Phosphorylation of $^{338}$SSYY$^{341}$ Regulates Specific Interaction between Raf-1 and MEK1*

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The present study characterizes the interaction between the Raf-1 kinase domain and MEK1 and examines whether the magnitude of their interaction correlates to the ability of Raf to phosphorylate MEK1. Here we show that the minimal domain required for the Raf kinase activity starts from tryptophan 342. Maximal binding of the Raf kinase domain to MEK1 and its kinase activity are achieved upon phosphorylation of the region $^{338}$SSYY$^{341}$ in response to $\beta$-12-0-tetradecanoylphorbol-13-acetate (TPA), or mutation of Y340T341 to aspartic acids. Conversely, the TPA-stimulated MEK binding and kinase activity are diminished when this region is deleted or Ser$^{338}$ and Ser$^{339}$ are mutated to alanines. We also show that the integrity of the Raf ATP-binding site is necessary for the interaction between Raf-1 and MEK1. Furthermore, two MEK-binding sites are identified; the first is localized between amino acids 325 and 349, and the second is within the region between amino acids 350 and 648. Separately, the binding of each site to MEK1 is weak, but in a cis context, they give rise to a much stronger association, which can be further stimulated by TPA. Finally, we find that tryptophan 342, which is conserved among the Raf family and other protein kinases, is essential for the Ser$^{338}$ phosphorylation of the full-length Raf and its binding to MEK1. Taken together, our results indicate that the phosphorylation of Ser$^{338}$ and Tyr$^{341}$ on Raf exerts an important effect on reconfiguring the two MEK-binding sites. As a result, these two sites coordinate to form a high affinity MEK-binding epitope, leading to a marked increase in Raf kinase activity.

Raf-1 is a key enzyme in transition from a tyrosine phosphorylation event at the plasma membrane to serine/threonine phosphorylation in the mitogen-activated protein kinase pathway (1, 2). Activation of Raf-1 engages multiple concerted steps and factors, such as Ras binding, phosphorylation, and dimerization (1, 2). Phosphorylation plays both positive and negative roles in regulation of Raf-1 kinase. In the inactive state, Raf Ser$^{338}$ is phosphorylated and bound by 14-3-3, thereby restraining spontaneous activation of Raf-1. In response to extracellular mitogens, Ras-GTP binds to a domain on Raf-1 amino-terminal to Ser$^{259}$, which destabilizes the interaction between Ser(P)$^{259}$ and 14-3-3 and allows dephosphorylation of this site and phosphorylation of other sites on Raf-1 to accomplish the activation process (3–7). Recent studies have shown that activation of Akt causes an inhibition of Raf-1 kinase in specific cell contexts such as muscle cell hypertrophy and differentiated myotubes (8–11). This cross-regulation occurs through phosphorylation of Raf-1 Ser$^{259}$ in accompany with an increase in 14-3-3 binding. Similar regulation also applies to B-Raf (12).

Compelling evidence has demonstrated that phosphorylation of the carboxyl moiety of Raf kinase plays an essential role in Raf-1 activation. This includes phosphorylation of Thr$^{491}$, Ser$^{494}$, Ser$^{497}$, and Ser$^{499}$ (13, 14) in the activation loop of the catalytic domain. The regulation of Raf by protein kinase C impinges on both the amino and carboxyl termini (14–18). On the one hand, it activates Ras leading to subsequent membrane recruitment and activation of Raf-1 (16, 17); on the other hand, it directly phosphorylates Ser$^{497}$ and Ser$^{499}$, resulting in an increase in its kinase activity (14, 15, 18). Thus, when the kinase domain of Raf-1 is expressed independently of its amino-terminal regulatory region, in contrast to growth factors, TPA1 causes potent activation of Raf-1 (15, 19).

Another set of residues whose phosphorylation is critical for Raf activation is Ser$^{238}$ and Tyr$^{241}$ located in the junction region between the regulatory and kinase domains (20–24) that is referred to as the negative regulatory region by Mason et al. (21). γ-PAN, an isoform of p21-activated kinase, is the first kinase identified to phosphorylate RafSer$^{238}$ (23), although the site may be phosphorylated by different kinases depending on the specific stimulus (24). Likewise, the Src family is responsible for phosphorylation of Tyr$^{240}$, Tyr$^{241}$ (20, 24, 25). Mason et al. (21) have described an incidence in which the mutation of Tyr$^{241}$ to Phe abrogates Raf-1 activation despite normal phosphorylation of Ser$^{338}$. Together with the observation that mutation of Ser$^{338}$ to Ala abolishes Raf kinase activity, they suggest that phosphorylation of Ser$^{338}$ is necessary but not sufficient for Raf activation. We have demonstrated that disrupting microtubules activates Raf-1, which occurs through activation of Rac/Cdc42/Pak pathway (26). In vivo, Pak strongly stimulates phosphorylation of Raf-1 Ser$^{338}$ oncomitant with an increase in the Erk activation (27), which can be further boosted by other stimuli (26). The physical association between Pak1 and Raf-1 occurs under physiological conditions and depending on the activation state of Pak1 (27). Furthermore, the Raf-binding site has been localized to the carboxyl-terminal kinase domain of Pak1. All of these data suggest that Pak serves as a physiological blocker for the Raf kinase domain.

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upstream kinase phosphorylating Raf Ser338.

Although the role of phosphorylation of Ser338 has been well accepted in Raf activation, the mechanism by which it regulates Raf kinase activity has yet to be addressed. The present study attempts to evaluate whether the ability of Raf to associate with MEK1 is regulated via phosphorylation of Ser338 during Raf activation. We demonstrate that the magnitude of Raf-1 kinase activation closely correlates with its ability to bind to MEK. Furthermore, two MEK-binding sites are present in Raf-1, and the interaction of these two sites on the cis context with MEK are greatly enhanced in response to phosphorylation of Ser338.

EXPERIMENTAL PROCEDURES

Materials—48-12-O-tetradecanoyl-phorbol-13-acetate (TPA) and the anti-Myc monoclonal antibody 9E10.2 were purchased from Sigma. The anti-GST and Raf (E10) monoclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). GSH-Sepharose was purchased from Amersham Biosciences. The rat monoclonal antibody against Raf-1 Ser338 was a gift from R. Marais. The anti-EE epitope antibody was from Dr. A. Makkink. cDNA for Raf-1, tagged amino-terminally by a Myc epitope, was inserted into the pMT2 plasmid. The rat monoclonal antibody against Raf-1 Ser338 was a gift from R. Marais. The anti-EE epitope antibody was from Dr. A. Makkink.

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Minimal Sequence Requirement for Raf Kinase Activity—Phosphorylated Ser338 and/or Tyr341 are considered a key element for the catalytic function of Raf-1 kinase. Thus, the Raf mutants S338A and Y341F are regarded as kinase-defective. Because these residues are located outside the conserved kinase core domain, the present study attempts to define the minimal domain required for Raf-1 kinase activity and explore the relationship between the phosphorylation of Ser338 and Tyr341 and the minimal kinase domain, as reflected by the binding of Raf to MEK and its MEK kinase activity. To determine the functional unit of Raf-1 containing the catalytic activity, a series of deletion and point mutations were made to the kinase domain, as depicted in Fig. 1.
Previous studies demonstrated that the activity of the Raf catalytic domain devoid of the amino-terminal regulatory region could still be up-regulated by the treatment of cells with agents such as phorbol ester (15, 19). To compare the activity of the carboxyl-terminal kinase domain (amino acids 325–648) with the full-length Raf-1, the recombinant Raf variants, as indicated in Fig. 2, were expressed as GST fusion proteins in HEK 293T cells and purified by GSH affinity beads, and the kinase activity was assayed in vitro by sequential addition of MEK and Erk. The specific activity was calculated by dividing the cpm of 32P-Erk bands with the scan densitometric unit of Raf polypeptide blot (one of a duplicate Raf blots is shown at the bottom). The results represent the means ± S.E. of duplicate experiments. B, HEK 293 cells were transfected, and the cell extracts were blotted with antibodies as indicated. IB, immunoblot.

Next, the activation of endogenous Erk was examined. The results (Fig. 2B) revealed that without TPA treatment, the full-length Raf only modestly increased Erk phosphorylation, whereas the Raf kinase domain induced a potent Erk activation that was obviously severalfold higher and equal to the level at which Erk was aroused by TPA in the GST-transfected cells (lanes 1–3 and 5). In addition, TPA stimulated the Erk phosphorylation approximately to the same level in both the Raf full-length and carboxyl-terminal kinase domain-transfected cells (lane 4 and 6). Together, the results in Fig. 2 demonstrated that the basal activity of the Raf kinase domain in vivo was much greater than that of the full-length Raf, although the in vitro assay revealed that the amino-terminal truncation only caused a marginal increase in Raf kinase activity.

Because the activity of the Raf kinase domain is regulated well by TPA, the present study focuses on characterization of the Raf kinase domain. To determine the minimal sequence required for the catalytic activity of Raf, deletion was made to amino acids 350, and the activity of this mutant was examined. As shown in Fig. 3A, whereas the Raf kinase domain containing
residues 325–648, as a control, was potently activated by TPA, truncation to amino acids 350 caused a complete loss of Raf kinase activity. Although Raf 350/648 contained the entire catalytic domain (Gly338-Xaa-Gly-Xaa-Xaa-Gly) according to sequence alignment with cAMP-dependent protein kinase, we could not exclude the possibility that the truncation had altered the proper folding of the first β sheet, a structural tag to the catalytic domain, which is important for the catalytic function. Thus, another set of truncations was made, and the results revealed (Fig. 3B) that deletion of the amino-terminal 341 amino acids blunted TPA-induced activation. One more amino acid deletion essentially inhibited all of the Raf kinase activity. These findings suggest that 1) Trp342 is involved in acquisition of a proper conformation of Raf-1 and 2) the negative charge of the region encompassing 338SSYY341 is necessary for the maximal activation of Raf-1.

To further evaluate the role of the region 338SSYY341, we assessed the kinase activity of the Raf kinase domain variants, wild type, S338A/S339A, and Y340D/Y341D. As shown in Fig. 4A, mutation of Ser338 or Tyr341 with or without aspartic acid substitution, as had the mutant 325/648-Y340D/Y341D, which was about 60% that of wild type (Fig. 4A). Immunoblot of endogenous Erk with an anti-phospho-Erk antibody showed parallel changes. The polyvinylidene difluoride membranes were subsequently probed with anti-GST monoclonal antibody after the kinase assay. The blot represents one example of duplicate experiments. The luciferase activity was normalized by densitometric units of Raf blot. The results also displayed some interesting findings wherein Raf variants 325/648-S338A/S339A, 338/648, and 338D/648 still possessed substantial amounts of kinase activity (30–60% that of wild type), whereas they lost the kinase activity as assayed in vitro (Fig. 4, A and B).

**Fig. 4. Impact of the negative charge of Ser338-Tyr341 on the Raf kinase activity.** A, the Raf 325/648 variants, wild type, S338A/S339A, and Y340D/Y341D, were transfected and the kinase activity was assayed as described in the legend to Fig. 2. Raf kinase activity was expressed as fold of the basal activity of the wild type Raf. Immunoblotting was performed with an anti-GST monoclonal antibody after the kinase assay. The blot represents one example of duplicate experiments. B, different deletion mutants as indicated were analyzed as in A. The polyvinylidene difluoride membranes were subsequently probed using anti-GST and anti-Ser(P)338 antibodies after the kinase assay. C, effect of Raf mutants on Elk activation. HEK 293T cells were transfected with pFA2-Elk1, pFR-Luc, and pSEA P2, and Raf variants, as indicated. Forty-eight hours after transfection, the luciferase activity was assayed as described under “Experimental Procedures.” The luciferase activity was normalized by densitometric units of Raf blot. The results represent the means ± S.E. of triplicate experiments. The lower panel of C is an example of immunoblots of endogenous Erk and recombinant Raf variants. IB, immunoblot.
Binding of Raf-1 to MEK Correlates to Its Activation—A loss of kinase activity resulting from deletion or point mutations of the negative region of Raf-1 could be attributed to the impairment of the physical interaction between Raf and MEK. To test this hypothesis, we first co-transfected plasmids bearing a cDNA for EE-tagged MEK1 into HEK 293T cells with Raf constructs encoding 325/648, 342/648, or 343/648 and compared their abilities to bind to MEK. The results (Fig. 5 A) showed that the interaction between Raf 325/648 and MEK was greatly potentiated by TPA, consistent with the increase in the kinase activity. Deletion beyond residue 341 abrogated the response of Raf to TPA in binding to MEK, although the mutants exhibited a modestly increased binding at the basal level.

In another experiment, we assessed the binding of Raf 325/648, 325/648-S338A/S339A, and 325/648-Y340D/Y341D to MEK1 (Fig. 5 B). Conversion of Ser338-Ser339 to Ala-Ala severely impeded the association under both basal and TPA-stimulated conditions, whereas the double mutant Y340D/Y341D displayed the strongest binding. Again, these data were in agreement with the activity assays on these mutants, suggesting a mechanism by which phosphorylation of Ser338 or Tyr341 facilitates an optimal conformation of Raf, allowing maximal binding and phosphorylation of MEK1.

To determine whether binding of Raf to MEK requires its kinase activity, lysine 375, a crucial residue for ATP binding and phospho-transfer, was replaced by methionine in the Raf kinase domain that already had the Y340D/Y341D substitution. The binding was then examined for comparison with its active counterpart. To our surprise, the result shown in Fig. 5 C revealed that the binding of Raf Y340D/Y341D to MEK was almost completely wiped out by the Lys375 to Met mutation. This clearly indicates that the integrity of the Raf ATP-binding site is necessary for Raf binding to MEK.

Two Elements in the Carboxyl-terminal Domain of Raf-1 Bind to MEK—The necessity of the Raf sequence from amino acids 325 to 349 for both Raf binding to MEK1 and its kinase activity inspired us to ask whether an MEK-binding site is located in this region. Thus, this Raf segment was co-expressed with EE-MEK1 into HEK 293T cells. The complex was isolated with anti-EE antibody (A) or GSH beads (B). EE-MEK1 and GST-Raf were blotted in both precipitates and cell extracts. C, two sites cooperatively bind to MEK1 in response to TPA. EE-MEK1 was co-expressed with Raf variants and treated as indicated. The complex was purified with the anti-EE antibody and examined as in A. IP, immunoprecipitation. IB, immunoblot.
as a GST fusion protein with MEK, and the binding was evaluated by reciprocal purification and immunoblot, as compared with the GST control. The kinase activity was assayed as described in the legend to Fig. 2 and expressed as fold of basal activity of the wild type Raf. Aliquots of the immunoprecipitates were blotted with anti-Ser(P)338 or anti-Myc antibody. The bars represent the averages ± S.E.

Effect of Trp342 to Ala Mutation on the Full-length Raf-1—Studies by deletion mutations indicate that Trp342 is important for the activity of the Raf kinase domain and its binding to MEK. In evaluating the impact of Trp342 on the function of full-length Raf-1, the residue was first converted to alanine, and the response of the resultant mutant to TPA was examined. As shown in Fig. 7 (A and B), whereas the wild type Raf was activated by TPA (about 5-fold), as well as by active mutants of Ras and Src, the mutation of Trp342 to Ala disabled Raf activation, concomitant with a loss of the Ser338 phosphorylation. Alternatively, when Trp342 was replaced with histidine in the active mutant Raf-1 Y340D/Y341D, the kinase also became totally inactive (Fig. 7C).

To exclude the possibility that the W342A mutation induced a conformational change in overall structure of Raf-1, we evaluated the binding of the wild type and mutant Raf to the two well-characterized interacting proteins, Ras and 14-3-3, which

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are known to be important for active conformation of Raf-1. In this assay, Myc-Raf-1, wild type or W342A, was co-expressed with GST-Ki-Ras (V12) or GST-14-3-3, and the binding was examined. The result (Fig. 8A) demonstrated that these two Raf functional domains were not disturbed by the mutation. In fact, overexpression of this mutant kinase could compete with endogenous Raf for activators (e.g. Ras), resulting in an inhibition of Raf activation by Ki-Ras (V12) and TPA (Fig. 8B).

Finally, to explore whether the inhibition of Raf activation by the mutation is due to a decrease in Raf/MEK binding, the cDNAs for the wild type or the mutant Raf were introduced into HEK 293T cells with MEK1, and the binding was determined. Fig. 8C shows that the mutation abolished TPA-stimulated binding of Raf to MEK, although it caused a mild increase in binding to MEK under the basal condition. This result was similar to those obtained with the truncation mutants (Fig. 5). Thus, we conclude that Trp342 is essential for both phosphorylation of Ser338 and TPA-stimulated binding to MEK1.

**DISCUSSION**

An important feature of the Raf/MEK/Erk signaling module is the accuracy of signal transmission. Although individual kinases possess homology with those at the same level in other mitogen-activated protein kinase pathways, they do not erroneously cross-phosphorylate substrates that are not in the same pathway. In the signal transduction cascade from Ras to Erk, many of the components are associated with each other through multiple interactions in addition to being tethered by scaffold proteins, so as to assure the accuracy and efficiency of signaling. The present study is the first to show that Raf-1 contains two sites that can independently bind to its substrate, MEK1. The results indicate that these two sites on the *cis* context cooperate to bind to MEK in response to TPA. Deletion of the amino-terminal site outside the core kinase domain abolishes Raf kinase activity because of loss of TPA-stimulated binding to MEK.

Previous studies using transformation assays have demonstrated that truncation from the amino terminus causes progressive activation of Raf-1 (32). In the present investigation, we have found that at the basal level, the *in vitro* activity of the Raf kinase domain is low, whereas it is highly active *in vivo*. Although it is not known what contributes to these different results, we could conceive several possibilities. First, the difference may be ascribed to the duration of the enzymatic reaction, because the *in vitro* kinase assay occurs in much less time (about 30 min), whereas the *in vivo* kinase reaction lasts more than 48 h. The latter is long enough to allow Raf phosphorylation of the endogenous substrate MEK to reach a high level. Hence, as long as it attains a threshold, even a small difference is sufficient to produce a significant change, leading to accumulation of considerable amount of active MEK and Erk. Secondly, the amino-terminal regulatory domain might restrain the interaction of the Raf kinase domain with MEK or with an adaptor protein between Raf and MEK, if it ever existed inside the cells. Thus, such an adaptor might bind better to the kinase domain than to the full length, resulting in potent activation of MEK. These possibilities are not mutually exclusive and should also be applicable to other carboxyl-terminal mutants, provided that they contain a functional unit of the catalytic domain (Fig. 4).

The present study reinforces the notion that maximal activation of Raf kinase requires the phosphorylation of the sites Ser338 and/or Tyr341. Accordingly, point mutation or deletion of these sites greatly impairs the *in vitro* enzymatic activity of Raf-1. Additionally, our study suggests that the negative charge on the residue 341 is more important than that of Ser338. Failure of the Raf mutant 338D/648 to respond to TPA (Fig. 4) implies that Tyr341 in these mutants is not phosphorylated, probably because of a loss of an additional sequence amino-terminal to Ser338 (residues 325–337) (Fig. 4). It is also possible that phosphorylated Ser338, rather than an acidic amino acid substitute, induces phosphorylation of Tyr341. Unfortunately, we were unable to detect it by using a Tyr(P) antibody and, instead, observed an increase in phosphorylation of Ser338 by mutation of Tyr338 to aspartic acid.

Our results demonstrate that the phosphorylation of Raf Ser338 and/or Tyr341 markedly stimulates binding of Raf to MEK. The binding magnitudes of the Raf kinase domain mutants closely correlate with their abilities to phosphorylate MEK. We noticed that deletion or mutation of Trp342 (Raf 342/648 and Raf FL W342A) slightly increased Raf binding to MEK but reduced the TPA-stimulated binding that is even lower than the basal level (Figs. 5A and 8C). The mechanism underlying this change remains unclear. Because the observed binding reflected the balance between association and dissociation, it is likely that Trp342 in cooperation with phosphorylated Ser338-Tyr341 residues, regulates the equilibrium with result of the increased binding. Conversely, the loss of such cooperation caused by the Trp342 mutation may favor the dissociation upon the treatment of cells with TPA. In other words, phosphorylation of other sites stimulated by TPA could facilitate the dissociation process in the absence of Trp342.

McPerson et al. (33) have recently reported a similar observation regarding the importance of Trp342, where its mutations to Ala, Asp, His, or Lys all eliminate the enzyme activity. The present study further demonstrates that Trp342 is necessary for phosphorylation of Ser338 and thus kinase activity. An *in vitro* study by using a synthetic peptide as a substrate by King et al. (25) showed that Trp342 is dispensable for the phosphorylation of Ser338 by Pak3/Y/pak. There might be two explanations for this discrepancy; first, the Ser338 kinase in response to TPA, Src and Ras may be different from Pak, and second, phosphorylation of the peptide substrate is different from that of the intact molecule. The latter is more restrictive.

It is intriguing to hypothesize that a tryptophan close to the amino terminus of the kinase domain plays a role in regulating the conformation of the kinase, because it can be found in almost all Raf family members and a number of other kinases, such as MEKK1 (34), MOS (35), KSR (36), and Src (37). The sequence surrounding Trp342 in Raf-1 shows the closest similarity to KSR and Src (QEWDI/mouse KSR, DAWEI/Src versus YYWEI/Raf-1). Although its role in KSR has not been addressed, tryptophan at the position 260 of Src exerts an inhibitory role by interacting with the Co-helix and constraining the conformation of the Src catalytic domain so as to inhibit the phospho-transfer reaction. In light of the sequence alignment between Raf-1 and Src, it is reasonable to speculate that the role of tryptophan depends on the negative charge of the residue immediately amino-terminal to it. Thus, loss of Raf kinase activity by the Tyr341 to Ala or Phe mutation may be a consequence of the transition of Raf conformation into the closed one, whereas mutation of Tyr341 to Ala does not have such an effect. In keeping with this, substitution of Tyr341 with Asp in the absence of additional amino-terminal sequence creates an active kinase. Likewise, we predict that mutation of Ala259 to Asp would generate a constitutively active form of Src.

We also show that, when Lys375, a critical residue for ATP binding and phospho-transfer reaction, is mutated to methionine, Raf fails to bind to MEK (Fig. 5C). This suggests that the Lys to Met mutation may disturb the tertiary structure of the Raf kinase domain, possibly because of loss of ATP binding. As a result, the kinase adapts an inactive, closed conformation that is unfavorable to interact with MEK. Alternatively, phos-
phorylation of Ser\textsuperscript{338}–Tyr\textsuperscript{341} may stimulate autophosphorylation of other sites such that they cooperate to expose two MEK-binding sites. These two possibilities are currently under investigation.

In summary, an important finding in the present study is the identification of Trp\textsuperscript{342} as the start point of the minimal Raf catalytic unit and two MEK-binding sites on Raf-1, which extends our knowledge on the double-lock feature in the mitogen-activated protein kinase pathway that confers specificity and efficiency in signaling. Binding of these two sites to MEK is greatly invoked by phosphorylation of Raf Ser\textsuperscript{338} and Tyr\textsuperscript{341} when they are on a single molecule. The stimulated MEK activity closely correlates with the increase in Raf kinase activity. This should serve as a framework for understanding phosphorylation of other substrates by Raf.

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Interaction between Raf and MEK 45003
Phosphorylation of $^{338}\text{SSYY}^{341}$ Regulates Specific Interaction between Raf-1 and MEK1

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