V,37G expression did not alter Raf1CAAX activity. Therefore, the ability of Ras to physically interact with Raf1 at the plasma membrane is an important determinant of Raf1 function in addition to membrane recruitment.

Cells transiently expressing membrane-targeted raf1 have
activated Rafl at the plasma membrane independently of ras activation (10, 19). With stably expressing cell lines, however, we have detected a Ras-dependent component of membrane-targeted Rafl activation. We suggest that there may be differences in the regulation of Rafl activity during transient versus stable expression. Rafl is a substrate for negative regulation by membrane bound phosphatases (32). Therefore, the effects of ras on membrane-targeted rafl activation that we observe in stable cell lines most likely reflect a balance between Rafl activation and deactivation. This balance may be very different in transient expression assays, particularly if the negative regulatory machinery must be induced. If Ras is not required for, but participates in, Rafl activation at the plasma membrane, then the absence of negative regulation coupled with high levels of expression of membrane-targeted Rafl may mask the contribution of Ras. Observations made in stably transfected cell lines may more closely mimic the cellular responses to oncogenic ras during tumorigenesis. Consistent with this, we have observed that native Rafl is transiently recruited to caveolae upon mitogen stimulation, but is constitutively associated with caveolae in oncogenic ras-transformed cells (12).

ras12V,37G retains productive interactions with effector molecules other than Rafl that mediate transforming activity which is independent of, but cooperates with, activation of the Raf/MAP kinase cascade (27, 33). Expression of Raf1CAAX or rafl257L/CAAX alone resulted in a low level of MAP kinase activation, while ras12V,37G cooperated with only rafl257L/CAAX in focus formation assays. The lack of cooperation between ras12V,37G and raflCAAX in these assays was unexpected, since we had previously observed cooperation between ras12V,37G and raflBXB (33), a constitutively active rafl variant lacking the amino-terminal regulatory domain (34). Raf1CAAX activity may be below the threshold required to cooperate with ras12V,37G to transform cells, possibly owing to negative regulation through the Rafl amino terminus. Consistent with this, significantly more Rafl kinase activity was detected in cells expressing both rafl257L/CAAX and ras12V,37G, where enhanced focus formation was also observed.

The mechanism by which Ras interaction with Rafl at the plasma membrane enhances Rafl activity remains to be determined. It may involve the induction of a conformational change in Rafl, exposing residues that are substrates for activating kinases (25). Alternatively, Ras may participate in the assembly of a signaling complex between Rafl and other proteins such as KSR1 and TPL2 which positively modulate Rafl activity (35, 36). Regardless of the mechanism, the results presented here demonstrate that the physical interaction of Ras and Rafl in caveolae membrane is important for sustained Rafl activation.

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