Membrane Localization, Oligomerization, and Phosphorylation Are Required for Optimal Raf Activation*

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Activation of the serine/threonine kinase c-Raf-1 requires membrane localization, phosphorylation, and oligomerization. To study these mechanisms of Raf activation more precisely, we have used a membrane-localized fusion protein, myr-Raf-GyrB, which can be activated by coumermycin-induced oligomerization in NIH3T3 transfectants. By introducing a series of point mutations into the myr-Raf-GyrB kinase domain (S338A, S338A/Y341F, Y340F/Y341F, and T491A/S494A) we can separately study the role that membrane localization, phosphorylation, and oligomerization play in the process of Raf activation. We find that phosphorylation of Ser-338 plays a critical role in Raf activation and that this requires membrane localization but not oligomerization of Raf. Mutation of Tyr-341 had a limited effect, whereas mutation of both Ser-338 and Tyr-341 resulted in a synergistic loss of Raf activation following coumermycin-induced dimerization. Importantly, we found that membrane localization and phosphorylation of Ser-338 were not sufficient to activate Raf in the absence of oligomerization. Thus, our studies suggest that three key steps are required for optimal Raf activation: recruitment to the plasma membrane by GTP-bound Ras, phosphorylation via membrane-resident kinases, and oligomerization.

The Ras/Raf/MEK1/MAPK signaling pathway plays a key role in regulating cell proliferation and differentiation in a variety of organisms (1). In the absence of stimulation, Ras is found in its inactive GDP-bound state. Recruitment of guanine nucleotide exchange factors to the plasma membrane by activated cytokine receptors promotes the exchange of GDP for GTP; this converts Ras to its active GTP-bound state. Ras-GTP activates a number of effector molecules including the serine/threonine kinase Raf-1. Subsequently, activated Raf-1 phosphorylates MEK, which in turn phosphorylates MAPK. Ultimately, this leads to activation of transcription factors in the nucleus that regulate downstream cellular processes.

The mechanisms responsible for Ras, MEK, and MAPK activation are well characterized. In contrast, our understanding of Raf activation remains incomplete. Previous results show that artificial farnesylation of Raf results in its activation (2, 3). This has led to the suggestion that membrane localization is sufficient for Raf-1 activation. Another proposed mechanism for Raf activation involves phosphorylation by membrane resident kinases (4). For example, previous studies (5, 6) have demonstrated that phosphorylation on both serine and tyrosine residues in the Raf kinase domain cooperate to activate Raf-1. Specifically, mutation of Tyr-341 or Ser-338 compromises Raf activation. These residues have been shown to be phosphorylated by membrane resident kinases such as Src/Lck (Tyr-341) or Pak1 and/or Pak3 (Ser-338) (4, 7, 8). A more recent finding by Chong et al. (9) suggests that two additional sites, Thr-491 and Ser-494, are also important residues in the kinase domain of Raf that contribute to its activation.

Although considerable evidence supports the notion that membrane localization is required for Raf activation, we and others have shown that this is not sufficient. For example, localization of Raf to the membrane via N-terminal myristylation and palmitylation sequences does not result in constitutive Raf activation (10, 11). These results suggest that additional events are required to activate Raf. Recent results have demonstrated that Raf oligomerization also plays an important role in its activation. For example, Inouye et al. (12) have shown that Ras exists as a dimer and that driving Ras dimerization activates the Raf pathway. Thus, Ras dimerization may lead to the formation of Raf dimers. Furthermore, in yeast the scaffolding protein Ste5 has been shown to organize signaling of the yeast orthologs of Raf (Ste11), MEK (Ste7), and MAP kinase (Fus3). Dimerization of Ste5 is both necessary and sufficient to activate Ste11 and its downstream targets Ste7 and Fus3 (13). Finally, we have demonstrated that dimerization of a membrane-localized form of Raf leads to its activation (11).

These findings suggest that three processes are needed for optimal Raf activation: membrane localization, phosphorylation, and oligomerization. However, the relationships between these processes are unclear. For example, it is possible that oligomerization and/or membrane localization of Raf is required for certain phosphorylation events. To study these processes in greater detail, we made use of a chemical-induced dimerization strategy to regulate Raf activity. This approach allows us to separate membrane localization, phosphorylation, and oligomerization of Raf, and independently monitor their effects on Raf activation.

We have utilized myr-Raf-GyrB fusion proteins that can be dimerized by coumermycin to study Raf activation (14). In this system, the myristylation and palmitylation sequences from lck have been fused to the N terminus of Raf. This targets Raf to the plasma membrane, but does not result in Raf activation. In addition, we have fused the N-terminal portion of bacterial...
Fig. 1. **Key mutation sites in c-Raf-1.** A, the structure of c-Raf-1 contains 3 regions: CR1 (cysteine finger-like domain and Ras binding domain), CR2 (serine-rich domain), and CR3 (kinase domain). Ser-338 and Tyr-341 are well characterized residues in the N-terminal kinase domain that are phosphorylated by Pak- and Src-family kinases, respectively. The Thr-491 and Ser-494 residues in the activation loop are phosphorylated by an unknown kinase. B, myr-Raf-GyrB mutants. The point mutations for each construct were created by PCR site-directed mutagenesis. Each mutant fusion protein was then cloned into the pSR expression vector as described under “Experimental Procedures.” RBD, Ras binding domain.

DNA Gyrase (GyrB) to the C terminus of the myr-Raf fusion protein. This GyrB domain binds the symmetrically dimeric antibiotic, coumermycin, with a stoichiometry of 2:1. Thus, coumermycin can be used to induce dimerization of myr-Raf-GyrB fusion proteins. Using these constructs, and a panel of myr-Raf-GyrB phosphorylation mutants, we have examined the effects of membrane localization and oligomerization on Raf phosphorylation and activation.

Consistent with previous findings, we observe that Ser-338 is an important phosphorylation site involved in Raf-1 activation because mutation of this residue greatly decreases downstream MEK and MAPK phosphorylation in our system. Herein, we demonstrate that membrane localization but not oligomerization of Raf is sufficient for phosphorylation of Ser-338. These studies conclusively demonstrate that membrane localization and Raf phosphorylation on Ser-338 are not sufficient for Raf activation. In contrast, mutation of Tyr-341 has a more modest effect on MEK and MAPK phosphorylation in our system, although when combined with the Ser-338 mutation (S338A/Y341F), Raf-1 activation was completely ablated. Taken together, these results suggest that three distinct events are necessary for c-Raf-1 activation: recruitment to the plasma membrane by GTP-bound Ras, phosphorylation of Ser-338 via membrane-resident protein kinases, and oligomerization of Raf.

**EXPERIMENTAL PROCEDURES**

**Construction of Mutant Myr-Raf-GyrB Fusion Proteins—**Construction of the myr-Raf-GyrB construct has been previously described (11). The S338A and S338A/Y341F myr-Raf-GyrB mutants were created by PCR site-directed mutagenesis using the primers listed below. The myr-Raf-GyrB mutants were then digested with XhoI and cloned into the XhoI site of the pSR expression vector. The Raf-GyrB Y340F/Y341F fusion protein has been previously described (14). Myr-Raf-GyrB

**PCR Primers—**PCR site-directed mutagenesis was used to create specific point mutations in the kinase domain of c-Raf-1. The primers that were used are listed as follows: S338A primers, 5′ primer GGA-GAGAGATACAGAGCTAATTGGGAA, 3′ primer TTCCACAAATATAGCTTGATCTCTGCTGGC, 5′ S338A/Y341F primers, 5′ primer GGA-GAGAGATACAGAGCTAATTGGGAA, 3′ primer TTCCACAAATATAGCTTGATCTCTGCTGGC. Calcium Phosphate Transfection—Tissues were carried out as previously described to generate stable NIH3T3 transfectants (11). The cells were selected for 2 weeks in high concentrations of G418 (1 mg/ml). Stable transfectants were then maintained in G418 at a concentration of 500 μg/ml.

**Quantitation of Myr-Raf-GyrB Fusion Proteins—**Whole cell lysates were made from the stable transfectants (5 × 10⁶ cells) using ice-cold buffer H containing 1% Triton (11). The cells lysates were centrifuged for 5 min at 14,000 rpm to remove cell debris/nuclei and the resulting supernatant was mixed with 4 volumes of polyvinylidene difluoride membranes. The membranes were blocked for 1 h in 5% milk, 1× TBS-0.05% Tween. Anti-c-Raf-1 monoclonal antibody (Cell Signaling Technologies) was incubated overnight at four degrees Celsius at a 1:1000 dilution. The primary antibody was then washed off (2× for 5 min with 1× TBS-Tween) and the secondary antibody (anti-mouse alkaline phosphatase; Amersham Biosciences) was incubated for 1 h at a 1:10,000 dilution. After two hours of washing with 1× TBS-Tween, the blot was developed with ECF reagent from Amersham Biosciences. The blot was then imaged with a STORM chemiluminescence scanner (Amersham Biosciences) to compare relative expression levels and analyzed using ImageQuant software. Relative expression levels for each construct were determined by dividing the value for the fusion protein by the value for endogenous Raf expression.

**MEK and MAPK Phosphorylation Assays—**Cells were plated in 100-mm tissue culture plates at 5 × 10⁵ cells/plate and allowed to adhere overnight. The following day, the cells were washed two times with 1× phosphate-buffered saline and once with serum-free media (Dulbecco’s modified Eagle’s medium containing 2 mM l-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin). The cells were serum starved overnight in serum-free media. The cells were then stimulated with appropriate amounts of Me₂SO, coumermycin, PMA, or 20% serum for 1, 5, or 15 min (11). Stimulation was stopped by adding ice-cold
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RESULTS

Raf has been divided into three functional domains called CR1, CR2, and CR3 (Fig. 1A). CR1 contains the Ras binding domain and is involved in recruitment to Ras-GTP (15–19). Less is known about the CR2 domain, but it contains multiple serine and threonine residues that can be phosphorylated, and act to positively or negatively regulate Raf function (20, 21). Finally, the CR3 region encompasses the kinase domain of Raf. This region contains four important phosphorylation sites including Ser-338, Tyr-341, Thr-491, and Ser-494 (4, 5, 7–9). To address the role between membrane localization, phosphorylation, and oligomerization, we constructed the following four myr-Raf-GyrB mutant fusion proteins: S338A, S338A/Y341F, Y340F/Y341F, and T491A/S494A (Fig. 1B).

The wild-type and mutant myr-Raf-GyrB fusion proteins were transfected into NIH3T3 cells. We confirmed that wild-type and mutant fusion proteins were expressed at comparable levels by immunoblotting using an anti-c-Raf-1 monoclonal antibody. The myr-Raf-GyrB fusion protein migrates around 98 kDa, whereas endogenous Raf migrates around 74 kDa. Protein levels were compared by dividing the expression of our fusion proteins by that of endogenous Raf, which serves as an internal control. As shown in Fig. 2, all myr-Raf-GyrB mutants were expressed at similar levels relative to the wild-type construct.

To examine Raf activation in our studies, we monitored MEK and MAPK phosphorylation levels because this has been correlated with Raf activation. As seen in Fig. 3, coumeycin stimulation of the wild-type myr-Raf-GyrB transfectant results in activation of both MEK and MAPK. MEK and MAPK activation were observed as early as 5 mins following coumeycin stimulation and Table I). To examine whether phosphorylation of MEK and MAPK when compared with the wild-type myr-Raf-GyrB construct (Fig. 4, middle panel, and Table I). This result suggests that the Tyr-340/Tyr-341 residues play a minimal role in Raf activation in our system. However, when both the Ser-338A and Tyr-341F mutations are combined, there is a 20-fold decrease in phosphorylation of MEK and MAPK (Fig. 4, bottom panel, and Table I). To examine whether Ser-338 and Tyr-341 are redundant residues required for Raf activation, we analyzed Raf function in the S338A mutant. As shown in Fig. 5, mutation of Ser-338 alone results in a 4.5-fold reduction in coumeycin-induced Raf activation (Table I).

Table I. Fold induction of P-MEK and P-MAPK for the wild-type and mutant myr-Raf-GyrB constructs

| Raf mutants | Me2SO | 5 min coumeycin | 15 min coumeycin | 30 min coumeycin | 15 min PMA |
|-------------|-------|------------------|------------------|------------------|----------|
| Wild-type   | 1.0 ± 0.1 | 4.2 ± 0.6 | 8.6 ± 2.4 | 4.9 ± 0.5 | 2.5 ± 0.2 |
| S338A       | 1.0 ± 0.2 | 3.5 ± 1.1 | 19.0 ± 3.5 | 12.9 ± 2.0 | 8.6 ± 2.8 |
| S338A/Y341F | 1.0 ± 0.2 | 0.75 ± 0.5 | 1.9 ± 0.3 | 0.25 ± 0.0 | 4.8 ± 0.59 |
| Y340F/Y341F | 1.0 ± 0.07 | 0.24 ± 0.1 | 0.4 ± 0.01 | 0.0 | 10.7 ± 1.54 |
| T491A/S494A | 1.0 ± 0.01 | 0.59 ± 0.3 | 0.45 ± 0.17 | 0.65 ± 0.33 | 3.0 |

* Phospho-MEK expression levels.

* Phospho-MAPK expression levels.

phosphate-buffered saline and the cells were then lysed in ice-cold buffer containing 1% Triton (11). Cell lysates were harvested with a cell scraper and cell debris/nuclei were removed by centrifugation for 5 min at 14,000 rpm. Supernatants were removed and mixed with 4× Laemmli buffer. The samples were then fractionated as described above, and transferred onto polyvinylidene difluoride membranes. The membranes were blocked as described above and incubated overnight with antibodies to phospho-MAPK (P-MAPK) or phospho-MEK (P-MEK) (Cell Signaling Technologies, Inc.) at a 1:10,000 dilution. After washing off the primary antibody, the secondary antibody (anti-rabbit alkaline phosphatase; Amersham Biosciences) was incubated for 1 h at a 1:10,000 dilution. The blot was then washed, developed, and imaged with a STORM chemiluminescence scanner as described above. The values obtained from ImageQuant were normalized by stripping the blot and reprobing with an anti-Erk-1 antibody to calculate relative expression of P-MEK and P-MAPK.

Fig. 3. Coumeycin-induced activation of myr-Raf-GyrB results in high levels of MEK and MAPK phosphorylation. Myr-Raf-GyrB transfectants were grown in 100-mm tissue culture dishes and used in the coumeycin stimulation assay as described. Cell lysates were examined for phospho-MEK (P-MEK) and phospho-MAPK (P-MAPK) expression. Phosphorylation levels were normalized to total MAP kinase expression. Fold induction was calculated relative to the Me2SO (DMSO) control. These findings are representative of seven experiments.
This is not due to a general defect in downstream activation of MEK or MAPK in the S338A transfectants because activation of endogenous Raf via PMA stimulation still results in robust MEK and MAPK activation. These results demonstrate that phosphorylation of Ser-338 plays the predominant role in Raf activation in our system, although modest effects of Tyr-341 could be observed.

One possible explanation for the above result is that Raf dimerization may directly result in Ser-338 phosphorylation. To test the role that Ser-338 may have during Raf activation, we utilized a panel of three different wild-type Raf fusion proteins: myr-Raf-GyrB, myc-tagged Raf-GyrB (myc-Raf-GyrB), and Raf-GyrB. Both the myc-Raf-GyrB and Raf-GyrB constructs lack membrane-targeting sequences and are thus localized to the cytoplasm, whereas myr-Raf-GyrB is localized to the plasma membrane. Stable transfectants expressing these distinct constructs were stimulated with coumermycin, and Ser-338 phosphorylation levels were quantitated using a phospho-specific Ser-338 antibody (5). As shown in Fig. 6, membrane localization itself was sufficient to induce Ser-338 phosphorylation (myr-Raf-GyrB unstimulated).

In contrast, coumermycin addition had no significant effect on Ser-338 phosphorylation in either membrane localized or cytoplasmic Raf-GyrB constructs. As a control, we demonstrated that phosphorylation of Tyr-341 could be observed.

DISCUSSION

Previous studies (2, 4–9, 11) have shown that membrane localization and phosphorylation are required for Raf activation. Herein, we demonstrate that these two processes are not sufficient for Raf to become fully activated. Specifically, we demonstrate that neither targeting of Raf to the plasma membrane, via a myristylation/palmitylation sequence, nor phosphorylation within Raf’s kinase domain of the critical serine.

FIG. 4. S338A/Y341F and T491A/S494A are unable to induce MAPK and MEK phosphorylation, whereas Y340F/Y341F is minimally affected. S338A/Y341F, T491A/S494A, and Y340F/Y341F cell lines were stimulated with either Me2SO (DMSO), coumermycin (cou.), or PMA as described under “Experimental Procedures.” Whole cell lysates were harvested and probed for anti-Phospho-MEK/MAPK expression. The blot was stripped, and reprobed with antibodies to total MAP kinase to normalize for differences in protein loading. These results are representative of three (S338A/Y341F), four (T491A/S494A), and seven (Y340F/Y341F) experiments.

FIG. 5. S338A shows a major defect in MEK and MAPK phosphorylation. Stable transfectants of the wild-type and S338A myr-Raf-GyrB constructs were generated as described. Cells were serum-starved and stimulated with the indicated agents for various times (cou., coumermycin; DMSO, Me2SO). Whole cell lysates were made as described, and a Western blot was carried out using anti-phospho-MEK and MAPK antibodies. The blot was stripped and reprobed with antibodies to total MAP kinase to normalize for differences in protein loading. These findings are representative of seven (wild-type) and four (S338A) experiments, respectively.

FIG. 6. Membrane localization, but not dimerization, is required for Ser-338 phosphorylation. Stable transfectants of Ser-338 were stimulated as described under “Experimental Procedures.” A phospho-specific Ser-338 polyclonal antibody was used to examine Ser-338 phosphorylation levels in each of the stable transfectants. Phosphorylation levels were normalized relative to total MAP kinase expression. Myr-Raf-GyrB constructs contain the amino acid sequences for palmitoylation and myristoylation, allowing localization of the fusion protein to the plasma membrane. The myc-Raf-GyrB and Raf-GyrB fusion proteins are not targeted to the plasma membrane. Myr-Raf-GyrB constructs include an N-terminal myc epitope tag. This experiment is representative of two performed. Cou., coumermycin.
residue, Ser-338, is sufficient to induce Raf activation. Rather, we demonstrate that a third mechanism, involving Raf oligomerization, works in conjunction with membrane localization and phosphorylation, and leads to maximal activation of the Raf pathway. In addition, our results suggest that the oligomerization step does not function by promoting transphosphorylation of key sites, such as Ser-338 or Thr-491/Ser-494, by Raf homodimers. Taken together our results suggest that at least three distinct events must occur for Raf to become activated: membrane localization, oligomerization, and phosphorylation of Raf.

Over the last several years evidence has begun to accumulate suggesting that Raf oligomerization may play an important role in its activation. For example, Inouye et al. (12) have demonstrated that Ras-GTP forms a dimeric complex that is capable of recruiting two molecules of Raf-1 to the plasma membrane. Similarly, in yeast the scaffolding protein Ste5, which helps assemble a homologous pathway to the Raf/MEK/MAP kinase cascade, has been shown to undergo dimerization upon activation (13). In the yeast system, dimerization of Ste5 is both necessary and sufficient for activation of the Raf ortholog Ste11. In mammalian systems, the identity of specific scaffolding proteins involved in Raf activation is less well defined. However, several proteins have been suggested to play a somewhat similar role. For example, the 14-3-3 proteins exist as homodimers and have been shown to promote the heterodimerization of a number of proteins (22). Finally, we and others have demonstrated that forced dimerization of Raf, promotes Raf activation, leading to the idea that Raf-1 itself may act as a dimer (14, 23). Taken together, these findings strongly suggest that Raf activation involves a key dimerization or oligomerization step.

A key question that remains is the mechanism by which Raf oligomerization promotes Raf activation. Two possibilities involve (i) transphosphorylation of specific Raf residues, such as Ser-338, or (ii) induction of specific conformational changes that promote Raf activation. We initially proposed that Raf oligomerization was involved in transphosphorylation of Ser-338 and thereby resulted in maximal Raf activation. However, our findings in this study do not support this hypothesis. Specifically, we found that induced dimerization of cytoplasmic forms of Raf (myc-Raf-GyrB or Raf-GyrB fusion proteins, see Fig. 6) did not result in phosphorylation of Ser-338. In contrast, we found that membrane localization of myr-Raf-GyrB constructs, in the absence of induced oligomerization, was sufficient to induce phosphorylation of Ser-338; importantly, this was not sufficient to induce Raf activation. Finally, although dimerizing a membrane-localized form of Raf (myr-Raf-GyrB) did result in maximal Raf activation, it did not enhance phosphorylation of Ser-338 above that observed upon membrane localization alone. These findings led us to two conclusions. First, the combination of membrane localization and Ser-338 phosphorylation is not sufficient to induce Raf activation. Second, Raf oligomerization does not result in transphosphorylation of Ser-338. Thus, phosphorylation on Ser-338 and oligomerization of Raf are independent events in Raf activation.

An alternative possibility is that Raf oligomerization is involved in promoting transphosphorylation of Thr-491 and Ser-494. These residues are located in the activation loop of the kinase domain of Raf. Thus, an intriguing possibility is that Raf oligomerization might lead to phosphorylation of these residues in trans, and thereby mimic the mechanism involved in the activation of receptor tyrosine kinases following their dimerization (24). Previous work (9) has demonstrated that Thr-491 and Ser-494 are required for Raf function and that they become phosphorylated upon stimulation of the Raf pathway. We have observed a similar effect using our inducibly activated form of Raf as myr-Raf-GyrB mutants, in which Thr-491 and Ser-494 are changed to alanine, show a dramatic decrease in Raf activation (Fig. 4). We have attempted to determine whether these residues are phosphorylated with antibodies previously used to detect phosphorylation of these residues (9). However, in our hands we find that these antibodies recognize Raf proteins in which Thr-491/Ser-494 have been mutated to alanine, suggesting that they may recognize a more general phospho-serine or phospho-threonine epitope. Thus, we have not been able to directly determine whether membrane localization is sufficient to induce Thr-491/Ser-494 phosphorylation. However, we have observed that dimerization of the myr-Raf-GyrB T491S/A494A mutants does lead to low level activation of the Raf signal transduction pathway (2-fold, see Table 1), indicating that oligomerization must be having an effect other than just promoting phosphorylation of these residues. Based on these results we suggest that oligomerization of Raf does not function by promoting transphosphorylation of the major serine/threonine phosphorylation sites found in the kinase domain of Raf.

Because oligomerization does not regulate activation of Raf by inducing transphosphorylation of specific serine and threonine residues, we speculate that it most likely functions by inducing or stabilizing conformational changes in Raf. For example, several studies have demonstrated that the N-terminal region of Raf (CR1/CR2) interacts with the kinase domain of Raf and prevents its activation (25). This is believed to involve interactions of 14-3-3 proteins with Ser-621 (a site that is constitutively phosphorylated and required for Raf activation) and Ser-259; this event is thought to fold Raf into an inactive conformation. Activation of Raf involves dephosphorylation of Ser-259, resulting in an altered conformation that allows for Raf phosphorylation and subsequent activation. It is unclear whether Raf oligomerization influences this process. However, it is tempting to speculate that Raf oligomerization may promote the formation of intermolecular 14-3-3 bridges. This could be mediated by binding of 14-3-3 proteins to Ser-621 residues on dimerized Raf molecules, thereby exposing Ser-259 for dephosphorylation and further stabilizing the Raf oligomeric complex. Consistent with this hypothesis, Weber et al. (22) have demonstrated that 14-3-3 proteins are involved in heterodimerization of c-Raf and the related kinase B-Raf, and that this interaction involves binding of 14-3-3 proteins to c-Raf on phospho-Ser-621. Finally, alternative mechanisms include the possibility that Raf oligomerization may alter the function of scaffolding proteins associated with Ras and Raf and thereby promote Raf activation. Additional studies will be required to sort out these possibilities.

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