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

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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 RasV12T35S and RasV12T35S mutants fail to activate Raf, yet retain the ability to interact with Raf. In this report, we showed that RasV12T35S 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 Rac 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.

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 MAPKKKK activation, such as Raf, is not fully understood (6).

One of the key events in Raf activation is growth factor-induced association with Raf (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
Activation of Raf

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\(^{509}\) 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 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.

**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. Human embryonic kidney 293 cells, COS-7 cells, and HeLa cells were transfected with plasmids prepared as described previously (26). The small GTPases (Rac1, Cdc42, and RhoA, all in the Myc-pRK5) and Raf-myc-pRK5 constructs were added 24 h following transfection. Cells were washed once with phosphate-buffered saline and then lysed in immunoprecipitation buffer containing 1% SDS, 0.5% deoxycholate, 50 mM NaCl, 1 mM dithiothreitol, and 0.5 mM EDTA. Kinase activities for ERK were measured as described previously (16). GST-Elk-1 (containing the N-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 500 ng of GST-SEK S220E/T224D or 250 ng of GST-SEK S220A/T224L mutants. The transfected 293 cells were starved for 12 h before being stimulated with 20 \(\mu\)g/ml anisomycin for 30 min. Then the cells were lysed and analyzed on a 10% polyacrylamide gel that was chemically fixed and dried. Proteins were transferred to a nitrocellulose membrane and probed with specific antibodies. The membranes were then incubated with 125\(^{I}\) labeled goat anti-rabbit antibodies and visualized by autoradiography. The amount of phosphorylated Elk-1 was quantitated by phosphorimage analysis.

**Activation of Raf**—Raf is a serine/threonine kinase expressed in response to growth factors and mitogens. Raf regulates the activity of the MAP kinase cascade by phosphorylating and activating MEK and ERK kinases. 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 Vitro Binding**—GST-PakRBD (Rac1-binding domain) and GST-Rock RBD were made and purified from E. coli. HEK 293 cells were transfected with wild type Myc-Rac1 (250 ng) and wild type RhoA (250 ng) with GST-PakRBD and GST-Rock RBD as baits and subjected to SDS-PAGE. Protein was detected by Western blotting with specific antibodies. The amount of phosphorylated Elk-1 from GST-PakRBD and GST-Rock RBD was quantitated by phosphorimage analysis.
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 mm 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.

Table I

### Activation of Raf

**Ras effector domain mutants in Raf binding and activation**

| Ras mutant | Raf binding activity | Raf activation activity |
|------------|----------------------|------------------------|
| RasV12     | +++++                | +++++                  |
| RasN17     | +++++                | -                      |
| RasV12Y32F | +++++                | +/-                    |
| RasV12T35S | +++++                | +/-                    |
| RasV12E37G | -                    | -                      |
| RacL61     | -                    | +/-                    |
| RhoAL63    | -                    | +/-                    |

#### 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. 1A). 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. 1B). 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 RasV12Y45E 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 GT-Pases (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 (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. 2A, lane 2). However, RacL61 could cooperate with RasV12Y32F or RasV12T35S to activate Raf kinase activity (Fig. 2A, lanes 3 and 5). In contrast, RacL61 was ineffective to cooperate with the RasV12E37G and no cooperation was observed between RacL61 and RacV12Y40C mutant (Fig. 2A, 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. 2A, 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
results indicate that Cdc42 acts similarly to Rac in cooperation with the RasV12Y32F and T35S mutants to stimulate ERK activity (Fig. 2C, 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. 2C, lane 6). Surprisingly, co-expression of RhoAL63 with the Ras effector domain mutants enhanced ERK activation (Fig. 2C, 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. 2D) and COS-7 cells (Fig. 2E). 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...
RhoAN19 are indicated. Raf activity was assayed (as in Fig. 2) in the presence or absence of RasV12. Co-transfections of RacN17 or RhoAN19 blocked Ras-dependent Raf activation. FLAG-Raf was transfected into HEK293 cells with dominant negative mutants. The dominant negative RacN17 or RhoAN19 blocked Ras-induced ERK activation. Expression of dominant negative RacN17 or RhoAN19 blocked Raf-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).

![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. 2A). 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 activity. 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).](Image 108x462 to 239x730)

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. 4C indicate that RacL61-F37G and RacL61-Y40C are compromised in the ability to cooperate with RasV12Y32F to stimulate Raf activity (Fig. 4B, lanes 7 and 8). Interestingly, similar mutations were defective in stimulating Raf 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 Raf/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 RafCAAX, which contains the C-terminal membrane targeting CAAX sequence of Ras. As previously reported, RafCAAX displays a much higher basal activity than the wild type Raf (Fig. 5A) (32). Interestingly, RacL61 alone could stimulate RafCAAX activity while under the same conditions no stimulation was observed with the wild type Raf (Fig. 5A). Similar experiments were performed with the active RhoAL63 mutant, which also activated RafCAAX (Fig. 5B). 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. 6A, lane 7). Co-expression of Pak-(165–544) and RasV12Y32F resulted in cooperation 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. 6A, 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. 6A, lane 17). In contrast, the kinase inactive Pak had no effect on the ability of RhoAL63 to enhance Raf activation (Fig. 6A, lane 18). These results support that Pak mediates the effects of Cdc42/Rac, but plays no significant role in RhoA to activate Raf. Neither wild type Pak nor RhoAL61 alone can activate Raf. Surprisingly, co-expression of RacL61 or Cdc42 and Pak resulted in significant activation of Raf (Fig. 6A, lanes 3 and 4). In contrast, co-expression of RhoAL63 and Pak failed to activate Raf (Fig. 6A, 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. 6B, 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...
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. 6C), 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 activity (Fig. 6A) and further support the role of Pak in Raf activation. We also tested whether the JNK pathway plays 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. 6D). Consistently, SEK(AL), a dominant negative mutant, does not block ERK activation by RasV12Y32F and RhoAL63 (Fig. 6D). As positive controls, SEK(ED) activates JNK while SEK(AL) blocked JNK activation by anisomycin (Fig. 6E). 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 Ser338 by Pak plays an important role in Raf activation (27). We determined the phosphorylation status of Ser338 during Raf activation by RasV12Y32F and RacL61. Phosphorylation of Ser338 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 Ser338 phosphorylation in Raf activation. Expression of RacL61 significantly enhanced phosphorylation of Ser338 during Raf activation by RasV12Y32F and RhoAL63. These results are consistent with the observations that active Pak1 cooperates with RasV12Y32F to stimulate Raf activity (Fig. 6A) and further support the role of Pak in Raf activation. We also tested whether the JNK pathway plays 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. 6D). Consistently, SEK(AL), a dominant negative mutant, does not block ERK activation by RasV12Y32F and RhoAL63 (Fig. 6D). As positive controls, SEK(ED) activates JNK while SEK(AL) blocked JNK activation by anisomycin (Fig. 6E). These data clearly demonstrated that JNK plays no significant role in Raf activation by RasV12Y32F and RhoAL63.

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as RacL61 in Raf activation. These observations indicate that Ser338 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-Ser338 antibody, supporting the specificity of the antibody. Although RacL61 and Cdc42L61 stimulated phosphorylation of Ser338 to a similar or higher extent as RasV12 (Fig. 7B, 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 Ser338 is not quantitatively correlated with Raf activity, consistent with observations by others (43), and Ras may induce modifications of Raf in addition to Ser338. Consistent with this model, active Pak1 induced a significant Ser338 phosphorylation (Fig. 7C, lane 4) but did not activate Raf (Fig. 6A, lane 7). The Ser338 phosphorylation of C-Raf was dramatically enhanced by co-expression of Pak and RacL61 or Cdc42L61 (Fig. 7B, lanes 7 and 8). In contrast, RhoAL63 enhanced Ser338 phosphorylation weakly even in the presence of Pak (Fig. 7B, lane 9), further supporting that RhoA activates Raf through a mechanism dif-
different from Rac or Cdc42. However, Ser338 is required for Raf activation (Fig. 7D). The Raf S338A mutant cannot be activated by RasV12Y32F and RhoAL63 or RacL61 (Fig. 7D, lanes 5 and 6).

The effect of PKN1 and PRK2 on Ser338 phosphorylation of Raf was also investigated. Both active PKN1 and PRK2 failed to stimulate Ser338 phosphorylation while active Pak1 was very effective (Fig. 7C, lanes 4–7). The above data demonstrate that Ser338 phosphorylation is a likely target of Cdc42/Rac1 and Pak1 in Raf activation. Furthermore, phosphorylation of Ser338 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 Ser338. The Rho family GTPases may signal downstream of Ras and upstream of Raf via protein kinases, such as Pak that can directly phosphorylate Ser338. 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. 6A). Phosphorylation of Ser338 was high under both conditions (Fig. 7, A).

**FIG. 7.** A, RacL61 stimulates Ser338 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-Ser338 antibody (upper panel) or anti-Raf antibody (lower panel). S338A denotes mutant Raf in which the Ser338 was mutated to alanine (lanes 8 and 9). B, Rac/Cdc42 stimulates Pak to phosphorylate Ser338 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 Ser338 phosphorylation of Raf. FLAG-Raf was co-transfected with various plasmids as indicated. Phosphorylation of Ser338 (upper panel) and protein levels (lower panel) of Raf were detected as in panel A. D, Ser338 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.
A possible interpretation is that Rac induces additional phosphorylation of Raf besides at residue Ser338. 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 Ser338 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 Gβγ, which directly couples to the pheromone receptor, regulates Cdc42 activity possibly via the Cdc24 nucleotide exchange factor (46). Gβγ may also regulate Ste11 via other components such as the Ste5 scaffold protein, which interacts with Gβ (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 Ser338 of C-Raf.

Raf activation is more complex than simply Ras binding and phosphorylation of Ser338 by Pak. It is clear that phosphorylation of Ser338 is not the sole event in Raf activation because a direct quantitative correlation between Ser338 phosphorylation and Raf activity is lacking. For example, active Pak dramatically stimulates Ser338 phosphorylation while it is not sufficient to activate Raf. In addition, expression of Pak with Rac or Cdc42 induces Ser338 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 Ser338 phosphorylation than Rac. These observations indicate that other events, likely phosphorylation or dephosphorylation, must occur in order to achieve full Raf activation.
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