THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 37, Issue of September 14, pp. 34728–34737, 2001

Printed in U.S.A.

Function of the Rho Family GTPases in Ras-stimulated Raf Activation*

Received for publication, April 19, 2001, and in revised form, July 5, 2001
Published, JBC Papers in Press, July 16, 2001, DOI 10.1074/jbc.M103496200

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Ras plays an essential role in activation of Raf kinase which is directly responsible for activation of the MEK-ERK kinase pathway. A direct protein-protein interaction between Ras and the N-terminal regulatory domain of Raf is critical for Raf activation. However, association with Ras is not sufficient to activate Raf *in vitro*, indicating that Ras must activate some other biochemical events leading to activation of Raf. We have observed that RasV12Y32F and RasV12T35S mutants fail to activate Raf, yet retain the ability to interact with Raf. In this report, we showed that RasV12Y32F and RasV12T35S can cooperate with members of the Rho family GTPases to activate Raf while alone the Rho family GTPase is not effective in Raf activation. A dominant negative mutant of Rac or RhoA can block Raf activation by Ras. The effect of Rac or Cdc42 can be substituted by the Pak kinase, which is a direct downstream target of Rac/Cdc42. Furthermore, expression of a kinase inactive mutant of Pak or the N-terminal inhibitory domain of Pak1 can block the effect of Rac or Cdc42. In contrast, Pak appears to play no direct role in relaying the signal from RhoA to Raf, indicating that RhoA utilizes a different mechanism than Rac/Cdc42. Membrane-associated but not cytoplasmic Raf can be activated by Rac or RhoA. Our data support a model by which the Rho family small GTPases play an important role to mediate the activation of Raf by Ras. Ras, at least, has two distinct functions in Raf activation, recruitment of Raf to the plasma membrane by direct binding and stimulation of Raf activating kinases *via* the Rho family GTPases.

Stimulation of cells with mitogenic growth factors results in activation of receptor tyrosine kinases which can lead to activation of the Ras GTPase (1). Ras plays an essential role in regulating a wide range of cellular functions, including cell proliferation, differentiation, oncogenic transformation, and development (2, 3). The GTP-bound, but not the GDP-bound, form of Ras directly binds to and regulates its downstream targets. Numerous downstream targets of Ras have been identified that mediate the signal transduction of Ras. Raf, a serine/threonine kinase, originally isolated as a viral oncogene contributing to cellular transformation, is one of the best characterized Ras effectors and activates the MEK[1]-ERK pathway (4–6).

A MAP kinase module consists of three kinases (MAPKKK, MAPKK, and MAPK) acting in sequence (7–10). The canonical MAP kinase cascade is found in many signal transduction pathways. Biochemical mechanisms of MAP kinase activation are completely conserved although different MAP kinase modules may couple to different signal transduction pathways in response to different extracellular stimuli. In mammalian cells, the Raf(MAPKKK)-MEK(MAPKK)-ERK(MAPK) pathway has been well characterized in response to a large number of mitogenic stimuli. The c-Jun N-terminal kinase (JNK) also known as the stress-activated protein kinase and the p38 kinase are activated by a kinase cascade very similar to the Raf-MEK-ERK pathway (7–9). The Raf-MEK-ERK cascade is a linear pathway and shows extremely high specificity. In contrast, the situation is more complex with JNK and p38. Numerous MAPKK and MAPKKK are implicated in activation of JNK or p38. Furthermore, some of the upstream kinases have been implicated in activation of both JNK and p38. MAP kinase modules have been discovered in all eukaryotes and are involved in many physiological responses. For example, in yeast, MAP kinase pathways are involved in regulation of mating pheromone response, sporulation, cell wall integrity, hyperosmotic stress, and pseudohyphal growth (11, 12). Similarly, MAP kinase pathways have been demonstrated to regulate many developmental programs in *Caenorhabditis elegans* (13) and *Drosophila* (14).

The biochemical mechanisms of activation of ERK and MEK are well understood. Raf directly phosphorylates and activates MEK via two conserved serine residues in the kinase activation loop of MEK (15, 16). These phosphorylations are necessary and sufficient to fully activate MEK. Activated MEK then directly phosphorylates a conserved tyrosine and threonine residue in the kinase activation loop of ERK (17). Again, these phosphorylations are necessary and sufficient for full ERK activation. Similar biochemical mechanisms of activation are employed by the other MAP kinase modules. In contrast, the biochemical mechanism of MAPKKK activation, such as Raf, is not fully understood (6).

One of the key events in Raf activation is growth factor-induced association with Ras (18–21). The interaction of Raf with Ras is a common and necessary feature of all Raf family members, however, is not sufficient for Raf activation. It is

* This work was supported by grants from the National Institutes of Health and the Walther Cancer Institute (to K. L. G.) and National Institutes of Health National Research Service Award 5-T32-GM07544 (to H. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: MEK, mitogen-activated protein kinase and extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ROCK, Rho-associated coiled-coil-containing kinase; PKN, protein kinase N; RBD, Ras-binding domain or Rac binding domain; GST, glutathione *S*-transferase; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis.

conceivable that a common biochemical mechanism (possibly phosphorylation) is involved in Raf activation by Ras. Raf kinase activity is regulated by multiple events, including positive and negative phosphorylations (6). Most of the studies performed with C-Raf demonstrated that phosphorylation of Ser^{338} is essential for Raf activation by Ras, growth factors, and phorbol 12-myristate 13-acetate (22–25). A synergistic activation of Raf was observed when both Ser^{338} and Tyr^{341} are phosphorylated. However, the above model (phosphorylation of Ser^{338} and Tyr^{341}) fails to explain the activation of other Raf family proteins because these two residues are not conserved. For example, the *C. elegans lin-45* Raf contains aspartate residues at positions corresponding to Ser^{338} and Tyr^{341} of C-Raf. Similarly, residue corresponding to Tyr^{341} is substituted by an aspartate residue in B-Raf. Recently, we have demonstrated that phosphorylation of Thr^{598} and Ser^{601} in B-Raf, which correspond to Thr^{491} and Ser^{494} of C-Raf, are essential for B-Raf activation (26).

Previous studies have demonstrated that Ser^{338} of C-Raf can be phosphorylated by Pak2 which is a direct downstream target of Cdc42 and Rac (27). However, the exact mechanism of Pak in Raf activation is not clear because expression of Pak alone is generally not sufficient to activate Raf or the ERK pathway. Furthermore, expression of active Cdc42 or Rac induces marginal Raf activation while expression of active Ras induces dramatic Raf activation. Interestingly, co-expression of Pak with active Rac can induce significant Raf activation (28), indicating a potential role of Cdc42/Rac and Pak in Raf regulation.

In this report, we investigated the mechanism of Raf activation by Ras. The effector domain of GTP-bound Ras is directly involved in protein-protein interaction with Raf. Mutations of the effector domain, such as E37G and Y40C, eliminate the ability of Ras to interact with Raf. Such effector domain mutants are also defective in Raf activation. We identified two Ras effector domain mutants, Y32F and T35S, that retain the ability to interact with Raf but are severely compromised in Raf activation. We observed that Rho family GTPases can cooperate with the RasV12Y32F or RasV12T35S to stimulate Raf activation although neither Rho nor Rac/Cdc42 alone can induce significant Raf activation. Furthermore, Pak1 can substitute the effect of Rac or Cdc42, but not Rho. Active Ras stimulates activation of Rac and RhoA while the RasV12Y32F or RasV12T35S mutant fail to stimulate activation of Rac or RhoA. In addition, dominant negative mutant of Rac or Rho can block Raf activation by Ras. Our results demonstrated that the Rho family GTPases play an important role in Raf activation by Ras.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human embryonic kidney 293 cells, COS-7 cells, and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37 °C. Transfection was done by LipofectAMINE (Life Technologies, Inc.) methods. Six-well plates were used for all the transfection experiments except where indicated. Vector such as pcDNA3 was used to balance the amount of DNA used for transfection. For stimulation or kinase assay, 24 h after transfection, cells were starved for an additional 12 h by incubating in Dulbecco's modified Eagle's medium with 0.1% serum. For immunoprecipitation, cells were cultured under the normal growth conditions for 48 h after transfection.

Plasmid Construction—HA-tagged Ki-Ras and ERK1 cDNA were subcloned into the vector pcDNA3HA. FLAG-Raf-1 is a kind gift from K. Pumiglia (29). Ki-Ras mutants, such as RasV12, RasV12Y32F, RasV12T35S, RasV12E37G, and RasV12Y40C, and Raf-1 mutant S338A were obtained by site-directed mutagenesis (Strategene). FLAG-Raf-1CAAX construct was made by adding the C-terminal 18 amino acid residues from the C terminus of Ki-Ras to the C terminus of Raf. The plasmids used for yeast two-hybrid interaction tests were made by polymerase chain reaction and their DNA fragments were subcloned into PVP16 or Plex Ade vector as targets or baits, respectively. The small GTPases (Rac1, Cdc42, and RhoA, all in the Myc-pRK5), Rat-myc-Pak1/pCMV5M (including wild type Pak1, 165–544 deletion mutant, 83–149 deletion mutant, and Pak1K299A), PRK2/pCMV2, PKN1/pRC/CMV, HA-JNK, GST-SEK S220E/T224D, and GST-SEK S220A/T224L mutants are the kind gifts from A. Hall, M. Cobb, L. Quilliam, Y. Ono, and J. Kyriakis. Rac effector domain mutants were obtained by polymerase chain reaction mutagenesis. The GST-PakRBD and GST-Rock RBD were made by subcloning the Rac-binding domain of Pak1 and the Rho-binding domain of Rock1 into pGEX-KG vector (30).

ERK Kinase Assay—The conditions for transfections were as described above. The amounts of DNA used in each transfection were as following: Myc-ERK1 (100 ng), RhoAL63 (50 ng), RacL61/Cdc42L61 (50 ng), Pak1 (200 ng), Pak1 deletion (83–149) (250 ng), RasV12 (20 ng), and its effector domain mutants (100 ng). The transfected 293, COS-7, and HeLa cells were serum-starved for 12 h before harvesting. Cells were washed once with phosphate-buffered saline and then lysed in immunoprecipitation buffer Nonidet P-40 (10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 50 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin). Cell lysates were incubated with 1 μg of HA antibody (anti-HA) for 1.5 h at 4 °C followed by addition of protein G-Sepharose (Pierce) for 1.5 h. Immunoprecipitated proteins were washed three times with lysis buffer followed by one wash with 25 mM HEPES, pH 7.5, 1 mM dithiothreitol, and 0.5 mM EDTA. Kinase activities for ERK were measured as described previously (16). GST-Elk-1 (containing the C-terminal amino acid residues 305–425) expressed in *Escherichia coli* and purified by glutathione affinity chromatography was used as a substrate in kinase activity assay.

JNK Kinase Assay—HEK 293 cells were co-transfected with 300 ng of HA-JNK and various plasmids, including 250 ng of GST-SEK S220E/T224D or 250 ng of GST-SEK S220A/T224L mutants. The transfected 293 cells were serum-starved for 12 h before being stimulated by 20 μg/ml anisomycin for 30 min. Then the cells were lysed in Nonidet P-40 buffer. The procedure of HA-JNK protein immunoprecipitation and kinase assay was similar as the ERK kinase assay. Except the GST-c-Jun was used as substrate in the kinase activity assay.

Raf Kinase Assay—HEK 293 cells were co-transfected with 150 ng of FLAG-Raf-1, FLAG-Raf S338A mutant, and various plasmids, including 50 ng of RasV12 or 100 ng its effector domain mutants, 50 ng of RacL61 or 100 ng its effector domain mutants, 50 ng of Cdc42L61, 50 ng of RhoAL63, 200 ng of Pak1, 500 ng of Pak1K299A, 500 ng of RacN17, 500 ng of RhoAN19, 200 ng of PKN1, 200 ng of PRK2, and 200 ng of ROCK1 plasmid DNA. 24 h following transfection, cells were incubated for 12 h in serum-free medium before harvesting. If stimulated, cells were treated with epidermal growth factor (50 ng/ml) for 5 min. Cells were washed once with phosphate-buffered saline and then lysed in about 300 μl of RIPA buffer (0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM sodium vanadate). After centrifugation, 250-μl supernatants were used for immunoprecipitation with 2 μg of FLAG antibody and 10 μl of protein G-Sepharose beads. Raf kinase activity was assessed by coupled MEK/ERK 1 kinase assays, according to previously described methods in which purified recombinant GST-MEK1, GST-ERK1, and GST-Elk1 were included in the reaction (26). In this coupled kinase assay, Raf activates MEK1 which activates ERK1 which then phosphorylates Elk1. Phosphorylation of Elk1 (by radioactivity) was quantitated by phosphoimage analysis.

Immunoprecipitation and Western Blot—293 cells were transfected for about 48 h, and washed with phosphate-buffered saline. Then the cells were lysed in immunoprecipitation buffer containing protease inhibitor (10 mM Tris-HCl, pH 7.5, 5 mM EGTA, 150 mM NaCl, 1% Nonidet P-40, 20 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Cell extracts were incubated with antibody for 2 h at 4 °C. The proper amount of protein A or protein G beads were added in immunoprecipitation solution, and incubated for an additional hour. Immunoprecipitates were collected by centrifugation, washed four times with immunoprecipitation buffer, and subjected to SDS-PAGE. Protein was detected by immunoblotting with specific antibodies. To determine specific phosphorylation on Raf-1, an immunoblot was conducted by using antibody which specifically recognizes Ser^{338}-phosphorylated Raf (New England Biolab).

In Vitro Binding—GST-PakRBD (Rac1-binding domain) and GST-Rock RBD were expressed and purified from *E. coli*. HEK 293 cells were transfected with wild type Myc-Rac1 (250 ng) and wild type RhoA (250

TABLE I
Ras effector domain mutants in Raf binding and activation

Raf binding was determined by the yeast two-hybrid assays. Raf activity was determined by in vitro coupled kinase assays using immunoprecipitated Raf (++++ denotes for 100% activity; +/− denotes for <10% activity).

	Raf binding	Raf activation
RasV12	++++	++++
RasN17	−	−
RasV12Y32F	+++	+/−
RasV12T35S	++++	+/−
RasV12E37G	−	−
RasV12Y40C	−	−
RacL61	−	+/−
RhoAL63	−	+/−

ng) in the presence of RasV12 (50 ng) or effector domain mutants (100 ng). Cells were lysed in binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 1% (v/v) Triton X-100, 5 mM $MgCl_2$, 25 μM $ZnCl_2$, and 0.2% (w/v) bovine serum albumin). The cleared cell lysates were incubated with 5 μg of GST-PakRBD or GST-Rock RBD for about 2 h and followed by addition of glutathione-Sepharose beads for an additional 1–2 h. The beads were washed four times with binding buffer, and once with binding buffer without bovine serum albumin. Then the beads were eluted with 10 mM glutathione. The elution was analyzed by SDS-PAGE and immunoblot with a Rac or RhoA antibody.

Interaction Test by the Yeast Two-hybrid System—Protein-protein interactions by the yeast two-hybrid experiments were performed as described (31).

RESULTS

Different Effector Domain Mutations in Ras Distinguish Raf Binding and Raf Activation—GTP-bound Ras is a potent activator of Raf. Artificial membrane attachment of Raf can lead to Raf activation (32). These observations suggest that membrane targeting of Raf by Ras is a key event for Raf activation. We examined several Ras effector domain mutants in Raf binding and activation (results summarized in Table I). Our results are consistent with the current model that binding is required for Ras to activate Raf. Mutation in the effector domain, such as E37G and Y40C, eliminated Raf binding and simultaneously abolished the ability of Ras to activate Raf (Table I). However, two effector domain mutants Y32F and T35S, which retain the ability to bind Raf, failed to activate Raf. T35S has been previously shown to bind Raf but not phosphoinositide 3-kinase (33), while Y32F has not been biochemically tested. We confirmed the interaction between RasV12Y32F and Raf by GST-RafRBD pull-down experiments *in vitro* (Fig. 1*A*). RasV12 and RasV12E37G were included as positive and negative controls, respectively. We also examined the activation of ERK by Ras as an *in vivo* assay for Raf activation. Both RasV12Y32F and RasV12T35S showed a much reduced ability to activate ERK when compared with RasV12 (Fig. 1*B*). The above data demonstrate that Ras-Raf binding is not sufficient to activate Raf. In addition to association, RasV12 must have additional activity required for Raf activation. Mutation of Y32F or T35S compromises the ability of RasV12 to stimulate Raf. Consistent with our observation, Tamada *et al.* (34) have previously reported that RasV12V45E effector domain mutant binds Raf but is severely compromised in its ability to activate Raf although the molecular mechanism is unknown (34).

The Rho Family Small GTPases (Rac, Cdc42, and RhoA) Can Restore the Defects of RasV12Y32F and T35S Effector Domain Mutants—We wanted to determine which downstream pathways of Ras might complement the defect of RasV12Y32F or T35S mutants. Interestingly, T35S is known to be defective in binding and activation of phosphoinositide 3-kinase that plays a positive role in the activation of Rac, which is an activator of Pak (33, 35). Since Pak has been implicated in Raf activation

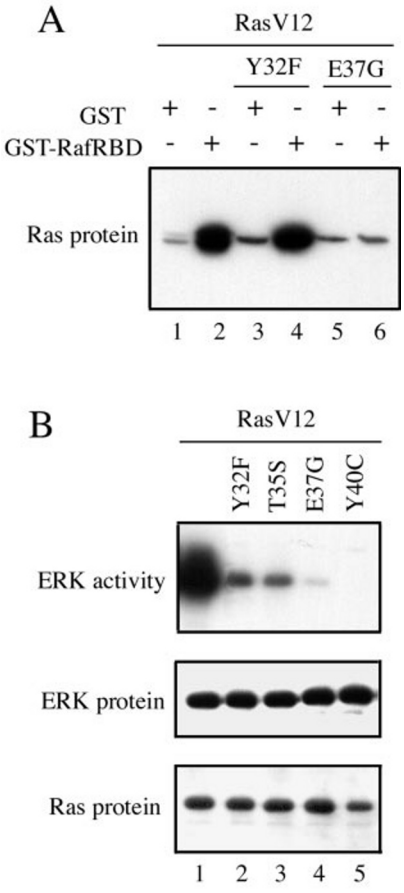

FIG. 1. *A,* RasV12Y32F binds Raf. *In vitro* binding of Ras effector domain mutants with GST-RafRBD was tested. Ras proteins were expressed in HEK293 cells by transfection. Cell lysates containing RasV12 (*lanes 1* and *2*), RasV12Y32F (*lanes 3* and *4*), or RasV12E37G (*lanes 5* and *6*) were incubated with 5 μg of GST-RafRBD or GST as indicated. The bound protein was eluted and detected by Western blotting with a Ras specific antibody. RasV12 or RasV12Y32F show strong binding to GST-RafRBD while RasV12E37G showed no binding. All data shown in this figure and subsequent figures are representative of at least three similar experiments. *B,* activation of ERK by Ras effector domain mutants. HA-ERK1 was co-transfected with RasV12 or different effector domain mutants in the RasV12 background as indicated. 36 h after transfection, cells were starved in 0.1% fetal bovine serum overnight before lysis and immunoprecipitated with anti-HA antibody for HA-ERK. Kinase activity of immunoprecipitated HA-ERK1 was assayed using GST-Elk1 as a substrate. The *top panel* indicates phosphorylation of GST-Elk1 substrate. The amount of ERK in immunoprecipitates and Ras in cell lysates are shown in the *middle* and *bottom panels*, respectively.

(27, 28), we tested whether an activated RacL61 mutant can cooperate with the Ras effector domain mutants in Raf activation. RacL61 alone was ineffective to activate Raf (Fig. 2*A, lane 2*). However, RacL61 could cooperate with RasV12Y32F or RasV12T35S to activate Raf kinase activity (Fig. 2*A, lanes 3* and *5*). In contrast, RacL61 was ineffective to cooperate with the RasV12E37G and no cooperation was observed between RacL61 and RasV12Y40C mutant (Fig. 2*A, lanes 7–10*), which has lost the ability to bind Raf (Table I). The effect of RacL61 was specific and not due to alterations of either Raf or Ras expression (Fig. 2*A, middle* and *bottom panels*). We also determined ERK activity in cells co-transfected with Ras effector domain mutants and RacL61. The ERK activation results are completely consistent with the Raf activation data (Fig. 2, *A* and *B*). RacL61 cooperates with the RasV12Y32F and T35S mutant to stimulate ERK activity.

Another Rho family member, Cdc42, is also known to activate Pak (35). Therefore, the effect of Cdc42 was examined. Our

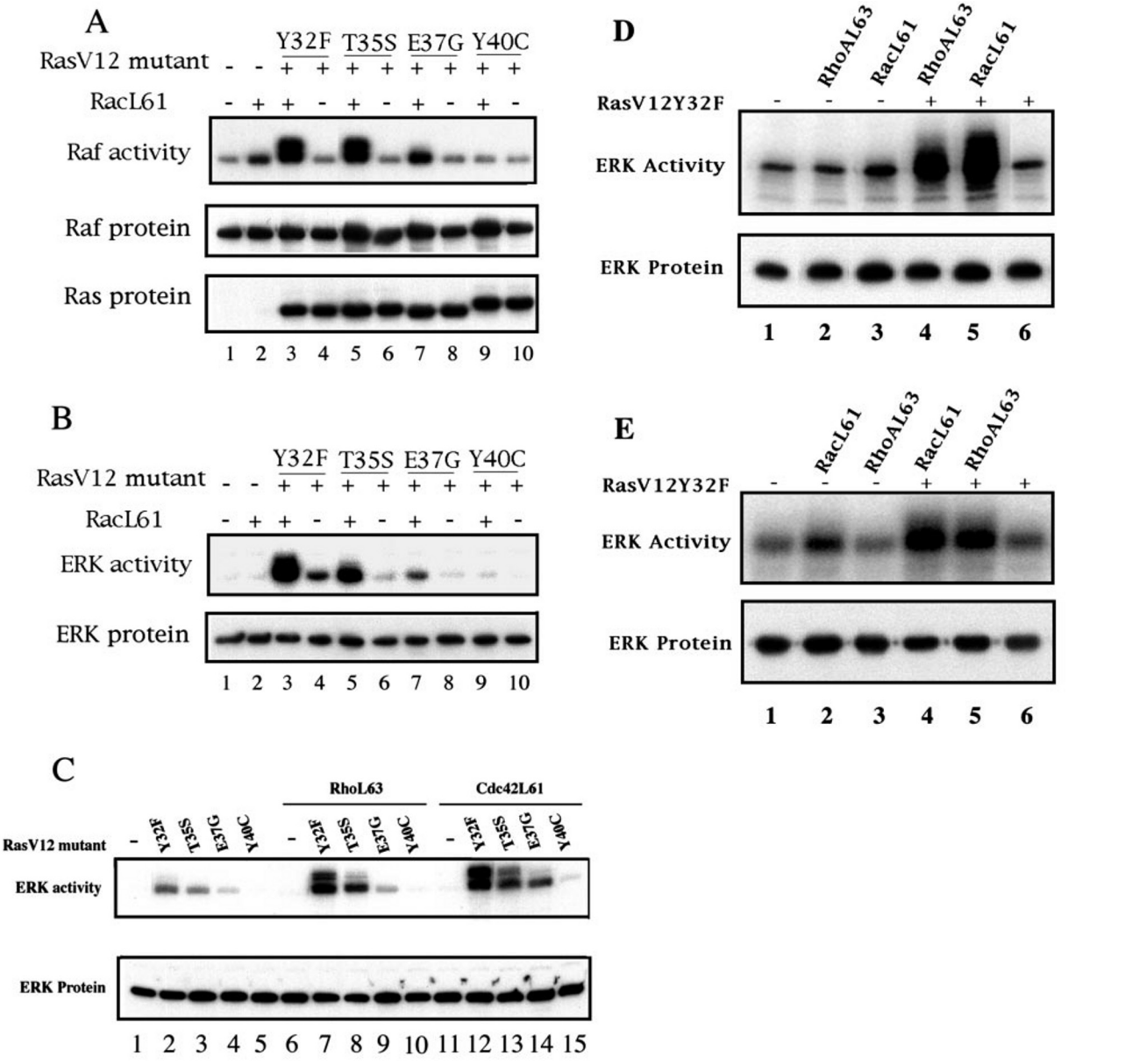

FIG. 2. *A,* Rac cooperates with Ras effector domain mutants to activate Raf. FLAG-Raf was transfected into HEK293 cells in the presence of various RasV12 effector domain mutants (RasV12Y32F, *lanes 3* and *4*; RasV12T35S, *lanes 5* and *6*; RasV12E37G, *lanes 7* and *8*; RasV12Y40C, *lanes 9* and *10*) as indicated. Co-transfection of RacL61 is indicated. 24 h after transfection, cells were starved in 0.1% fetal bovine serum overnight before harvesting. Immunoprecipitation of FLAG-Raf was performed and the immunoprecipitated Raf activity was determined in an *in vitro* coupled kinase assay as described under "Experimental Procedures." Raf kinase activity (indirectly determined by phosphorylation of GST-Elk1) is shown in the *top panel*. Raf protein used for kinase assays and Ras protein in cell lysates are shown in the *middle* and *bottom panels*, respectively. Neither RacL61 alone (*lane 2*) nor RasV12Y32F (*lane 4*) induced a significant Raf activation while co-transfection of both induced a dramatic Raf activation (*lane 3*). Similar results were obtained with RasV12T35S and RacL61. *B,* cooperation between Rac and Ras effector domain mutants in ERK activation. Experiments are similar to those described in *panel A* except FLAG-Raf was replaced by HA-ERK. The data show that RacL61 cooperated with RasV12Y32F or RasV12T35S to stimulate activation of the co-transfected HA-ERK. *C,* Cdc42 and RhoA cooperate with Ras effector domain mutants. Experiments were similar to *panel B*. Co-transfections of RhoAL63 or Cdc42L61 with different RasV12 effector domain mutants are indicated. ERK activity and protein are shown in the *upper* and *lower panels*, respectively. *D,* activation of ERK in HeLa cells. Experiments are similar to those in *panel B* except transfections were performed in HeLa cells. ERK kinase activity and protein levels are shown in the *top* and *bottom panel*, respectively. Co-transfections of RasV12Y32F and RacL61 or RhoAL63 are indicated on top of each lane. *E,* activation of ERK in COS-7 cells. Experiments are identical to *panel D* except transfections were performed in COS-7 cells.

results indicate that Cdc42 acts similarly to Rac in cooperation with the RasV12Y32F and T35S mutants to stimulate ERK activity (Fig. 2*C, lanes 12* and *13*). We wanted to test whether RhoA, a Rho family member unable to activate Pak, could cooperate with the Ras effector domain mutants in ERK activation. RhoAL63 alone did not activate ERK (Fig. 2*C, lane 6*). Surprisingly, co-expression of RhoAL63 with the Ras effector domain mutants enhanced ERK activation (Fig. 2*C, lanes 7* and *8*). This result indicates that Pak may not be the only downstream effector responsible for Raf activation. Our results demonstrate that the Rho family GTPases play a role in Raf activation by Ras.

Activation of ERK in COS-7 and HeLa cells was determined. Co-expression of RacL61 or RhoAL63 cooperates with the RasV12Y32F mutant in stimulation of ERK activity in both HeLa (Fig. 2*D*) and COS-7 cells (Fig. 2*E*). These results indicate that the functions of the Rho family GTPases in Raf activation are independent of cell type.

Rho and Rac Are Required for Raf Activation by Ras—The role of the Rho family small GTPases in Raf activation was further

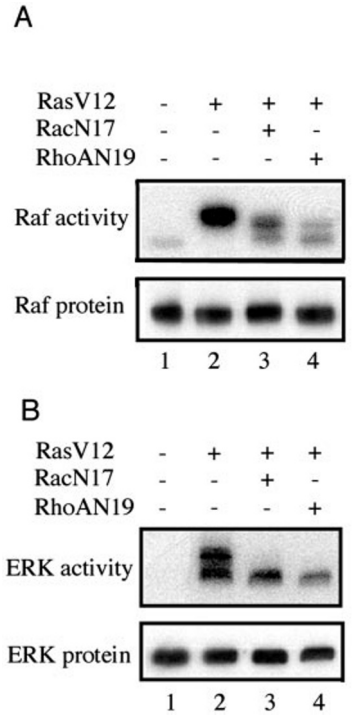

FIG. 3. *A,* dominant negative RacN17 or RhoAN19 blocked Ras-dependent Raf activation. FLAG-Raf was transfected into HEK293 cells in the presence or absence of RasV12. Co-transfections of RacN17 or RhoAN19 are indicated. Raf activity was assayed (as in Fig. 2*A*). The amount of Raf protein used in the kinase assay was determined by Western blotting with anti-C-Raf antibody. *B,* dominant negative RacN17 or RhoAN19 blocked Ras-induced ERK activation. Experiments were similar to *panel A* except HA-ERK was used in the experiments. HA-ERK protein used in kinase assay was detected by anti-ERK Western blotting (*lower panel*).

tested using dominant negative mutants. The dominant negative mutants of RacN17 and RhoAN19 exist constitutively in GDP-bound form. Dominant negative small GTPases can block activation of endogenous GTPases, possibly by sequestering upstream activators. Expression of dominant negative RacN17 or RhoAN19 effectively blocked Raf activation by RasV12 (Fig. 3*A*). Similarly, activation of ERK by RasV12 is also largely inhibited when dominant negative RacN17 or RhoAN19 was coexpressed (Fig. 3*B*). These results provide strong evidence that activation of the endogenous Rho family small GTPases is required for full Raf activation by Ras.

The Ability of Ras Effector Domain Mutants to Stimulate Rac and RhoA Activation Is Correlated with Their Ability to Activate Raf—To further elucidate the mechanisms of Ras effector domain mutants, we determined activation of co-transfected wild type Rac1 by Ras. The level of GTP-bound Rac was measured by using the RBD of Pak, which specifically interacts with the GTP form, but not the GDP form of Rac. Co-expression of RasV12 caused a detectable increase of Rac-GTP as determined by the GST-PakRBD pull-down experiments (Fig. 4*A, lanes 1–3*). In contrast, RasV12Y32F and T35S mutants failed to activate wild type Rac in co-transfection experiments (Fig. 4*A, lanes 4* and *5*). Similar experiments were performed with RhoA. Levels of GTP-bound RhoA were measured by pull-down experiments using the RhoA-binding domain of Rock. The results in Fig. 4*B* shows that RasV12 but not RasV12Y32F nor RasV12T35S activated RhoA in co-transfection experiments. These results support that the inability of Ras effector domain mutants to activate Raf may be due to the defect in Rac/Rho activation.

Rac also contains an effector domain, which is responsible for the interaction with downstream targets. We examined several Rac effector domain mutants to determine their ability to cooperate with RasV12Y32F. The data in Fig. 4*C* indicate that RacL61-F37G and RacL61-Y40C are compromised in the ability to cooperate with RasV12Y32F to stimulate Raf activity (Fig. 4*B, lanes 7* and *8*). Interesting, similar mutations were defective in stimulating Pak activity (36), further indicating a role of Pak in Raf activation.

Activation of Membrane-associated Raf by Rac and Rho—Our results suggest that Ras stimulates Raf by two separate activities: 1) Ras recruits Raf to plasma membrane and 2) Ras activates Rac/Rho. Both of these functions are required for full Raf activation. Based on this hypothesis, one can predict that Rac may be able to activate membrane-associated Raf. Membrane targeting of Raf could partially substitute for the effect of Ras binding and elevate basal Raf kinase activity. We tested the effect of active RacL61 on the activity of Raf*CAAX*, which contains the C-terminal membrane targeting C*AAX* sequence of Ras. As previously reported, Raf*CAAX* displays a much higher basal activity than the wild type Raf (Fig. 5*A*) (32). Interestingly, RacL61 alone could stimulate Raf*CAAX* activity while under the same conditions no stimulation was observed with the wild type Raf (Fig. 5*A*). Similar experiments were performed with the active RhoAL63 mutant, which also activated Raf*CAAX* (Fig. 5*B*). These data support that active Rho and Rac can activate membrane localized, but not cytoplasmic localized Raf.

Pak Mediates the Effects of Rac/Cdc42 but Not Rho—Pak is a likely candidate to mediate the positive effect of Rac on Raf activation. We determined whether an active form of Pak-(165–544) (37), a deletion of the N-terminal inhibitory domain, could substitute for the effect of active Rac. Pak-(165–544) alone was not sufficient to activate Raf (Fig. 6*A, lane 7*). Co-expression of Pak-(165–544) and RasV12Y32F resulted in co-operation in Raf activation (*lane 11*). This observation indicates that active Pak can substitute for Rac to stimulate Raf activation. To further test the role of Pak in mediating the Rac signal to activate Raf, we examined the kinase inactive Pak mutant which may function as dominant negative. Co-expression of Pak-K299A significantly blocked the activation of Raf by RacL61 and RasV12Y32F (Fig. 6*A, lane 16*), suggesting that Pak is essential for Rac to cooperate with RasV12Y32F in Raf activation. Similarly, the effect of Cdc42L61 was also blocked by kinase inactive Pak (Fig. 6*A, lane 17*). In contrast, the kinase inactive Pak had no effect on the ability of RhoAL63 to enhance Raf activation (Fig. 6*A, lane 18*). These results support that Pak mediates the effect of Cdc42/Rac, but plays no significant role in RhoA to activate Raf. Neither wild type Pak nor RacL61 alone can activate Raf. Surprisingly, co-expression of RacL61 or Cdc42 and Pak resulted in significant activation of Raf (Fig. 6*A, lanes 3* and *4*). In contrast, co-expression of RhoAL63 and Pak failed to activate Raf (Fig. 6*A, lane 5*).

We also examined the effect of other potential downstream effectors of RhoA. PKN1 and PRK2, two kinases known to be activated by RhoA (38, 39), were tested in cooperation with RasV12Y32F. Our results demonstrated that these two kinases had minimal effect in cooperating with RasV12Y32F to stimulate Raf activity (Fig. 6*B, lanes 6, 7*, and *10*). Another downstream target of RhoA, ROCK1 (40), was tested in similar experiments and found not to cooperate with RasV12Y32F (data not shown). These results indicate that activation of Raf by RhoA is likely mediated by an as yet unidentified effector (possibly a kinase) of RhoA.

The kinase dead mutant of Pak1-K299A binds Rac/Cdc42 and may interfere activation of other downstream targets of Rac/Cdc42. To further establish the role of Pak in Raf activation, we tested the Pak1 fragment between amino acid residues

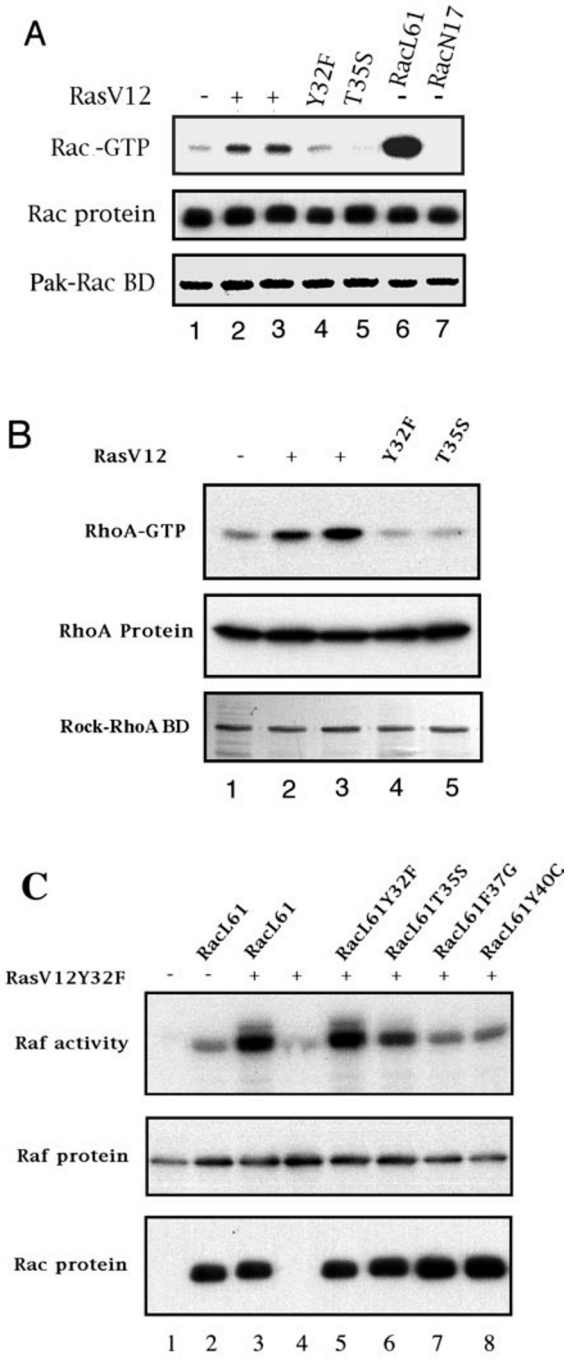

FIG. 4. *A,* activation of Rac by RasV12. Myc-Rac (wild type) was transfected into HEK293 cells (*lanes 1–5*) in the presence of RasV12 (*lanes 2* and *3*, identical duplicates), RasV12Y32F (*lane 4*), and RasV12T25S (*lane 5*). Myc-RacL61 (*lane 6*) and Myc-RacN17 (*lane 7*) were included as positive and negative controls, respectively. Cell lysates were incubated with purified GST-PakRBD, which specifically interacts with the GTP-bound Rac. The bound Rac was eluted and detected by anti-Myc Western blotting for Myc-Rac (*top panel*). The expressions of Rac in cell lysates are shown in the *middle panel*. GST-PakRBD used in binding reactions was detected by Coomassie Blue staining (*bottom panel*). *B,* activation of RhoA by RasV12. Wild type RhoA was transfected into HEK293 cells in the presence of control vector (*lane 1*), RasV12 (*lanes 2* and *3*), RasV12Y32F (*lane 4*), and RasV12T35S (*lane 5*) as indicated. Cell lysates were incubated with GST-Rock RBD, which binds to RhoA-GTP. The bound RhoA was detected by Western blotting (*top panel*). The levels of RhoA expression in cell lysates are shown in the *middle panel*. GST-Rock RBD was detected by Coomassie Blue staining (*bottom panel*). *C,* the effector domain of Rac is important for its ability to cooperate with RasV12Y32F mutant. RacL61 and different effector domain mutants were tested for their ability to cooperate with RasV12Y32F to stimulate Raf activity. Raf kinase activity is shown in the *top panel*. The expressions of Raf and different Rac effector domain mutants are shown in *middle* and *bottom panels*, respectively.

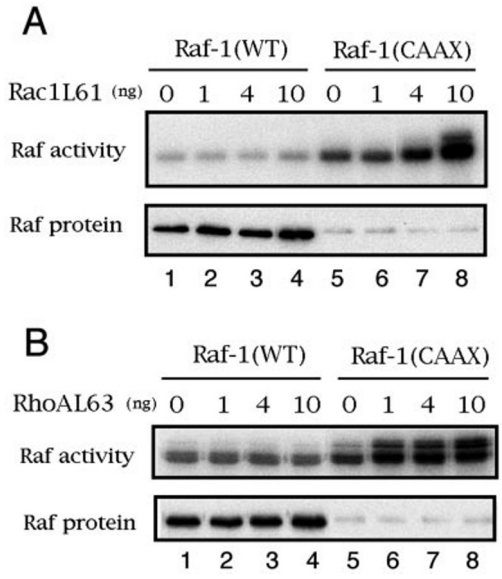

FIG. 5. *A,* activation of membrane targeted Raf by RacL61. Wild type FLAG-Raf (100 ng, *lanes 1–4*) or FLAG-Raf*CAAX* (10 ng, *lanes 5–8*) was co-transfected with increasing amounts of RacL61 as indicated. FLAG-Raf was immunoprecipitated and kinase activity was measured (*upper panel*). The level of FLAG-Raf expression is shown in the *lower panel* by anti-Raf Western blot. *B,* activation of membrane-targeted Raf by RhoAL63. Experiments were similar to those described in *panel A*. RhoAL63 stimulated Raf*CAAX* (*lanes 5–8*) but not wild type Raf (*lanes 1–4*).

83 and 149, which does not bind to Cdc42/Rac but still can inhibit Pak activity (41). ERK activation by RasV12Y32F and RacL61 was significantly inhibited by co-expression of Pak1-(83–149) (Fig. 6*C*), indicating that inhibition of Pak alone is sufficient to block the ability of Rac to cooperate with RasV12Y32F. These results are consistent with the observations that active Pak1 cooperates with RasV12Y32F to stimulate Raf activation (Fig. 6*A*) and further support the role of Pak in Raf activation. We also tested whether the JNK pathway play a role in Raf activation in response to RhoA, which is reported to activate JNK in HEK 293 cells (42). JNK is activated by the upstream kinase, SEK. Our results showed that SEK(ED), a constitutively active mutant, did not cooperate with RasV12Y32F (Fig. 6*D*). Consistently, SEK(AL), a dominant negative mutant, does not block ERK activation by RasV12Y32F and RhoAL63 (Fig. 6*D*). As positive controls, SEK(ED) activates JNK while SEK(AL) blocked JNK activation by anisomycin (Fig. 6*E*). These data clearly demonstrated that JNK plays no significant role in Raf activation by RasV12Y32F and RhoAL63.

RhoA and Rac Use Different Mechanisms to Stimulate Raf Activity in Cooperation with Ras Effector Domain Mutants—Phosphorylation of Ser^{338} by Pak plays an important role in C-Raf activation (27). We determined the phosphorylation status of Ser^{338} during Raf activation by RasV12Y32F and RacL61. Phosphorylation of Ser^{338} in C-Raf was increased by either RasV12 or epidermal growth factor stimulation (Fig. 7, *A, lane 7,* and *B, lane 2*). This result is consistent with previous observations (24) and supports a role of Ser^{338} phosphorylation in Raf activation. Expression of RacL61 significantly enhanced the Ser^{338} phosphorylation (Fig. 7*A, lane 2*). The phosphorylation of Raf can be further increased by cooperation of RacL61 and RasV12Y32F (Fig. 7*A, lanes 2* and *4*). In contrast, RhoAL63 had much less effect on Ser^{338} phosphorylation (*lane 3*). Furthermore, the enhancement of Ser^{338} phosphorylation by RhoAL63 was much less than RacL61 in the presence of RasV12Y32F (*lanes 4* and *5*) although RhoAL63 is as effective

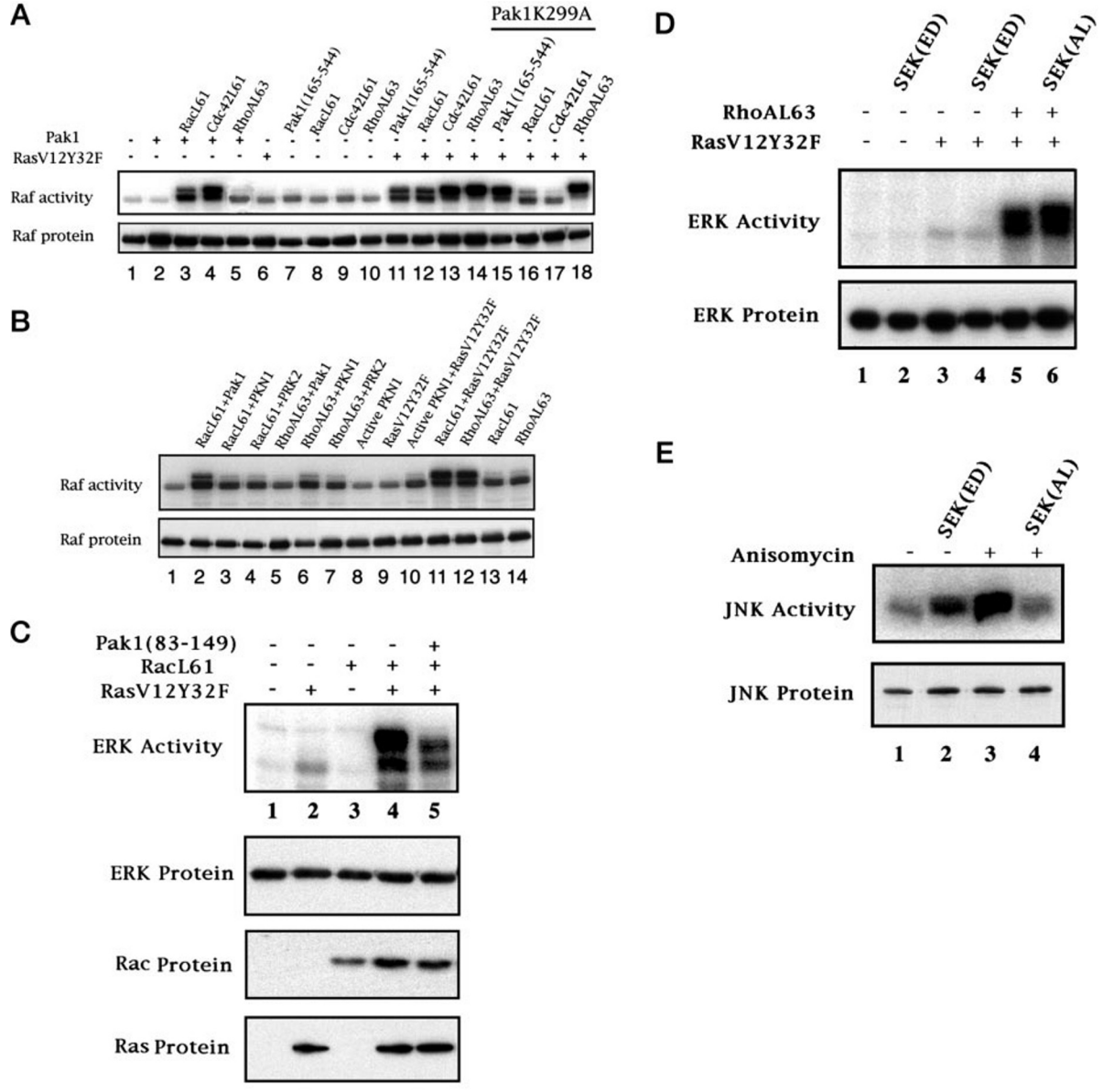

FIG. 6. *A,* Pak functions downstream of Rac/Cdc42 to stimulate Raf activation. FLAG-Raf was co-transfected with various other plasmids as indicated. Rho family members or constitutively active Pak-(165–544) (truncation of the N-terminal 164 residues) was transfected alone or in combination with RasV12Y32F. Co-transfections with wild type Pak1 (*lanes 2–5*) or kinase inactive Pak mutant (Pak1-K299A, *lanes 15–18*) are indicated. FLAG-Raf was immunoprecipitated and kinase activity was determined (*upper panel*). FLAG-Raf protein levels were determined by anti-Raf Western blot (*lower panel*). *B,* lack of effect of PKN1 and PRK2 on Raf activation. The effect of PKN1 and PRK2 on the cooperation between RhoA and RasV12Y32F was tested. Experiments were similar to *panel A*. FLAG-Raf was co-transfected with various plasmids as indicated on *top* of each lane. Raf activity and protein are shown in the *upper* and *lower panels*, respectively. *C,* activation of Pak is required for the cooperation between RasV12Y32F and RacL61. HA-ERK was co-transfected with the indicated plasmids and immunoprecipitated. ERK kinase activity and protein levels are shown in the *top panel* and the *second panel* from the *top*, respectively. The expression levels of Rac and Ras are shown in the *second panel* from the *bottom* and the *bottom panel*, respectively. Co-transfection of Pak1-(83–149), which can inhibit Pak activity but cannot bind Rac, was included in *lane 5*. *D,* SEK plays no significant role in the cooperation between RasV12Y32F and RhoAL63. HA-ERK was co-transfected with various plasmids in HEK293 cells as indicated. Constitutively active SEK(ED) mutant was co-transfected in *lanes 2* and *4*. Dominant negative SEK(AL) mutant was co-transfected in *lane 6*. ERK activity and protein level are presented in the *top* and *bottom panels*, respectively. *E,* activation of JNK. HA-JNK was co-transfected with SEK(ED) (*lane 2*) or SEK(AL) (*lane 4*) in HEK293 cells. Anisomycin (20 μg/ml) was used to activate JNK for 30 min (*lanes 3* and *4*). HA-JNK was immunoprecipitated and kinase activity was determined using GST-c-Jun as a substrate (*top panel*). SEK(ED) stimulated JNK activity (*lane 2*) while SEK(AL) blocked JNK activation by anisomycin.

as RacL61 in Raf activation. These observations indicate that Ser^{338} is not the major target of RhoA which likely stimulates Raf via another phosphorylation site. Mutation of Ser^{338} to alanine completely eliminated the recognition by the anti-phospho-Ser^{338} antibody, supporting the specificity of the antibody.

Although RacL61 and Cdc42L61 stimulated phosphorylation of Ser^{338} to a similar or higher extent as RasV12 (Fig. 7*B, lanes 2–4*), Raf activity was not highly activated by RacL61 or Cdc42L61 whereas RasV12 induced a dramatic Raf activation. These results indicate that phosphorylation of Ser^{338} is not quantitatively correlated with Raf activity, consistent with observations by others (43), and Ras may induce modifications of Raf in addition to Ser^{338}. Consistent with this model, active Pak1 induced a significant Ser^{338} phosphorylation (Fig. 7*C, lane 4*) but did not activate Raf (Fig. 6*A, lane 7*). The Ser^{338} phosphorylation of C-Raf was dramatically enhanced by co-expression of Pak and RacL61 or Cdc42L61 (Fig. 7*B, lanes 7* and *8*). In contrast, RhoAL63 enhanced Ser^{338} phosphorylation weakly even in the presence of Pak (Fig. 7*B, lane 9*), further supporting that RhoA activates Raf through a mechanism dif-

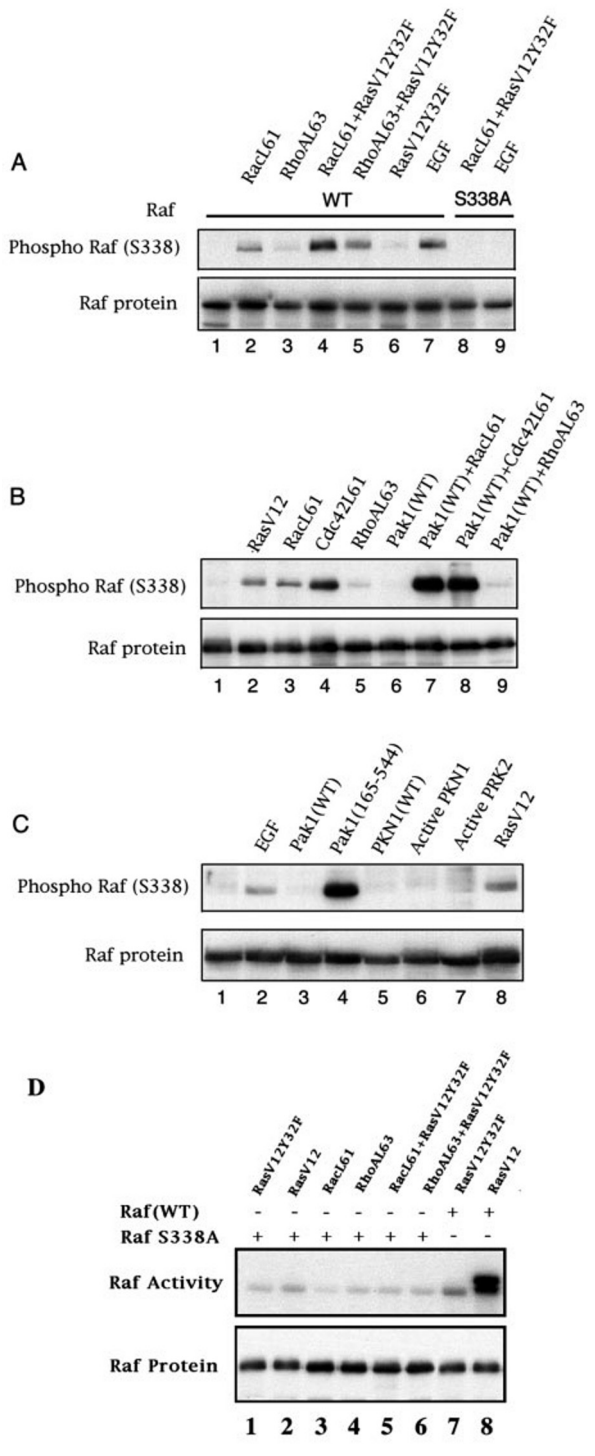

FIG. 7. *A,* RacL61 stimulates Ser^{338} phosphorylation of Raf. Wild type FLAG-Raf (*lanes 1–7*) was co-transfected with RacL61 or RhoAL63 in the absence or presence of RasV12Y32F as indicated on *top* of each lane. Epidermal growth factor stimulation (50 ng/ml for 5 min) was included as a positive control (*lane 7*). FLAG-Raf was immunoprecipitated and subjected to SDS-PAGE followed by Western blotting with anti-phospho-Ser^{338} antibody (*upper panel*) or anti-Raf antibody (*lower panel*). S338A denotes mutant Raf in which the Ser^{338} was mutated to alanine (*lanes 8* and *9*). *B,* Rac/Cdc42 stimulates Pak to phosphorylate Ser^{338} of Raf. FLAG-Raf was co-transfected with various plasmids as indicated. Experiments were performed similar as described in *panel A. C,* active Pak1 but not PKN1 nor PRK2 stimulates Ser^{338} phosphorylation of Raf. FLAG-Raf was co-transfected with various plasmids as indicated. Phosphorylation of Ser^{338} (*upper panel*) and protein levels (*lower panel*) of Raf were detected as in *panel A. D*, Ser^{338} of Raf is required for activation. FLAG-Raf-S338A (*lanes 1–6*) and FLAG-Raf (*lanes 7* and *8*) were transfected into HEK293 cells. Raf activity and protein levels are shown in *top* and *bottom panels*, respectively.

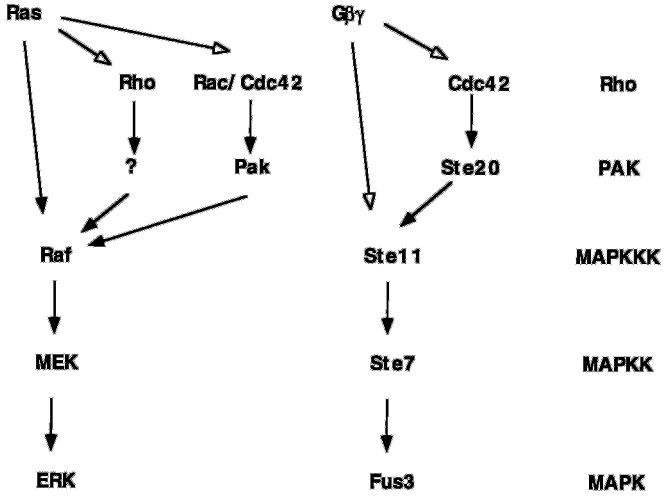

FIG. 8. **A proposed model of Raf activation by Ras.** *Solid arrows* indicate a direct protein-protein interaction or phosphorylation while *open arrows* indicate an indirect connection. Comparisons of the mammalian Ras-Raf pathways with the yeast mating pheromone response pathways are presented. This model predicts that Ras has at least two effects (direct binding to the N-terminal region, and indirect phosphorylation via Rac/Cdc42 and Pak) on Raf activation.

ferent from Rac or Cdc42. However, Ser^{338} is required for Raf activation (Fig. 7*D*). The Raf S338A mutant cannot be activated by RasV12Y32F and RhoAL63 or RacL61 (Fig. 7*D, lanes 5* and *6*).

The effect of PKN1 and PRK2 on Ser^{338} phosphorylation of Raf was also investigated. Both active PKN1 and PRK2 failed to stimulate Ser^{338} phosphorylation while active Pak1 was very effective (Fig. 7*C, lanes 4–7*). The above data demonstrate that Ser^{338} phosphorylation is a likely target of Cdc42/Rac1 and Pak1 in Raf activation. Furthermore, phosphorylation of Ser^{338} is not sufficient to fully activate Raf.

DISCUSSION

Genetic and biochemical studies have unequivocally demonstrated that Ras is the major upstream activator of Raf. Activation of Raf by Ras requires a direct interaction between Ras and Raf. However, purified Ras protein is unable to activate purified Raf, indicating that other events are involved *in vivo*. This report demonstrated that the Rho family GTPases play an important role in Raf activation.

We propose a model where Ras plays at least two important roles in Raf activation (Fig. 8). First, Ras directly binds to the N-terminal region of Raf. The binding of Ras to Raf could have two consequences. Ras recruits Raf to the membrane proximity where Raf activation occurs. This effect can be mimicked by artificial membrane targeting of Raf, which partially activates Raf. Furthermore, binding of Ras may relieve the inhibitory effect of the N-terminal region on the C-terminal kinase domain. Consistent with this notion, deletion of the N-terminal region activates Raf. Second, Ras activates a kinase(s) responsible for phosphorylation of activating sites in Raf or a phosphatase(s) responsible for dephosphorylation of inhibitory sites. Once activated, Raf remains active even when Ras is dissociated. One of the Ras-stimulated phosphorylation sites is Ser^{338}. The Rho family GTPases may signal downstream of Ras and upstream of Raf via protein kinases, such as Pak that can directly phosphorylates Ser^{338}. Ras could modulate Rho and Rac activity indirectly via effectors, such as phosphoinositide 3-kinase (44, 45). However, the model in Fig. 8 may be too simple. For example, it does not explain why co-expression of Rac and Pak partially activates Raf while co-expression of active Pak1-(165–544) does not (Fig. 6*A*). Phosphorylation of Ser^{338} was high under both conditions (Fig. 7,

B and *C*). A possible interpretation is that Rac induces additional phosphorylation of Raf besides at residue Ser^{338}. Another interpretation is that Pak1-(165–544) phosphorylates Raf in the cytoplasm whereas the Rac-activated Pak1 phosphorylates Raf in the membrane proximity where other modifications for Raf activation can occur.

Our data indicate that Pak can mediate the positive signal from Cdc42/Rac to Raf activation. Pak has been previously shown to phosphorylate Ser^{338} of C-Raf and stimulate Raf activation. We showed that constitutively active Pak cooperates with RasV12Y32F in the activation of Raf. Furthermore, kinase inactive Pak mutant blocks the cooperation between RasV12Y32F and active Cdc42 or Rac. In contrast, Pak is unlikely to play a role in the cooperation between Rho and RasV12Y32F because dominant negative Pak does not block the cooperation. These observations are consistent with the fact that Pak is not activated by Rho. We examined Rho activated kinases, including ROCK1, PRK2, and PKN1. However, we failed to observe a cooperation between these kinases and RasV12Y32F in Raf activation. We also excluded the involvement of the JNK pathway in Raf activation. Therefore, the downstream kinase mediating the signal from RhoA to Raf is currently unknown and requires further investigation.

The role of Pak in Raf regulation shares similar features with the yeast mating pheromone response MAP kinase pathway (46). In this MAP kinase module, Ste11 is the MAPKKK acting at the same level as Raf in the ERK pathway (Fig. 8). Ste11 is believed to be activated by Ste20 which is a member of the Pak family. Ste20 is regulated by the Cdc42 gene product in yeast. Both genetic and biochemical evidence have indicated that $G\beta\gamma$, which directly couples to the pheromone receptor, regulates Cdc42 activity possibly via the Cdc24 nucleotide exchange factor (46). $G\beta\gamma$ may also regulate Ste11 via other components such as the Ste5 scaffold protein, which interacts with $G\beta$ (47) and forms a complex with Ste11, Ste7, and Fus3. The model in Fig. 8 underscores that remarkable conservation of the MAP kinase module exists not only at the level of MAPK and MAPKK but also at the level of MAPKKK and upstream (Fig. 8).

Extensive genetic studies failed to identify Rho family members or Pak kinases as upstream activators of Raf. This could be due to the fact that there are functional redundancies. Our results demonstrate that one of the Rho family members is sufficient to relay the signals from Ras to Raf. Therefore, single mutation of Rho, Rac, or Cdc42 is not sufficient to eliminate the positive signal from Ras to Raf. Similarly, at the level of Pak, multiple members of the Pak family may prevent the genetic isolation of these kinases as positive regulators of Raf. It is worth noting that the function of Rac-Pak in Raf activation may not be conserved in *C. elegans*. The *C. elegans lin-45* Raf contains an aspartate residue at a position corresponding to Ser^{338} of C-Raf.

Raf activation is more complex than simply Ras binding and phosphorylation of Ser^{338} by Pak. It is clear that phosphorylation of Ser^{338} is not the sole event in Raf activation because a direct quantitative correlation between Ser^{338} phosphorylation and Raf activity is lacking. For example, active Pak dramatically stimulates Ser^{338} phosphorylation while it is not sufficient to activate Raf. In addition, expression of Pak with Rac or Cdc42 induces Ser^{338} phosphorylation stronger than that induced by RasV12, yet RasV12 is a more potent activator of Raf. Furthermore, RhoA is as effective as Rac to cooperate with RasV12Y32F in Raf activation whereas RhoA induces a much weaker Ser^{338} phosphorylation than Rac. These observations indicate that other events, likely phosphorylation or dephosphorylation, must occur in order to achieve full Raf activation. C-Raf is known to be phosphorylated on multiple sites (6). We have recently observed that multiple phosphorylations are required for C-Raf activation (48). Ras may stimulate phosphorylation of Raf on different sites via different downstream effectors. In addition, phosphorylation of Ser^{259} in C-Raf has an inhibitory effect. It is also possible that Ras may promote dephosphorylation of the inhibitory site by activating a specific phosphatase. Future studies to examine the relationship among all the phosphorylation sites in Raf are needed for a complete understanding of the molecular mechanism of Raf regulation.

Acknowledgments—We thank T. Zhu for technical assistance; Drs. K. Pumiglia, A. Hall, M. Cobb, J. Frost, L. Quilliam, J. Kyriakis, and Y. Ono for plasmids; and T. Lanigan and H. Vikis for critical reading of the manuscript.

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