Microtubule Integrity Regulates Pak Leading to Ras-independent Activation of Raf-1

INSIGHTS INTO MECHANISMS OF Raf-1 ACTIVATION*

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Growth factors activate Raf-1 by engaging a complex program, which requires Ras binding, membrane recruitment, and phosphorylation of Raf-1. The present study employs the microtubule-depolymerizing drug nocodazole as an alternative approach to explore the mechanisms of Raf activation. Incubation of cells with nocodazole leads to activation of Pak1/2, kinases downstream of small GTPases Rac/Cdc42, which have been previously indicated to phosphorylate Raf-1 Ser338. Nocodazole-induced stimulation of Raf-1 is augmented by co-expression of small GTPases Rac/Cdc42 and Pak1/2. Dominant negative mutants of these proteins block activation of Raf-1 by nocodazole, but not by epidermal growth factor (EGF). Thus, our studies define Rac/Cdc42/Pak as a module upstream of Raf-1 during its activation by microtubule disruption. Although it is Ras-independent, nocodazole-induced activation of Raf-1 appears to involve the amino-terminal regulatory region in which the integrity of the Ras binding domain is required. Surprisingly, the Raf zinc finger mutation (C165S/C168S) causes a robust activation of Raf-1 by nocodazole, whereas it diminishes Ras-dependent activation of Raf-1. We also show that mutation of residues Ser338 to Ala or Tyr340-Tyr341 to Phe-Phe immediately amino-terminal to the catalytic domain abrogates activation of both the wild type and zinc finger mutant Raf by both EGF/β-12-Octadecanoylphorbol-13-acetate and nocodazole. Finally, an in vitro kinase assay demonstrates that the zinc finger mutant serves as a better substrate of Pak1 than the wild type Raf-1. Collectively, our results indicate that 1) the zinc finger exerts an inhibitory effect on Raf-1 activation, probably by preventing phosphorylation of Ser338; 2) such inhibition is first overcome by an unknown factor binding in place of Ras-GTP to the amino-terminal regulatory region in response to nocodazole; and 3) EGF and nocodazole utilize different kinases to phosphorylate Ser338, an event crucial for Raf activation.

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The proto-oncogene raf-1, first identified as a cellular counterpart of the oncogene v-raf, encodes a serine/threonine protein kinase. Raf-1 is ubiquitously expressed and plays an important role in cell proliferation and differentiation (1). The mechanism by which Raf-1 is activated by growth factors is still incompletely understood, although it is known to be preassembled as a complex with 14-3-3 and heat shock proteins 90/50 (2–6). It involves multiple steps including Ras-GTP binding, membrane recruitment, and phosphorylation.

Raf-1 consists of an amino-terminal regulatory domain and a carboxyl-terminal kinase domain. The amino-terminal moiety of Raf-1 exerts an inhibitory effect on the catalytic activity, since amino-terminal truncations lead to progressive increases in its transforming ability (7, 8). The amino-terminal regulatory region of Raf-1 contains a Ras binding domain (RBD) and a cysteine-rich zinc finger domain (CRD), both of which participate in binding to Ras. The first interaction engages Raf RBD ranging from aa 50 to 150 and the effector loop of Ras-GTP, which is essential for activation of Raf-1 (9–11). Raf CRD, located between aa 139 and 184, binds to an epitope involving Ras residues Asn26 and Val315 outside the effector loop in prenylated Ras in a GTP-independent manner (12–15). The strength of this second interaction is much lower than the first one (12, 13). Nevertheless, it is crucial for the formation of a productive complex, as the mutation of residues necessary for this interaction on either proteins abolishes their functions, such as the ability to transform cells (14) and to activate MAPK kinase (15). Thus, these studies suggest that growth factor-stimulated GTP charging of Ras initiates the association of the Raf RBD with the effector loop of Ras, which ensures the second productive interaction between the CRD and Ras.

Considerable evidence indicates that phosphorylation plays an important role in Raf activation. Incubation of Raf-1 activated in vivo with serine/threonine or tyrosine protein phosphatases leads to inactivation of Raf-1 (16–19). Indeed, a number of serine/threonine and tyrosine protein kinases have been implicated in the activation of Raf-1 (3). Raf-1 Ser259 and Ser261 are the major phosphorylation sites which are critical for Raf activation.
determinants for binding to 14-3-3 (20). It appears that binding of 14-3-3 to the amino-terminal site (SerP\(^{258}\)) plays an inhibitory role, whereas the binding to the carboxyl-terminal site (SerP\(^{621}\)) is indispensable for the Raf kinase activity (21–23).

Other crucial residues include Ser\(^{338}\), Ser\(^{341}\), whose mutation to alanine or phenylalanine severely inhibits Raf-1 activation and to aspartic acid or glutamic acid results in an increase in the basal Raf-1 activity (24–27). In B-Raf, the two residues corresponding to Raf-1 Tyr\(^{204}\) and Tyr\(^{207}\) are replaced by aspartic acids, which may account for the increased kinase activity (27).

p21-activated kinases (Paks), mammalian homologs of Ste20-like SerThr protein kinases, are activated by signals that increase the level of GTP-bound form of Rac and Cdc42 GTPases, although the GTPase-independent pathway also exists (28, 29). The Pak family, consisting of Pak1 (Pako), Pak2 (Pak\(\gamma\), Pak3 (Pak\(\beta\)), and Pak4, has been implicated in a variety of cellular functions, including regulation of cell proliferation, apoptosis, the cell cycle, stress response, oxidant generation, cell adherence and motility, and cytoskeletal dynamics. Pak1 participates in V12Ras-induced transformation (30, 31) and cooperates with Raf-1, leading to a maximal activation of MEK1 by phosphorylating the latter (32). Recent data reveal that Pak2 can phosphorylate Ser\(^{358}\) (33) and contribute to Raf-1 activation by V12Ras or a constitutively active mutant of phosphatidylinositol 3-kinase (34). In addition, integrin-induced activation of Raf-1 has been shown to be mediated by Pak1 (35).

The requirement of multiple factors for receptor tyrosine kinase-stimulated activation of Raf-1 has greatly challenged us in precise elucidation of its regulation. Recent studies on Raf-1 activation by disrupting microtubule integrity that can elude the Ras binding and membrane recruitment may shed a light on uncovering this mystery (36, 37). We have shown that nocodazole, a microtubule-depolymerizing drug, activates Raf-1 and increases its binding to 14-3-3, while inducing mitosis and hyperphosphorylation of Raf-1 (38). Furthermore, the activation of Raf-1 is necessary for entry of the cell cycle into mitosis (38). In the present study, we have characterized this Ras-independent, nocodazole-induced activation of Raf-1. Here we first show that nocodazole utilizes Rac/Cdc42/Pak to activate Raf-1, in which a crucial step is the phosphorylation of Ser\(^{358}\) by Pak, while EGF activates Raf-1 by a different Ser\(^{358}\) kinase. We also find that, although Raf-1 activation by nocodazole is Ras-independent, the integrity of the Raf RBD, but not the CRD, is still required. Moreover, mutation of Cys\(^{165}\), Cys\(^{168}\) to Ser-Ser within the CRD causes a robust activation of Raf-1 by nocodazole and an increased ability of Raf to be phosphorylated in vitro by Pak, suggesting that the zinc finger structure plays an inhibitory role in Raf activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Nocodazole and 4\(\beta\)-12-O-tetradecanoylphorlan-13-acetate (TPA) were purchased from Sigma. Human recombinant epidermal growth factor (EGF) was from Calbiochem (San Diego, CA). Glutathione (GSH)-Sepharose 4B was purchased from Amersham Pharmacia Biotech. Monoclonal antibody against Raf-1 (E10), monoclonal antibody against GST (B14), and horseradish peroxidase-conjugated second antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against Raf-1 (E10), monoclonal antibody against GST (B14), and horseradish peroxidase-conjugated second antibody were from Santa Cruz Biotechnology (Beverly, MA).

**DNA Construction**—cDNAs encoding Raf-1 variants, wild type, Ras binding site mutant (84–87AAA), zinc finger mutant (165S/168S) and kinase-dead mutant (K375M) were constructed into pMT2Myc as described previously (15). cDNAs for wild type Raf, amino-terminally truncated kinase domain (BXXRaf, aa 1–25/303–648) (39), amino-terminus regulatory region (C4, aa 1–259) (40), and Pak1 variants were inserted into pEGK (40). cDNA for Raf-1 84–87AAA was also inserted into pBMPFPKS in which Src myristoylation sequence was tagged to the amino terminus of three copies of FKBP followed by the cDNA encoding a hemagglutinin epitope and the Raf mutant (41). Double site mutants, 165S/168S-380A/339A and 165S/168S-340F/341F, were made by replacing the wild type Ser fragment in pMTMyc-Raf 165S/168S

**Raf-1 Kinase Assay**—Raf-1 kinase activity was measured by a coupled enzyme assay in which bacterially expressed recombinant GST-MEK1 and kinase-dead mutant of ERK2 were sequentially added to the Raf preparation in the presence of [\(^{32}\)P]ATP (100 \(\mu\)M, 2000 cpm/pmol), as described previously (40). The reaction was stopped by the addition of a SDS-PAGE sample buffer, and the labeled mixture was resolved by 8\% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and visualized by autoradiography. The radiolabeled Erk2 bands were excised and quantified by liquid scintillation counting.

**Pak Kinase Assay**—Recombinant GST-Pak1 and Myc-tagged Pak2 were transiently expressed in HEK293T cells. After stimulation with 300 nm nocodazole for 1 h, cell lysates were prepared as described above. GST-Pak1 was purified by GSH beads and Myc-Pak2 immunoprecipitated with anti-Myc antibody (9E10). Pak activity was analyzed at 30 °C for 30 min using 1 \(\mu\)g of myelin basic protein (MBP) as a direct substrate. For in vitro phosphorylation of Raf-1, recombinant GST-Pak1 was purified by GSH beads and eluted with 5 mM GSH. The immunoprecipitated Myc-Raf-1 was incubated with Pak1 and ATP. The phosphorylation was visualized by anti-SerP\(^{358}\) blotting (27).

**RESULTS**

**Nocodazole Activates Raf-1 through Ras-independent Mechanism**—Recently, we have shown that nocodazole activates Raf-1 in a time-dependent manner and such activation is necessary for transition of the cell cycle from G\(_2\) to M phase (38). To further study the mechanism of nocodazole-induced Raf activation, we first determined the dose effect of nocodazole on activation of Raf-1. In doing so, cDNAs encoding the wild type and the kinase-defective mutant (K375M) of Raf-1 were transiently expressed in HEK293T cells, and Raf-1 kinase activity was assayed after cells were treated with nocodazole in different doses as indicated in Fig. 1A. Raf-1 was progressively activated by increasing doses of nocodazole with a maximal effect at 300 \(\mu\)M under the condition of equal expression of the polypeptides. The inability of the kinase-defective mutant Raf
Wild type (WT) were treated with or without nocodazole (300 nM) for 1 h or EGF (20 ng/ml) for 10 min. Raf-1 was co-expressed with a dominant negative mutant of Ras, N17Ras, and empty vector was transfected into HEK293T cells. After serum starvation, cells were treated with or without increasing doses of nocodazole (NZ) for 1 h as indicated or with EGF (20 ng/ml) for 10 min. Raf-1 was immunoprecipitated with anti-Myc antibody (9E10), and the kinase activity was assayed as described under “Experimental Procedures.”

32P incorporation into Erk bands after autoradiography, and immunoblotting was carried out using Raf antibody (E10). Upper part represents results of three experiments (means ± S.E.) expressed as fold of the wild type basal activity, and lower part shows an example of Raf-1 blots after immunoprecipitation. B, Ras-independent activation of Raf-1 by nocodazole. Myc-Raf-1 was co-expressed with a dominant negative mutant of Ras, N17Ras, or amino-terminal regulatory domain C4, GST-RafC4 in COS7 cells. Cells were treated with or without nocodazole (300 nM) for 1 h or EGF (20 ng/ml) for 10 min, and Raf kinase was assayed as in A. The bottom panel shows Myc-Raf-1 immunoblot after immunoprecipitation. This figure represents one of three independent experiments.

Fig. 1. Nocodazole stimulates Raf-1 kinase activity in a Ras-independent mechanism. A, activation of Raf-1 kinase by nocodazole. Wild type (WT) Raf-1 and kinase-dead mutant Raf K375M (KD) in pMT-Myc and empty vector were transfected into HEK293T cells. After serum starvation, cells were treated with or without increasing doses of nocodazole (NZ) for 1 h as indicated or with EGF (20 ng/ml) for 10 min. Raf-1 was immunoprecipitated with anti-Myc antibody (9E10), and the kinase activity was assayed as described under “Experimental Procedures.” 32P incorporation into Erk2 was quantified by liquid scintillation counting of the Erk bands after autoradiography, and immunoblotting was carried out using Raf antibody (E10). Upper part represents results of three experiments (means ± S.E.) expressed as fold of the wild type basal activity, and lower part shows an example of Raf-1 blots after immunoprecipitation. B, Ras-independent activation of Raf-1 by nocodazole. Myc-Raf-1 was co-expressed with a dominant negative mutant of Ras, N17Ras, or amino-terminal regulatory domain C4, GST-RafC4 in COS7 cells. Cells were treated with or without nocodazole (300 nM) for 1 h or EGF (20 ng/ml) for 10 min, and Raf kinase was assayed as in A. The bottom panel shows Myc-Raf-1 immunoblot after immunoprecipitation. This figure represents one of three independent experiments.

to be activated indicated that the nocodazole-increased kinase activity was not due to kinases copurifying with Raf-1. The dose-dependent activation of Raf-1 is consistent with the ability of nocodazole to disrupt the microtubule structure (data not shown). To differentiate nocodazole-induced activation of Raf-1 from the Ras-dependent one by EGF, Myc-tagged Raf-1 was co-expressed with RafC4 (aa 1–250), the amino-terminal regulatory region containing RBD and CRD, or N17HaRas, a dominant negative mutant of Ras. This experiment was done in COS7 cells since this cell line exhibits better response to EGF than HEK293T cells. As shown in Fig. 1B, co-expression of C4 or N17Ras reproducibly inhibited EGF activation of Raf-1 by about 40%, which is similar to our previous findings (41). In contrast, these mutants did not have significant effect on Raf-1 activation by nocodazole, despite the fact that the immunoblot showed equal expression of C4 or N17Ras under these two treatments (data not shown). Interestingly, we observed a 40% increase in Raf activation by nocodazole in the presence of N17 Ras, as compared with that in its absence (Fig. 1B, lanes 1 and 3). Overall, our results indicate that nocodazole up-regulates Raf kinase via a Ras-independent mechanism, which is consistent with previous publications (36, 37).

Rac/Cdc42 GTPases and Pak Participate in Raf-1 Activation by Nocodazole—Since Ser338 has been previously shown to be phosphorylated by Pak (33–35), we attempted to evaluate the role of Rac/Cdc42 and Pak in nocodazole-induced Raf-1 activation. In the first assay, we introduced GST-Pak1 and Myc-Pak2 into HEK293T cells and examined activation of these kinases by nocodazole (Fig. 2A). The recombinant Pak1 was activated by 3-fold, whereas Pak2 activity was increased by about 50%. The lesser activation of Pak2 might be due to its high basal activity or partial activation caused by the basic substrate MBP (49).

In the next experiment, a constitutively active mutant of Rac, V12Rac was co-expressed with the wild type Raf and its activation was examined after treatment of cells with nocodazole. Coexpression of V12Rac led to about 2.5-fold increase in basal kinase activity of Raf (Fig. 2B, lanes 1 and 4) and more potent activation by nocodazole so that the Raf activity was increased by an additional 7-fold (Fig. 2B, lanes 1, 4, and 5). Similar activation profiles were obtained by expression of a constitutively active mutant of Cdc42, Pak1, and Pak2 (Fig. 2, C and D).

To further establish the role of Rac/Cdc42/Pak pathway in activation of Raf-1 by disrupting the microtubule integrity, we next co-transfected Raf with a dominant negative mutant of Rac, N17Rac, or a kinase-defective mutant of Pak2. Fig. 3 shows that, whereas the mutants had no effect on EGF-induced activation of Raf-1, they strongly inhibited Raf-1 activation by nocodazole. The same inhibitory effect was also achieved by co-expression of dominant negative mutants of Cdc42 and Pak1 with Raf-1 (data not shown). These results clearly place the Rac/Cdc42/Pak lineage as an upstream module in the activation of Raf-1 by microtubule depolymerization and suggest that growth factor-dependent activation of Raf-1 occurs through different regulators.

To evaluate the role of Ser338, Ser339 and Tyr340, Tyr341 in nocodazole-induced activation of Raf-1, we transfected Raf mutants 338A/339A and 340F/341F into COS7 cells and examined their kinase activities, as compared with the wild type Raf-1 (Fig. 4A). Both mutations greatly inhibited the activation of Raf-1 by EGF and nocodazole. Additionally, co-expression of Pak was without effect on Raf-1 activity if Ser338 was mutated to Ala (Fig. 4B). Thus, these results suggest that phosphorylation of this region is critical to both Ras-dependent and independent activation of Raf-1.

To verify whether Raf residue Ser338 is phosphorylated during Raf activation, endogenous Raf-1 was immunoprecipitated and blotted with phospho-Ser338 antibody. Fig. 5A shows that the phosphorylation of Ser338 was enhanced by both TPA and nocodazole. When Raf-1 was co-expressed with the active mutant of Pak2, Raf Ser338 was highly phosphorylated (Fig. 5B), which held with its stimulatory role in Raf-1 activation by nocodazole (Figs. 2 and 3). The phosphorylation of Raf-1 by Pak seemed to be site-specific, as the phosphorylation of the 14-3-3 binding sites was not altered under the same conditions (Fig. 5B). To ascertain whether the phosphorylation and activation of Raf-1 by nocodazole is a specific cellular event, we examined the phosphorylation of Akt Ser473, an indicator for its activation. Fig. 5C shows that phosphorylation of Akt Ser473 was not changed in HEK 293T cells treated with nocodazole and TPA, while MAPK/Erk was significantly activated. Another piece of experimental evidence was the failure of nocodazole to activate cAMP-dependent protein kinase (data not shown).
Distinct Requirement of RBD and CRD for Raf Activation by Both Ras-dependent and -independent Mechanisms—To assess whether the amino-terminal moiety is necessary for nocodazole-induced Raf-1 activation, we compared the activation of full-length Raf-1 and BXB-Raf-1 containing the entire carboxyl-terminal kinase domain. Fig. 6A shows that the full-length Raf-1 was activated well by both EGF and nocodazole, whereas the activity of BXB-Raf-1 was barely affected by these agents. The same results were obtained by using both HEK293T and COS7 cells. Thus, our findings demonstrate that the amino-terminal regulatory domain encompassing the Ras binding site is also necessary for Ras-independent activation of Raf-1.

We next tested whether RBD is required for the action of nocodazole. To this regard, Raf-1 with the 84KLAK87 to AAAA mutation in the RBD was assayed for its kinase activity. Although the wild type Raf-1 was activated normally by both TPA and nocodazole, the mutation blunted its activation (Fig. 6B), even in the presence of co-expressed Pak1 (Fig. 6C). To ascertain whether the inability of this mutant to respond to nocodazole was due to the propagated misfolding of the catalytic domain engendered by the mutation, we engineered a cDNA expressing a fusion protein (see "Experimental Procedures") containing Raf 84–87AAAA tagged by the Src myristoylation sequence, which enables Raf to be constitutively targeted to the plasma membrane. When this mutant was expressed in cells (Fig. 6D), its activity was dramatically stimulated by nocodazole, indicating that mutation has not altered the conformation of the catalytic domain. Therefore, we conclude that RBD is required for both Ras-dependent and independent activation of Raf.

In addition to Ras binding, the inhibitory role of the zinc finger in Raf activation was uncovered by the ability of the zinc finger mutant Raf to be potently activated by nocodazole. When C165S/C168S mutant was expressed in HEK 293T cells, the response of the mutant to TPA and nocodazole was different. Whereas the activation of the mutant by TPA was decreased (Fig. 7A, columns 2 and 5; \(p < 0.01\)), nocodazole caused a marked increase in the activity of the mutant Raf which was 74% greater than the wild type Raf (Fig. 7A, columns 3 and 6; \(p < 0.01\)). Similar results were obtained in COS7 cells by...
comparing nocodazole with EGF and TPA. The results again demonstrate that nocodazole and EGF/TPA activate Raf-1 through different mechanisms, and the integrity of CRD is necessary for Ras-dependent, but not for Ras-independent activation of Raf-1. They also suggest that the zinc finger plays an inhibitory role in Raf activation.

According to the observation that mutation of the CRD resulted in an increased response of Raf to nocodazole and while the S338A/339A or Y340F/Y341F mutation severely impaired Raf activation, we attempted to explore the interrelationship between these two sites by considering two possibilities. First, the CRD exerts its inhibitory effect on the catalytic domain and such inhibition could be counteracted by phosphorylation of Ser338/Ser339 or Tyr 340/Tyr341. If so, phosphorylation of this region would not be necessary for the activation of the CRD mutant. To test this, we made double site mutations, S338A/339A or Y340F/Y341F. The result in Fig. 7B revealed that the double site mutants were not activated at all, in contrast to the control, Raf-1 165S/168S, which was greatly stimulated by nocodazole. This suggests that the outcome of phosphorylating these residues is not primarily to relieve the inhibition of catalytic function of Raf-1 imposed by...
CRD and instead is required for the secondary step for Raf activation. Second, the zinc finger might inhibit phosphorylation of SSYY341, which is necessary for the next or final step for Raf activation. To test this hypothesis, in vitro phosphorylation of Raf-1 variants, wild type, 165S/168S, and 165S/168S/338A was performed by incubation with recombinant Pak1. As shown in Fig. 8, the wild type Raf was moderately phosphorylated by Pak1, while phosphorylation of the zinc finger mutant was 40% higher than that of the wild type Raf (phospho-signal/Raf-1 polypeptide determined by scan densitometry, $p = 0.05$). However, the Raf kinase activity was not correspondingly altered after phosphorylation in vitro (data not shown), suggesting that phosphorylation of Ser 338 is not the final step, albeit necessary, for Raf activation.

**DISCUSSION**

Recent studies have demonstrated that such small GTPases as Ki-Ras and Rac associate with microtubules (43, 44), and a novel guanine nucleotide exchange factor specific for Rac and RhoA has been isolated and documented to bind to microtubules (45). However, the biological significance of these associations has yet to be determined. We are the first to show here that disrupting microtubules activates Pak and hence leads to an activation of Raf-1. Thus, our studies provide new evidence for the link between the functionally undefined associations of microtubules with the small GTPases and Raf/MEK/MAPK pathway as well as microtubule dynamics. An important and challenging task in this line of research will be to elucidate the

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**FIG. 6.** The aminoterminal regulatory region is required for nocodazole (NZ) activation of Raf-1. A, nocodazole activates the full-length but not the carboxyl kinase domain of Raf-1. Full-length and the kinase domain of Raf-1 were expressed as GST fusion proteins in HEK293T cells and purified with GSH-beads. Kinase activity was assayed after cells were incubated with EGF or nocodazole as in Fig. 1. The bottom panel shows Raf immunoblot with crude extracts. B, mutation of Ras-binding domain abolishes nocodazole activation of Raf-1. HEK293T cells expressing Myc-Raf variants, wild type (WT), and the Ras binding site mutant 84–87AAA, were stimulated with or without TPA or nocodazole. The Raf immunoprecipitates were assayed for the kinase activity and immunoblotted with E10. C, mutation of the Ras binding domain abrogates the effect of Pak1 on nocodazole activation of Raf-1. Raf kinase activity was assayed from HEK293T cells co-expressing Myc-Raf-1 or Myc-Raf 84–87AAA with wild type GST-Pak1. After autoradiography, Raf-1 was blotted with E10. D, myristoylated Raf mutant 84–87AAA in pBJMFPK3E was transfected into HEK293T cells. Recombinant Raf was immunoprecipitated with anti-HA antibody (12CA5) and assayed for the kinase activity after cell treatment as indicated. Relative levels of Raf-1 in the immunoprecipitates were assessed by anti-HA blot. Raf activity (top) was expressed as in Fig. 3. This figure represents one of three independent experiments.

**FIG. 7.** Role of the zinc finger in Raf-1 activation. A, the zinc finger mutant responds to TPA and nocodazole differently. Wild type (WT) Raf and the zinc finger mutant Raf 165S/168S were transiently expressed in HEK 293T cells. Cell extracts containing equal amount of the Raf polypeptide were used for immunoprecipitation and kinase assay. Raf specific activity was expressed as percentage of the wild type stimulated with nocodazole (NZ). Results represent mean ± S.E. of five independent experiments. B, mutation of Ser338-Ser339 to Ala-Ala or Tyr340-Tyr341 to Phe-Phe prevents activation of Raf 165S/168S by nocodazole. cDNAs encoding Raf mutants, 165S/168S, 165S/168S-338A/339A, and 165S/168S-340F/341F, were transfected into HEK293T cells. Raf kinase activity was assayed after immunoprecipitation of recombinant Rafs from cells treated with TPA or nocodazole or untreated. These data represent one of three independent experiments.
mechanism by which Raf-1 is regulated by microtubule integrity.

The complexity of the interrelationship between microtubules and intracellular signaling events has been indicated recently (46). Microtubules can act as a scaffold for relay of signals from cell surface to nucleus or to other intracellular compartments or sequestering the bound signaling modules to repress their functions. Conversely, intracellular signaling events also regulate the dynamic balance between polymerization and depolymerization of microtubules. The application of the microtubule-interfering drugs has advanced our understanding of the role of microtubules in intracellular signaling events. However, it also raises the concern about the specificity of these drugs and correlation of their effects with the microtubule integrity, since they influence so many signaling molecules and some of these events are redundant. This question will be fully understood only when the mechanism by which the individual signaling pathway is regulated by different drugs is elucidated. Nonetheless, existing evidence indicates that different microtubule-interfering drugs exert their effects specifically and differently. 1) Paclitaxel, a microtubule-polymerizing drug, activates JNK/SAPK only in cells when its binding site in microtubules is intact (47). 2) Vincristine, another microtubule-depolymerizing drug, and paclitaxel promote apoptosis of tumor cells by inducing hyperphosphorylation of the antiapoptotic protein Bcl2, an event mediated by cAMP-dependent protein kinase (48). In contrast, nocodazole is not proapoptotic and fails to activate cAMP-dependent protein kinase (Ref. 48; data not shown). 3) Vincristine and paclitaxel have been shown to stimulate Ras binding to GTP, one of the avenues leading to activation of JNK/SAPK pathway (47), whereas our current data indicate that nocodazole activates Raf-1 through a Ras-independent mechanism. 4) The present results indicate that the phosphorylation of Akt is not stimulated by nocodazole treatment.

The mechanism by which the zinc finger structure regulates Raf-1 activation is still poorly defined. In addition to binding to processed Ras (15), the CRD interacts with 14-3-3 (49) and phosphatidylserine (50). Several recent studies suggest that it imposes an inhibition on the catalytic domain (49, 51, 52), probably through an intramolecular interaction (50). Thus, it is rational to propose that Ras-GTP plays dual role in Raf activation, on the one hand, by recruiting Raf to the plasma membrane to allow additional modifications, and on the other hand, by interacting with the Raf RBD and CRD such that the tight structure of Raf is held open and Raf then becomes accessible to other regulators such as kinases. It is difficult to dissect these processes by using mammalian cells, however, mainly for two reasons. First, the loss-of-function mutation of CRD sequesters Raf in the cytosol by disrupting its interaction with prenylated Ras, separating it from a growth factor-regulated kinase in the plasma membrane. As a result, the activation of Raf is inhibited, even though the mutant Raf-1 might serve as a better substrate for the kinase. Second, it is almost impossible to achieve the trans-inhibition of Raf catalytic domain by the amino-terminal regulatory region when they are both co-expressed in mammalian cells, as the expression level cannot be manipulated to be as great as that in Xenopus oocytes by microinjection (51). Our current study employs the approach to disrupt the microtubule integrity to bypass the requirement of Ras and the membrane recruitment for Raf activation and demonstrates that the C165S/C168S mutation does not cause a significant change in the basal kinase activity, but yields a more robust activation of Raf-1 by nocodazole (more than 74% increase above the wild type level on average) (Fig. 7A), suggesting that the CRD inhibits Raf activation. This argument is strengthened by the finding that the ability of the Raf to be phosphorylated by Pak was increased by the zinc finger mutation (Fig. 8).

In the present study, we find that mutation of \(^{84}KALK^{87}\) to AAAA in RBD inhibits Raf activation by nocodazole (Fig. 6), similar to its response to EGF (15). Our results also show that, in contrast to the full-length Raf-1, the catalytic kinase domain is not activated by nocodazole, suggesting that the amino-terminal regulatory region is required for Raf activation by nocodazole. However, overexpression of the amino-terminal regulatory region does not affect nocodazole activation of Raf-1 as it does the EGF-induced activation, implying a weak interaction between the amino-terminal regulatory region with the nocodazole-induced activator such that the inhibition is effective only when it is overwhelmingly expressed.

We have noticed that our results differ somewhat from previous publications (35, 36). The authors showed that another Ras binding site mutant, Raf R89L, was activated by fourteen-hour nocodazole treatment (35), whereas activation of Raf 165S/168S was inhibited (36). Two factors may account for the discrepancy. First, the difference may be attributed to nocodazole incubation time. We have observed that Raf-1 activity reaches maximum after brief (1–3 h) exposure to nocodazole and decreases with extended incubation, followed by hyperphosphorylation and increased ability to bind to 14-3-3 (38). Thus, Raf may be regulated differently after prolonged incubation with nocodazole. Second, it is possible that mutations of Raf \(^{84}KALK^{87}\) to AAAA and Arg\(^{89}\) to Leu might have different effects, which can be distinguished by nocodazole, even though...
both the mutations impede Ras-dependent activation of Raf-1.

Our study establishes Rac/Cdc42/Pak as an upstream module for Raf-1 activation in association with the integrity of microtubules, but not for EGF activation of Raf. A study by Frost et al. (32) has demonstrated that Pak1 can phosphorylate MEK1, thereby enhancing MEK binding to Raf-1 and consequently its ability to be phosphorylated by Raf-1. However, dominant negative mutants of Pak and Rac has no effect on EGF activation of MAPK in their studies, suggesting that Rac and Pak1 do not operate upstream of MAPK in the EGF pathway. Investigations by other laboratories suggest that Rac/Cdc42/Pak could serve as an upstream regulator for Raf under certain circumstances (30, 31, 33–35). The inability of Pak to mediate EGF in activation of Raf implies that EGF recruits a different kinase to phosphorylate Ser338 of Raf-1 during its activation. In keeping with this, Mason et al. (27) have shown that mutation of Raf Tyr341 to Ala does not affect phosphorylation of Ser338 in response to EGF and TPA, but it abolished the Ser338 phosphorylation induced by expression of constitutively active mutants of Ras and Src. This argues that the Raf Ser338 kinase in response to EGF and TPA is different from that regulated by constitutively expressed V12Ras and v-Src. The latter may be Paks. Although many studies including ours indicate that Pak participates in Raf activation by phosphorylating Ser338, our results demonstrate that in vitro phosphorylation of Raf Ser338 by Pak1 is not as impressive as the in vivo study (Figs. 5B and 8; in vivo data for Pak1 not shown). This difference could be attributed to two reasons; one is that efficient phosphorylation by Pak may depend on a scaffold protein, and another one is that the amino-terminal portion of Raf-1 inhibits its phosphorylation (e.g. by the zinc finger domain).

Although all reports agree that phosphorylation of Ser338 or Tyr341 is a critical step for Raf activation, it is not the final step, since (1) conversion of Ser338 or Tyr340-Tyr341 to acidic residues does not result in full activation of Raf-1, despite an increase in the basal activity and these mutants can be further activated by V12Ras and v-Src (24–27), (2) in vitro phosphorylation of Ser338 by Pak does not change Raf kinase activity (data not shown) and (3) co-expression of active mutant of Pak only leads to about 2-fold increase of Raf activity although phosphorylation of Ser338 is markedly stimulated (Fig. 5), while Raf activity is elevated by co-expression of v-Src by more than 20-fold, a level that can only be achieved by both active mutant of Pak2 and nocodazole treatment (data not shown).

Regarding the impact of phosphorylating Ser338/SSY341 on the Raf catalytic function, Cutler et al. (51) have shown that mutation of Raf Tyr340 to aspartic acid in the catalytic domain offsets the trans-inhibitory effect of CRD on its stimulation of germinal vesicle breakdown when the amino-terminal regulatory domain and catalytic domain are co-microinjected into Xenopus oocytes. Thus, it is possible that phosphorylation of Ser338/SSY341 is to de-repress the catalytic domain imposed by the CRD. The present results reveal that Raf 165S/168S becomes unresponsive to nocodazole when Ser338 is mutated to Ala or Tyr341-Tyr341 to Phe-Phe, implying that phosphorylation of this region is not solely to override the inhibition of the catalytic domain by CRD, but is rather required for secondary steps in Raf activation. Therefore, based on our data and others, we postulate as depicted in Fig. 9 that Raf is activated through the following sequence: (a) binding of a modulator (e.g. Ras-GTP at the plasma membrane or X factor in the cytoplasm in response to microtubule depolymerization) to the amino-terminal regulatory region changes Raf conformation so as to overcome the inhibition by CRD, which sets the next step in motion; (b) phosphorylation of the Ser338/SSY341 region by kinases such as Pak or Src family provides a prerequisite for additional modifications, that is; (c) phosphorylation of other sites locks Raf in an active state.

In summary, a key event in mitogenic signaling is the activation of Raf-1. This is a complex process that requires participation of Ras-GTP, 14-3-3, and kinases (such as Pak, Src, protein kinase C, KSR, and/or yet unidentified kinases) that directly phosphorylate Raf-1(3). However, it is still not clear how these factors act in concert to activate Raf-1. The present study has employed nocodazole and EGF/TPA to evaluate the importance of the structural elements, the RBD, the CRD, and Ser338/SSY341, in Ras-dependent and independent activation of Raf-1. We find the RBD to be necessary for both Ras-dependent and independent activation of Raf-1. In contrast, mutation of the CRD results in nocodazole-stimulated Raf activity that was significantly higher than that of the wild type Raf-1, but the same mutation diminishes Ras-dependent activation of Raf-1. The results indicate that the zinc finger plays an inhibitory role in Raf activation, at least in part through inhibition of phosphorylation of Ser338. Our study also defines Rac/Cdc42/Pak as an upstream module for Raf-1 in response to microtubule depolymerization.

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