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Activity of Plasma Membrane-recruited Raf-1 Is Regulated by Ras via the Raf Zinc Finger*

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Ras recruits Raf to the plasma membrane for activation by a combination of tyrosine phosphorylation and other as yet undefined mechanism(s). We show here that the Raf zinc finger is not required for plasma membrane recruitment of Raf by Ras but is essential for full activation of Raf at the plasma membrane. Membrane targeting cannot compensate for the absence of the zinc finger. One facet of the zinc finger activation defect is revealed using a constitutively activated Raf mutant. Targeting Raf Y340D,Y341D to the plasma membrane increments activity, but full activation requires coexpression with activated Ras. This sensitivity to regulation by Ras at the plasma membrane is abrogated by mutations in the Raf zinc finger but is unaffected by mutation of the minimal Ras binding domain. These data show for the first time that Ras has two separate roles in Raf activation: recruitment of Raf to the plasma membrane through an interaction with the minimal Ras binding domain and activation of membrane-localized Raf via a mechanism that requires the Raf zinc finger.

The mitogen-activated protein kinases ERK 1 and ERK 2 are activated by tyrosine kinases in a highly conserved signaling pathway. The Raf-1 serine threonine kinase activates the dual specificity kinase MEK[1] by phosphorylating two regulatory serine residues. In turn, MEK activates the mitogen-activated protein kinases by phosphorylating regulatory threonine and tyrosine residues (1). Genetic and biochemical data have established Raf-1 as a Ras effector, but in contrast to the simple activation mechanisms of MEK and mitogen-activated protein kinase, the mechanism of activation of Raf-1 is complex, involving more than the simple binding of Ras.

The minimal Ras binding domain (RBD) of Raf-1 spans residues 51–131, which are located predominantly in conserved region 1. *In vitro*, the interaction of Ras with this isolated RBD is of high affinity, is GTP-dependent, and is blocked by mutations in the Ras switch I effector loop that abrogate biological activity *in vivo* (2–4). The crystal structure of a complex between Rap1, which has an identical effector loop to Ras, and this minimal RBD has been solved, confirming the direct interaction of the RBD with the Ras effector loop (5). A mutation, R89L, in the minimal RBD is sufficient to abrogate binding to Ras *in vitro* and blocks activation of Raf-1 by Ras *in vivo* (6). More recently, the cysteine-rich domain (CRD) in conserved region 1 has also been shown to interact with Ras. The CRD spans residues 139–184 and includes a zinc finger delineated by cysteine residues 165 and 168, although the NMR structure reveals significant differences between the zinc fingers of Raf and protein kinase C (7). Mutations within the Raf-1 zinc finger (C165S,C168S) abrogate binding of the isolated CRD to Ras (8–11). Studies with mutant Ras proteins implicate Ras residues Asn-26 and Val-45 and switch II as the critical interacting residues. There are conflicting data as to whether the interaction of Ras with the CRD is GTP-dependent and whether Ras needs to be prenylated to bind (8–12). The CRD also binds phosphatidylserine and, *in vitro*, mediates the interaction of Raf with phosphatidylserine-containing liposomes (13, 14).

Ras binding to Raf-1 *in vitro* does not allosterically modulate Raf-1 kinase activity. However, coexpression with oncogenic mutant Ras *in vivo* results in recruitment of Raf-1 from the cytoplasm to the plasma membrane and activation (15). Localization of Raf-1 to the plasma membrane is critical because targeting Raf-1 to the plasma membrane independent of Ras is sufficient for activation (16, 17). Moreover, whereas Raf-1 cannot be activated *in vitro* by purified Ras and Src, partial activation can be achieved using membranes from Ras- and Src-transformed cells (18, 19). The nature of the membrane events that actually activate Raf-1 remain unclear, although phosphorylation of tyrosine residues 340 and 341 in conserved region 3 is a critical event (20, 21). *In vivo*, all activated tyrosine-phosphorylated Raf-1 is found sequestered at the plasma membrane (22), probably in caveolae, since sucrose gradient fractionation of plasma membranes shows that Ras and Raf-1 cofractionate with caveolin (23). This is consistent with our previous observation that membrane-recruited Raf-1 is found almost exclusively in a detergent-insoluble membrane fraction that is enriched for caveolin (17).

Given the critical role of the plasma membrane in activating Raf, we have developed assays to measure Raf activity in intact plasma membranes. Using these and other assays, we examined the role of the Raf zinc finger in membrane recruitment and activation. A recent study has shown that the activation of Raf by the epidermal growth factor receptor is critically dependent on an intact zinc finger but did not determine whether this was a result of failure of membrane recruitment or failure of a plasma membrane activation mechanism (12). Our study directly addresses this issue and shows that the RBD, not the zinc finger, is required for plasma membrane recruitment, but that the zinc finger is critical for the activation of plasma

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[1] The abbreviations used are: MEK, mitogen-activated protein kinase kinase; RBD, Ras binding domain; CRD, cysteine-rich domain; PCR, polymerase chain reaction; PAGE; polyacrylamide gel electrophoresis; ERK, extracellular-regulated protein kinase; MBP, myelin basic protein; RafCAAX, Raf with the C-terminal membrane targeting sequences of K-ras(B); DD, Y340D,Y341D double mutant; FF, Y340F,Y341F double mutant; CCSS, C165S,C168S double mutant.

membrane-recruited Raf. In addition, we present evidence that full activation of membrane-recruited Raf requires a negative charge on residues 340 or 341, an intact zinc finger, and activated Ras. Thus this study for the first time demonstrates that Ras has an additional role in the activation of Raf other than the recruitment of Raf to the plasma membrane.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—The Myc/Glu-Glu N-terminal epitope tags on the RafCAA*X* cDNA described previously (17) was replaced with a FLAG epitope tag using PCR primers that simultaneously introduced a new, translationally silent *Pst*I site by mutating codon 92 and either mutated codon 89 to leucine or left as wild type arginine. The Y340D,Y341D (DD) and Y340F,Y341F (FF) double mutations were introduced using a PCR mutagenesis strategy that simultaneously introduced a new, translationally silent *Nhe*I site by mutating codons 346 and 347. The mutated sequences were subcloned as *Acc*B7/*Nhe*I fragments into wild type FLAG RafCAA*X* and FLAG RafCAA*X*R89L. The C165S,C168S double mutant (CCSS) was generated using PCR mutagenesis, and the mutant sequence was subcloned as a *Pst*I/*Bsp*HI fragment into FLAG RafCAA*X*. FLAG RafCAA*X*CCSSDD was generated by recombination. All PCR products were completely sequenced before subcloning. The FLAG RafCAA*X* mutant cDNAs were cloned into the mammalian expression vector EXV-3. To generate nonmembrane targeted versions of each mutant, a stop codon was introduced by a Klenow fill-in of the *Eco*RI site at the 5′ end of the cDNA cassette encoding the K-ras polybasic domain and CAA*X* motif.

COS Cell Transfections and Fractionation—COS cells were electroporated as described previously (24). After 54 h, cells were switched to serum-free medium for a further 18 h before being harvested. Cells were washed and scraped on ice into 0.5 ml of Buffer A (10 mM Tris-HCl, pH 7.5, 25 mM NaF, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 100 μM $NaVO_4$). After 10 min on ice, cells were homogenized with 50 strokes in a tight-fitting dounce homogenizer, and the nuclei were removed by low speed centrifugation. The post-nuclear supernatants were spun at 100,000 × *g*. The supernatant (S100) was removed, and the sedimented fraction (P100) was rinsed and then sonicated for 5 min in 100 μl of ice-cold Buffer A. The S100 fraction and resuspended P100 fractions were snap-frozen and stored at −70 °C in aliquots after measuring protein content by the Bradford reaction.

Nonidet P-40 Solubility Assay—P100 fractions were normalized by protein content and adjusted to 15 μl in Buffer A. An equal volume of Buffer A containing 2% Nonidet P-40 was added, and the sample was sonicated at 4 °C for 5 min. After centrifugation at 100,000 × *g*, the Nonidet P-40-soluble supernatant was removed and added to 6 μl of 5 × SDS-PAGE sample buffer, and the Nonidet P-40-insoluble fraction was taken up in 36 μl of 1 × SDS-PAGE sample buffer. Equal volumes of each sample were resolved by SDS-PAGE and Western-blotted with anti-FLAG monoclonal antiserum.

Western Blotting—Expression and subcellular localization of Raf-1 and Ras proteins were determined using immunoblotting. Sample loading was normalized on S100 protein content, and equal proportions of the S100 and P100 fractions of each lysate were then used for blotting. Samples were resolved on 10% (Raf) or 15% (Ras) SDS-PAGE gels and transferred to polyvinylidene difluoride membranes using semidry transfer. Western blots were probed with M2 anti-FLAG monoclonal (Eastman Kodak Co.) or Y13-259 and developed using enhanced chemiluminescence or quantitated by phosphorimaging (Bio-Rad) after development with rabbit anti-mouse or rabbit anti-rat immunoglobulin (as appropriate) and ^{125}I-protein A. The linearity of this quantitative ^{125}I Western assay is demonstrated in Fig. 1.

Raf-1 Kinase Assays—We reported previously that Raf activity can be measured in intact plasma membranes using a coupled MEK/ERK activation assay (17). For this report we modified membrane preparation protocols to minimize copurification of MEK and ERK and introduced sonication steps to improve membrane suspension.

P100 samples were normalized for Raf-1 content using quantitative ^{125}I Western blotting. P100 aliquots (typically 10–30 μg of total protein) were adjusted to 100 μl in Buffer A, and the membranes were pelleted at 100,000 × *g* and resuspended in 36 μl of Buffer B (50 mM Tris-HCl pH 7.5, 75 mM NaCl, 5 mM $MgCl_2$, 25 mM NaF, 5 mM EGTA, 100 μM $NaVO_4$, 1% Nonidet P-40, 1 mM dithiothreitol) in a sonicating water bath for 2 min at 4 °C. Sonication does not remove any membrane-associated Raf-1.[2] To 10 μl of sonicated P100 fraction, 6 μl of Buffer A containing 0.25 μg MEK, 1 μg of ERK, and 4 μl of 0.5 mM ATP in 40 mM $MgCl_2$ was added, and the mix was vortexed at 30 °C. After 20 min, the sample was placed on ice, and 10 μl was diluted into 40 μl of ice-cold Buffer C (50 mM Tris-HCl, pH 7.5, 75 mM NaCl, 5 mM $MgCl_2$, 25 mM NaF, 5 mM EGTA, 100 μM $NaVO_4$, 1 mM dithiothreitol).

10 μl of this diluted sample was taken into a second incubation with 5 μl of MBP (16 μg) and 10 μl of an ATP mix containing 0.5 mM ATP, 50 mM $MgCl_2$, [γ^{32}P]ATP (2,400 cpm/pmol). After 10 min the reaction was stopped by adding 6 μl of 5 × SDS-PAGE sample buffer, and the reaction products were resolved on 15% SDS-PAGE. The radioactivity incorporated into MBP was measured by scintillation counting of gel slices of stained, fixed gels. MBP phosphorylation in the second 10-min incubation is linear with respect to P100 input and incubation time of the first incubation over ranges of 5–30 μg and 5–30 min, respectively (Fig. 1).

To determine background counts due to any remaining P100-associated MEK or ERK, control tubes were set up for each P100 assay without MEK or without MEK and ERK, respectively (each was <5% of the total activity). To determine background counts due to untransfected cells, P100 fractions prepared from COS cells transfected with empty vector (EXV-3) were analyzed. To verify Raf-1 normalization, 10 μl of the initial reaction incubation with MEK and ERK was analyzed by quantitative ^{125}I Western blotting.

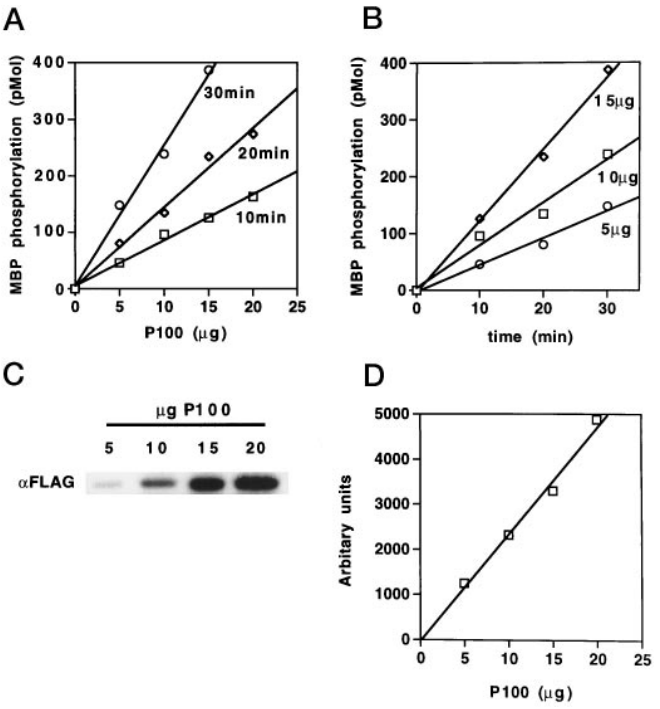

FIG. 1. **Demonstration of the linearity of the coupled Raf activity assay and the FLAG Western blotting assay.** Membrane fractions (P100) from COS cells expressing FLAG Raf + RasG12V were assayed for Raf activity. Aliquots of 5, 10, 15, and 20 μg of P100 fraction were incubated with MEK, ERK, and cold ATP for 10, 20, and 30 min. The reactions were then diluted, and an aliquot of the diluted reactions was incubated for 10 min with MBP and [γ^{32}P]ATP. The radioactivity incorporated into MBP was determined by scintillation counting of gel slices after the reaction products were resolved by SDS-PAGE. MBP phosphorylation due to MEK and ERK co-purifying with Raf was measured simultaneously in parallel reactions without MEK and without MEK and ERK. These counts (each <5% of the total) were subtracted from the total counts. The results are plotted against P100 input in *panel A* and against time in *panel B*. These data are from a representative single experiment and show that the activity assay is linear with respect to the time of the first incubation over 10–30 min and with respect to COS cell P100 input over 5–20 μg. Aliquots of 5, 10, 15, and 20 μg of the same P100 fraction were immunoblotted with M2 (anti-FLAG) monoclonal antibody and developed using rabbit anti-mouse immunoglobulin and ^{125}I-protein A. The immunoblot was autoradiographed (*panel C*) and then quantitated by phosphorimaging (Bio-Rad). The radioactivity bound to FLAG-Raf (expressed in arbitrary phosphorimaging units) is graphed in *panel D*. These data are from a representative single experiment and show that the Western blotting assay is linear with respect to Raf input.

[2] S. Roy and J. F. Hancock, unpublished data.

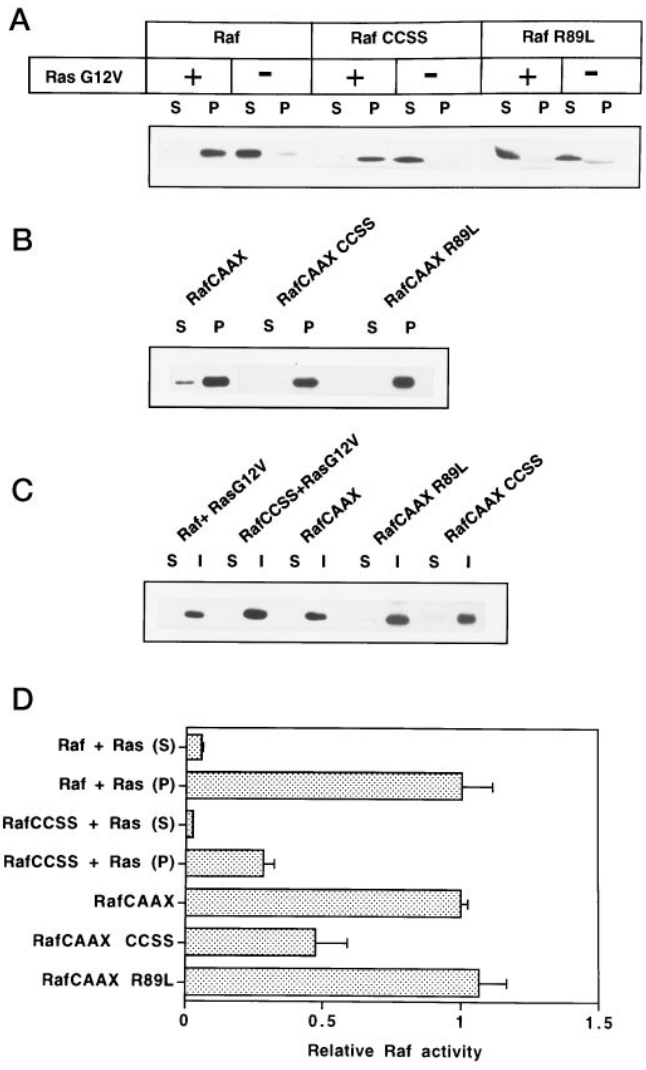

FIG. 2. **The Raf zinc finger is not required for membrane recruitment by RasG12V but is critical for Raf activation.** *A*, EXV plasmids encoding FLAG epitope-tagged Raf, RafR89L, and RafCCSS were electroporated into COS1 cells with (+) and without (−) EXV RasG12V. *B*, EXV plasmids encoding FLAG epitope-tagged RafCAA*X*, RafCAA*X*R89L, and RafCAA*X*CCSS were electroporated into COS1 cells without EXV RasG12V. Cells were harvested 72 h later after an 18-h incubation in serum-free medium and fractionated into cytosolic (S100) and membrane (P100) fractions. 20 μg of each S100 fraction (*S*) and an equal proportion of the corresponding P100 fraction (*P*) were resolved by SDS-PAGE, immunoblotted with an anti-FLAG monoclonal antibody, and developed using enhanced chemiluminescence. *C*, P100 fractions from COS cells expressing RafCAA*X*, RafCAA*X*R89L, and RafCAA*X*CCSS and coexpressing RasG12V + Raf or RasG12V + RafCCSS were adjusted to 1% Nonidet P-40, sonicated, and centrifuged at 100,000 × *g*. Equal proportions of the detergent-soluble supernatant fraction (*S*) and detergent-insoluble pellet fraction (*I*) were resolved by SDS-PAGE, immunoblotted with an anti-FLAG monoclonal antibody, and developed using enhanced chemiluminescence. *D*, membrane fractions (*P*) from COS cells expressing RasG12V, Raf + RasG12V, RafCCSS + RasG12V, RafCAA*X*, RafCAA*X*CCSS, and RafCAA*X*R89L and cytosolic fractions (*S*) from COS cells expressing Raf + RasG12V and RafCCSS + RasG12V were normalized for Raf content using ^{125}I immunoblotting and assayed for Raf activity. The expression of Ras was also measured by ^{125}I immunoblotting. Raf activity was measured in a coupled MEK/ERK assay, and the radioactivity incorporated into MBP was determined by scintillation counting of gel slices (as described under "Experimental Procedures"). MBP phosphorylation due to MEK and ERK co-purifying with Raf was measured and subtracted from total counts (each <5% of the total). The amount of endogenous Raf recruited and activated by RasG12V was determined by assaying membrane fractions, normalized for Ras content, of COS cells expressing RasG12V with no transfected Raf. This activity was subtracted from the total measured Raf activity to derive the activity of transfected Raf. To allow comparisons between successive experiments, a RafCAA*X* transfection was included in each independent experiment, and Raf activity in each sample was expressed relative to the activity of RafCAA*X* (=1) in that experiment. The graph shows mean relative activity (± S.E., n = 4–13) of Raf assayed in membranes (*P*) or Raf immunoprecipitated from cytosol (*S*). The mean activity of RafCAA*X* over all experiments was 120 ± 6 pmol of phosphate transferred to MBP/10 min/10 μg of membrane (mean ± S.E., n = 30). The mean correction made to the raw activity values of Raf proteins transfected with RasG12V to account for activation of endogenous Raf was 7 ± 2 pmol of phosphate transferred to MBP/10 min/10 μg of membrane (mean ± S.E., n = 20).

This assay measures P100-associated Raf activity, but membranes prepared from COS cells transfected with empty vector have minimal activity (≤2pmol of phosphate transfer to MBP/10 μg of P100/10 min). Therefore the measured Raf activity in P100 fractions containing transfected RafCAA*X* is an accurate measure of RafCAA*X* activity. Raf activity in P100 fractions containing transfected Raf and RasG12V represents the activity of membrane-recruited transfected Raf, plus membrane-recruited endogenous Raf. The activity of endogenous Raf recruited and activated by RasG12V was determined by assaying P100 fractions of COS cells transfected with RasG12V and no Raf, which contained an equal amount of Ras (determined by quantitative ^{125}I Western blotting). This endogenous Raf activity was subtracted from the total measured Raf activity to derive the activity of transfected Raf. The mean correction made to the raw values for transfected Raf proteins to account for activation of endogenous Raf was 7 ± 2 pmol of phosphate transferred to MBP/10 min/10 μg of membrane (mean ± S.E., n = 20).

To measure the activity of cytosolic Raf, S100 fractions were normalized for Raf content by quantitative ^{125}I Western blotting and immunoprecipitated with anti-FLAG-Sepharose beads for 2 h at 4 °C. After washing the immunoprecipitates three times in Buffer B, the immunoprecipitates were split into three aliquots and incubated with MEK and ERK and without MEK and without MEK and ERK, exactly as described above. The beads were then collected, washed, and immunoblotted to verify the amount of Raf present in the assay.

RESULTS

The Role of the Raf Zinc Finger in Membrane Recruitment and Activation—We first determined whether the Raf zinc finger is required for plasma membrane recruitment. The mutations C165S,C168S, and R89L were introduced into FLAG-Raf (Raf with an N-terminal FLAG epitope tag), and the constructs were coexpressed with oncogenic mutant RasG12V in COS cells. The cells were fractionated into cytosolic and membrane fractions, and the transfected Raf was detected by immunoblotting for the FLAG tag. The immunoblot in Fig. 2*A* shows that RafCCSS is recruited to the plasma membrane (P100 fraction) by RasG12V to the same extent as wild type Raf. In contrast, RafR89L remains localized to the cytosolic S100 fraction when coexpressed with RasG12V. Wild type Raf and RafCCSS recruited to the plasma membrane by Ras are both insoluble in 1%Nonidet P-40 (Fig. 2*C*). These results indicate that the Raf zinc finger is not required for plasma membrane recruitment of Raf. Membrane fractions from COS cells coexpressing RasG12V and either wild type Raf or RafCCSS were then prepared and normalized for Raf content, and Raf activity was measured in a coupled MEK/ERK assay. Fig. 2*D* shows that the activity of plasma membrane-recruited RafCCSS is approximately 25% that of plasma membrane-recruited wild type Raf. Thus, although the zinc finger mutation does not compromise membrane recruitment, it does significantly abrogate Raf activation.

If, as these experiments suggest, the role of the zinc finger is restricted to mediating Raf activation, then constitutive plasma membrane targeting of RafCCSS would not be expected to rescue activity. To test this prediction, we introduced the C165S,C168S mutations into RafCAA*X*. Figs. 2, *B* and *C*, shows that when expressed in COS cells, RafCAA*X*, RafCAA*X*R89L, and RafCAA*X*CCSS all localize to the Nonidet P-40-insoluble P100 fraction. Membrane fractions normalized

for Raf content were then assayed for activity. Fig. 2*D* shows that the activity of RafCAA*X*R89L is equivalent to that of RafCAA*X*, whereas that of RafCAA*X*CCSS is approximately 45% that of RafCAA*X*. Thus membrane targeting silences the R89L mutation but not the activation defect of the zinc finger mutation.

We conclude that the role of the RBD may be restricted to membrane recruitment, whereas Ras-zinc finger interactions or membrane-zinc finger interactions must function in some other aspect of the Raf activation process. To address this hypothesis, we investigated to what extent mutations in the zinc finger compromised the activity of constitutively activated Raf.

The Zinc Finger Is Required for a Membrane Activation Process Subsequent to Tyrosine Phosphorylation—Replacement of tyrosines 340 and 341 with aspartic acid activates Raf kinase activity by mimicking the negative charge normally imparted by phosphorylation (20). We therefore introduced the Y340D,Y341D mutations into wild type Raf (RafDD) and Raf-CCSS (RafCCSSDD) and coexpressed these Raf mutants with RasG12V in COS cells. Subcellular fractionation and Western blotting showed that RafDD and RafCCSSDD were recruited to the P100 fraction by RasG12V to the same extent as wild type Raf (Figs. 2*A* and 3*A*). The specific activities of Raf recruited to the P100 fraction and Raf remaining in the cytosol (S100 fraction) were then measured in coupled MEK/ERK assays. Fig. 3*B* shows that the RafDD immunoprecipitated from the cytosol was activated 10-fold over wild type Raf immunoprecipitated from the cytosol. Thus the DD mutations alone increase Raf activity to 80% that achieved by plasma membrane localization (compare RafDD + Ras (S100) with RafCAA*X* in Fig. 3*B*). Interestingly, the CCSS mutation has no effect on the activity of cytosolic RafDD (Fig. 3*B*). This contrasts with the effect of the CCSS mutation on cytosolic Raf where it reduces the already low activity by 50% (Fig. 2*D*).

The activity of RafDD is increased a further 7-fold when recruited to the plasma membrane by RasG12V (compare RafDD + Ras (S100) with RafDD + Ras (P100) in Fig. 3*B*). The activity of RafCCSSDD is also increased on recruitment to the plasma membrane by RasG12V but to less than 50% that of RafDD (Fig. 3*B*). We conclude that constitutive negative charge on tyrosines 340 and 341 suppresses the inhibitory effect of the zinc finger mutation on cytosolic Raf activity but does not fully suppress a defect in a plasma membrane-catalyzed activation process.

The Zinc Finger Is Required for a Ras-dependent Membrane Activation Process—To investigate the nature of the membrane activation of Raf DD in more detail, we introduced the Y340D,Y341D mutations into RafCAA*X* and expressed the mutant protein in COS cells. Subcellular fractionation and Western blotting showed that RafCAA*X*DD localized to the P100 fraction to the same extent as RafCAA*X* (Fig. 4*A*). Membrane fractions from COS cells were then prepared and normalized for Raf content, and Raf activity was measured in a coupled MEK/ERK assay. We and others have shown previously that Raf recruited to the plasma membrane by RasG12V is activated to the same extent as membrane-targeted RafCAA*X* and that the activity of RafCAA*X* is not further increased by coexpression of RasG12V (Refs. 16 and 17; Fig. 4*B*). In marked contrast, the activity of membrane-targeted RafCAA*X*DD was found to be significantly lower than the activity of RafDD recruited to the plasma membrane by RasG12V (Fig. 4*B*), and coexpression of RasG12V with RafCAA*X*DD was required to increase the activity of RafCAA*X*DD to that of RafDD recruited to the plasma membrane by RasG12V (Fig. 4*B*). It should be emphasized that this enhancement of RafCAA*X*DD activity is not due to any further recruitment of RafCAA*X*DD to the plasma membrane because RafCAA*X*DD is already P100-localized and the Raf assays are normalized for Raf content.

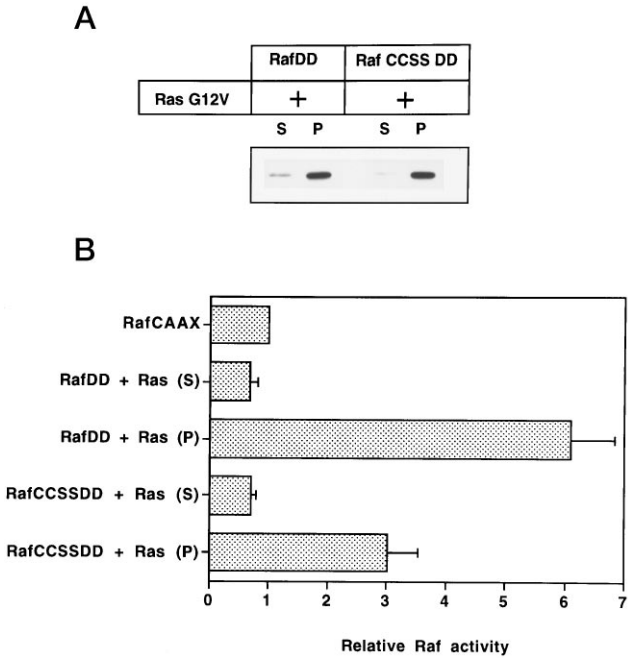

FIG. 3. **Zinc finger mutations compromise the activation of RafDD at the plasma membrane.** *A*, EXV plasmids encoding FLAG epitope-tagged Raf, RafDD, and RafCCSSDD were electroporated into COS1 cells with (+) and without (−) EXV RasG12V. Cells were harvested 72 h later after an 18-h incubation in serum-free medium and fractionated into cytosolic (S100) and membrane (P100) fractions. 20 μg of each S100 fraction (*S*) and an equal proportion of the corresponding P100 fraction (*P*) were resolved by SDS-PAGE, immunoblotted with an anti-FLAG monoclonal antibody, and developed using enhanced chemiluminescence. *B*, membrane fractions (*P*) from COS cells expressing RafCAA*X*, RafDD + RasG12V, RafCCSSDD + RasG12V, and cytosolic fractions (*S*) from COS cells expressing RafDD + RasG12V and RafCCSSDD + RasG12V were normalized for Raf content using ^{125}I immunoblotting and assayed for Raf activity. The expression of Ras was confirmed by immunoblotting. The graph shows mean activity (± S.E., n = 12–14) of Raf assayed in membranes (*P*) or Raf immunoprecipitated from cytosol (*S*) relative to the activity of membrane-localized RafCAA*X* (=1) measured in each independent experiment. For further experimental details, see the legend to Fig. 2*D* and "Experimental Procedures."

This result suggested that, in addition to recruiting Raf to the plasma membrane for tyrosine phosphorylation, Ras has a subsequent role in effecting full activation of membrane-localized, tyrosine-phosphorylated Raf or membrane-localized RafDD. If so, Raf mutations that interfere with Ras binding would be expected to adversely affect the activation of RafCAA*X*DD by RasG12V. To test this hypothesis, R89L and C165S,C168S mutations were introduced into RafCAA*X*DD, and the mutant proteins were expressed in COS cells with and without RasG12V. Membrane fractions were prepared, normalized for Raf content, and assayed for Raf activity. Fig. 4*B* shows that the specific activities of RafCAA*X*R89LDD and RafCAA*X*-CCSSDD are both reduced to approximately 70% that of RafCAA*X*DD. However, the response of these mutants to RasG12V is strikingly different. Coexpression with RasG12V increases the activity of RafCAA*X*R89LDD some 3-fold, close to the activities of RafCAA*X*DD coexpressed with RasG12V and RafDD recruited to the membrane by RasG12V. In contrast, coexpression with RasG12V has no effect on the activity of RafCAA*X*-CCSSDD. We conclude that Ras plays a critical role in the activation of membrane-localized RafDD (and by analogy tyrosine-phosphorylated membrane-localized Raf) and that this

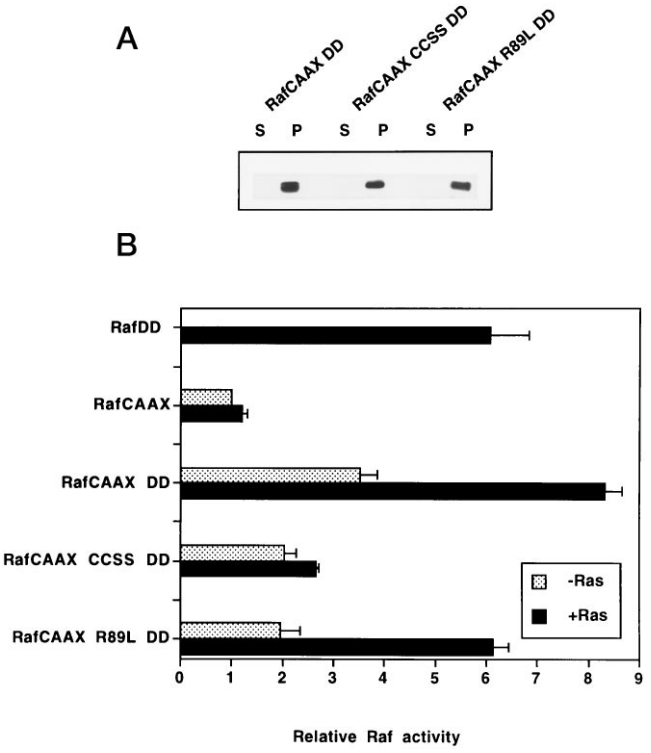

FIG. 4. **The Raf zinc finger is required for Ras-dependent membrane activation.** *A*, EXV plasmids encoding FLAG epitope-tagged RafCAA*X*DD, RafCAA*X*CCSSDD, and RafCAA*X*R89LDD were electroporated into COS1 cells without EXV RasG12V. Cells were harvested 72 h later after an 18-h incubation in serum-free medium and fractionated into cytosolic (S100) and membrane (P100) fractions. 20 μg of each S100 fraction (*S*) and an equal proportion of the corresponding P100 fraction (*P*) were resolved by SDS-PAGE, immunoblotted with an anti-FLAG monoclonal antibody and developed using enhanced chemiluminescence. *B*, membrane fractions from COS cells expressing RafDD + RasG12V, RafCAA*X* ± RasG12V, RafCAA*X* DD ± RasG12V, RafCAA*X*-CCSSDD ± RasG12V, and RafCAA*X*89LDD ± RasG12V were normalized for Raf content using ^{125}I immunoblotting and assayed for Raf activity. The expression of Ras was confirmed by immunoblotting. The graph shows mean activity (± S.E., n = 4–27) of Raf relative to the activity of membrane-localized RafCAA*X*−RasG12V (=1) measured in each independent experiment. For further experimental details, see the legend to Fig. 2*D* and "Experimental Procedures."

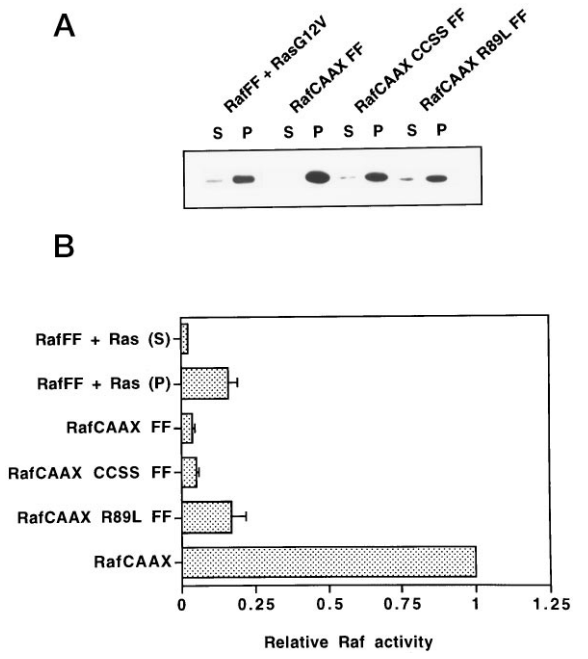

FIG. 5. **RafFF is minimally activated following plasma membrane localization.** *A*, EXV plasmids encoding FLAG epitope-tagged RafCAA*X*FF, RafCAA*X*CCSSFF, and RafCAA*X*R89LFF were electroporated into COS1 cells without EXV RasG12V. Cells were harvested 72 h later after an 18-h incubation in serum-free medium and fractionated into cytosolic (S100) and membrane (P100) fractions. 20 μg of each S100 fraction (*S*) and an equal proportion of the corresponding P100 fraction (*P*) were resolved by SDS-PAGE, immunoblotted with an anti-FLAG monoclonal antibody, and developed using enhanced chemiluminescence. *B*, membrane fractions (*P*) from COS cells expressing RafCAA*X*, RafFF + RasG12V, RafCAA*X*FF, RafCAA*X*CCSSFF, RafCAA*X*R89-LFF, and cytosolic fractions (*S*) from COS cells expressing RafFF + RasG12V were normalized for Raf content using ^{125}I immunoblotting and assayed for Raf activity. The graph shows mean activity (± S.E., n = 8–15) of Raf assayed in membranes (*P*) or Raf immunoprecipitated from cytosol (*S*) relative to the activity of membrane-localized RafCAA*X* (=1) measured in each independent experiment. For further experimental details, see the legend to Fig. 2*D* and "Experimental Procedures."

Ras- mediated activation mechanism requires an intact Raf zinc finger.

Finally we examined whether Raf that cannot be phosphorylated on tyrosines 340 or 341 could still be activated by membrane recruitment. The mutations Y340F,Y341F were introduced into Raf, RafCAA*X*, RafCAA*X*CCSS, and RafCAA*X*R89L, and the constructs were expressed in COS cells with or without RasG12V. RafFF was recruited to the plasma membrane by RasG12V to the same extent as wild type Raf and all of the RafCAA*X*FF proteins localized to the P100 fraction. Fig. 5 shows that recruitment of RafFF to the P100 fraction by RasG12V resulted in minimal activation; similarly RafCAA*X*FF, RafCAA*X*R89LFF, and RafCAA*X*CCSSFF all had very low activities. Coexpression of RasG12V with RafCAA*X*FF had no effect on RafCAA*X*FF activity (data not shown). We conclude that tyrosine residues 340 or 341 may be critical for all plasma membrane-mediated Raf activation mechanisms.

DISCUSSION

The interaction of Ras with the N terminus of Raf can be disturbed by a mutation in the minimal Ras binding domain or by mutations of the zinc finger in the adjacent CRD. Crystallography and NMR analysis suggest that the RBD (Raf residues 51–131) and the CRD (Raf residues 139–184) represent discrete Raf protein domains (5, 7, 25). There is general agreement that the effector loop of Ras binds the RBD in a GTP-dependent, prenyl-independent manner (2–4). Conversely, there is debate as to the sites of interaction of the CRD with Ras. Brtva *et al.* (9) conclude that the isolated zinger finger (Raf residues 139–186) interacts with Ras switch 1, is GTP-dependent, and does not require Ras to be prenylated. Drugan *et al.* (11) show that Ras switch 2 also interacts with the isolated Raf zinc finger (residues 139–186) and does not require Ras to be prenylated. Hu *et al.* (10) show GTP-independent binding of prenylated Ras to the isolated zinc finger (residues 132–206) and show that switch 1 mutations had no effect on this interaction. Most recently, Luo *et al.* (12) also show that high affinity interaction of full-length Raf with Ras required an intact zinc finger and Ras prenylation. Taken together, these data argue for two sites of interaction between Ras and Raf, the RBD binding to Ras switch 1 in a GTP conformation, and a more complex binding site for the zinc finger that may comprise switch 2 sequences flanking switch 1 and the C terminus of Ras.

The current model for the activation of Raf-1 by Ras envisages the recruitment of Raf from the cytosol to the plasma membrane where Raf is activated by a combination of tyrosine phosphorylation on Tyr-340 and Tyr-341 and other mechanisms (21) that may include dimerization (26, 27) or interaction with 14-3-3 proteins (28–31). There are two separate aspects of

Raf activation that can therefore be measured: the recruitment of Raf from the cytosol to the plasma membrane and the subsequent activation of plasma membrane-localized Raf. In this study we addressed the relative contribution of the two sites of Ras/Raf interaction to membrane recruitment and Raf activation *in vivo*.

The role of the RBD is limited to that of recruiting Raf to the plasma membrane. The mutation R89L completely abrogates the ability of the RBD to bind RasGTP and has been shown previously to block activation of Raf by oncogenic mutant Ras (6). In mammalian cells, the R89L mutation also blocks activation of Raf by activated Src (21). However, RafR89L targeted to the plasma membrane using Ras C-terminal localization signals is activated to the same extent as wild type Raf recruited by Ras or plasma membrane-targeted wild type Raf (this study and Ref. 21). Thus, once Raf is recruited to the plasma membrane, the RBD is dispensable for subsequent activation. The R89L mutation itself has no detrimental effect on the kinase domain because RafR89L is activated by membrane targeting to the same extent as wild type Raf. In contrast, the Raf zinc finger is completely dispensable for plasma membrane recruitment by oncogenic mutant Ras. These results exclude any significant role for the zinc finger in mediating membrane interactions *in vivo*.

Although the zinc finger is not required for plasma membrane recruitment, it serves at least two functions in Raf activation. We found that the basal kinase activity of cytosolic RafCCSS is 50% that of wild type Raf and that membrane localization of RafCCSS, either by RasG12V or by membrane targeting, increases kinase activity significantly less than wild type Raf. The basal activity of Raf in the cytosol is governed by the level of background phosphorylation of Tyr-340 and Tyr-341; phosphorylation of Tyr-340 and Tyr-341 is moderately increased when Raf is recruited to the plasma membrane and colocalized with relatively quiescent tyrosine kinases and further increased if specific tyrosine kinases, *e.g.* Src are activated (21). The reduction in the basal activity of cytosolic RafCCSS and the suboptimal activation on membrane recruitment could therefore reflect a reduction in the basal level of tyrosine phosphorylation and a reduced increase in tyrosine phosphorylation on membrane localization. This interpretation is supported by our observation that the elevated basal activity of Raf with aspartic acid substitutions at tyrosines 340 and 341 is unaffected by mutations of the zinc finger. This result also shows that the Raf kinase domain is not distorted by aberrant folding resulting from the C165S,C168S mutations. The same conclusion was reached by Luo *et al.* (12), who show that the whole Raf zinc finger can be replaced with the zinc finger of protein kinase Cγ without any compromise of the Raf kinase domain. Taken together, these data indicate that the Raf zinc finger may be required either for optimal interaction with tyrosine kinases or for inhibiting interaction with phosphatases. In this context, it is interesting to note that the CRD may provide a binding site for 14–3-3 proteins (32), which, when complexed with activated Raf, can prevent dephosphorylation and deactivation by tyrosine and serine phosphatases (33).

Maximum activation of Raf kinase is achieved by a combination of Y340D,Y341D mutations to simulate tyrosine phosphorylation plus recruitment to the plasma membrane by RasG12V. The different activities of the RafDD mutants described here can be accounted for if there are two separate plasma membrane activation mechanisms. One mechanism accounts for the greater activity of RafDD recruited to the membrane by RasG12V over membrane-targeted RafCAA*X*DD. This mechanism may reflect a direct interaction between Ras and the Raf zinc finger because coexpression of RasG12V fully activates RafCAA*X*DD but only if the zinc finger is intact. A striking control is provided by RafCAA*X*R89LDD, which is competent for Ras interaction with the zinc finger but not with the RBD. RafCAA*X*R89LDD is activated by RasG12V to approximately the same extent that RafCAA*X*DD is activated by RasG12V. RafCCSSDD recruited to the membrane by Ras has the same activity as RafCAA*X*DD because, although Ras is present, the zinc finger is not, and the necessary interaction between Ras and RafCCSSDD cannot occur. The second mechanism accounts for the increment in activity between cytosolic RafDD and RafCAA*X*DD and results directly from membrane recruitment. The recruitment is Ras- and RBD-dependent, but the actual activation at the membrane is Ras- and zinc finger-independent since the activities of RafCAA*X*CCSSDD and RafCAA*X*R89LDD and RafCAA*X*DD are similar. Dent *et al.* (18) demonstrate a similar Raf activation mechanism *in vitro*. We contend that these activation mechanisms reflect the membrane regulation of tyrosine-phosphorylated Raf-1. However, B-Raf has aspartic acid in place of tyrosine at residues 447 and 448 (homologous to 340 and 341 in Raf-1) and in consequence has an elevated basal kinase activity. The same activation mechanisms may therefore operate when B-Raf is recruited to the plasma membrane by RasG12V as operate for RafDD.

Consistent with previous reports (20, 21), we show here that replacement of tyrosines 340 and 341 with phenylalanine to prevent their phosphorylation almost completely abolishes the basal activity of RafFF. Moreover, minimal activation occurs when RafFF is targeted to the plasma membrane or recruited by RasG12V. These data indicate that 340 and 341 must be available for phosphorylation for any significant activation to occur at the plasma membrane. Whereas other interpretations cannot be excluded, it seems most likely that the first event that occurs on recruitment of Raf to the plasma membrane by Ras or by membrane targeting is tyrosine phosphorylation. If, as discussed above, zinc finger mutations compromise both initial tyrosine phosphorylation and subsequent activation by RasGTP of membrane-localized tyrosine-phosphorylated Raf, then the activation of Raf-1 by the epidermal growth factor receptor would be severely compromised by a zinc finger mutation even though plasma membrane recruitment is unaffected. The recent study by Luo *et al.* (12) confirms this prediction.

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