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Phosphorylation of Raf-1 Serine 338-Serine 339 Is an Essential Regulatory Event for Ras-Dependent Activation and Biological Signaling

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Activation of the Raf serine/threonine protein kinases is tightly regulated by multiple phosphorylation events. Phosphorylation of either tyrosine 340 or 341 in the catalytic domain of Raf-1 has been previously shown to induce the ability of the protein kinase to phosphorylate MEK. By using a combination of mitogenic and enzymatic assays, we found that phosphorylation of the adjacent residue, serine 338, and, to a lesser extent, serine 339 is essential for the biological and enzymatic activities of Raf-1. Replacement of S338 with alanine blocked the ability of prenylated Raf-CX to transform Rat-1 fibroblasts. Similarly, the loss of S338-S339 in Raf-1 prevented protein kinase activation in COS-7 cells by either oncogenic Ras[V12] or v-Src. Consistent with phosphorylation of S338-S339, acidic amino acid substitutions of these residues partially restored transforming activity to Raf-CX, as well as kinase activation of Raf-1 by Ras[V12] or v-Src. Two-dimensional phosphopeptide mapping of wild-type Raf-CX and Raf-CX[A338A339] confirmed the presence of a phosphoserine-containing peptide with the predicted mobility in the wild-type protein which was absent from the mutant. This peptide could be quantitatively precipitated by an antipeptide antibody specific for the 18-residue tryptic peptide containing S338-S339 and was demonstrated to contain only phosphoserine. Phosphorylation of this peptide in Raf-1 was significantly increased by coexpression with Ras[V12]. These data demonstrate that Raf-1 residues 338 to 341 constitute a unique phosphoregulatory site in which the phosphorylation of serine and tyrosine residues contributes to the regulation of Raf by Ras, Src, and Ras-independent membrane localization.

The Ras GTPases are crucial components of a transmembrane signaling apparatus involved in the control of development and cell division (3). Deregulation of the Ras signaling cascade can result in oncogenic cellular transformation through the constitutive activation of one or more downstream effectors (4, 28). Although a growing number of signaling proteins have been implicated as being subject to direct activation by Ras-GTP, the Raf serine/threonine kinases are considered to be the primary Ras effectors involved in the proliferation of animal cells (2, 9). The Raf protein kinases are best characterized for their role as upstream regulators of the vertebrate MAP kinase (ERK) signaling cascade (28). Raf-1 directly phosphorylates and activates the protein kinase MEK, which in turn phosphorylates and activates ERK1 and ERK2.

The three Raf genes, *raf-1*, B-*raf*, and A-*raf*, have been identified as viral and cellular oncogenes capable of inducing a Ras-like transformed phenotype (18). The three Raf family members share three homologous structural domains designated conserved region 1 (CR1), CR2, and CR3. CR1 contains a Ras binding domain and an adjacent zinc coordination site. Isoprenylated Ras interacts with Raf-1 through multiple contacts in both the Ras binding domain and the zinc binding region, resulting in the plasma membrane localization and activation of Raf (6, 8, 19). CR2 is a site of regulatory phosphorylation and association with the 14.3.3 protein (32). CR1 and CR2 are located in the amino-terminal 300 amino acids of Raf-1 and have been shown to constitute a negative regulatory domain. Removal of CR1 and CR2 results in oncogenic activation of Raf-1 (17). CR3 is the actual kinase domain and also associates with 14.3.3 (15, 34).

Normally localized in the cytosol in an inactive form, Raf-1 associates with Ras at the plasma membrane following growth factor-induced Ras guanine nucleotide exchange (39). Genetically engineered Raf-1, localized to the plasma membrane through posttranslational modification with myristate or isoprenoid, is active in the absence of Ras-GTP. This observation suggests that Ras functions primarily as a plasma membrane docking protein (17, 24, 38). Once at the membrane, Raf-1 becomes catalytically activated through a complex and still largely undefined mechanism. These activation steps probably include both phosphorylation of CR3 and a conformational change which relieves the inhibition imposed by the Raf-1 amino terminus (11, 18, 32). Lastly, lipid factors may bind to partially activated Raf-1, resulting in full activation (11).

Raf-1 activation requires phosphorylation, since treatment of active Raf-1 with protein phosphatases specific for either phosphoserine or phosphotyrosine results in loss of kinase activity (10, 20). Raf-1 is predominantly phosphorylated in vivo on serines 43, 259, and 621 (32). Phosphorylation of serines 43 and 259 has negative regulatory functions related to inhibition by protein kinase A and 14.3.3 binding. Serine 621 appears to have multiple roles. It is essential for Raf activation, but phosphorylation of this residue by protein kinase A or Raf-1 itself is associated with down regulation of Raf kinase activity (30). Phosphorylated serine 621 has been proposed to bind 14.3.3 (34). Raf-1 activation might then involve rearrangement of 14.3.3 binding sites accompanying or inducing a conformational change.

Potential inducible activators of Raf include protein kinase Cα (PKCα) and Src family tyrosine kinases (21, 32). PKCα

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phosphorylates Raf-1 on S499, which results in stimulation of catalytic activity in vitro. A potential physiological role for PKCα in the Raf pathway is indicated by the ability of Raf-1 and PKCα to transform fibroblasts when transfected together (21). However, the in vitro activation of Raf-1 by membranes from cells transformed by Ras and Src does not require either S499 or PKCα (11, 26). A more definitive regulatory role has been demonstrated for the Src-dependent tyrosine phosphorylation of Raf-1 residues 340 and 341 (14, 27). Phosphorylation of Y340 and Y341 strongly activates the kinase and transforming activities of full-length Raf-1. v-Src-dependent phosphorylation of Raf-1 Y340-Y341 has been shown to be dependent upon colocalization with Ras-GTP at the plasma membrane. However, replacement of Y340 and Y341 with phenylalanine has no effect on the ability of a truncated Raf-1 CR3 fragment to transform fibroblasts, although v-Src-dependent phosphorylation dramatically increases the catalytic activity of this fragment (14, 27). Furthermore, the physiological significance of Y340 and Y341 phosphorylation is uncertain since phosphotyrosine is difficult to detect on Raf-1 activated by growth factors in mammalian tissue culture.

Based on these results, we speculated that there may be other phosphorylation sites within CR3 which fully regulate kinase activity. Potential sites of serine phosphorylation were identified at positions 338 and 339 on the basis of sequence conservation among Raf family amino acid sequences. In this study, we found that serines 338 and 339 form an essential site of regulated phosphorylation important for both the biological and catalytic functions of Raf-1. We propose that Raf-1 residues 338 to 341 constitute a unique site of coordinate serine and tyrosine phosphorylation important for the regulation and biological function of the Raf-1 protein kinase.

MATERIALS AND METHODS

DNA manipulations: cloning and mutagenesis. Raf-1 was tagged at the amino terminus with a Myc epitope (EQKLISEEDL) (22). Some constructs also contained a Ki-Ras membrane localization sequence (KDGKKKKKKSKTKCVIM) at the carboxy terminus (16). All mutations in Raf-1 were made by using the Altered Sites Mutagenesis kit (Promega Corp.) and confirmed by DNA sequencing with Sequenase (Amersham Life Science Inc.) or *Taq* polymerase (Promega Corp.). Raf-1 constructs were subcloned into mammalian expression vector pBabepuro (31) or pcEXV-3 (29).

Cell culture. Rat-1 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum and 5% calf serum. The focus-forming ability of Raf-1 constructs was assayed by transfecting Rat-1 cells with 10 μg of desired DNA with a calcium phosphate transfection kit (Life Technologies, Inc.). Cells were split 2 days after transfection by transferring 1/10 of their volume to a plate containing medium supplemented with puromycin (2.5 μg/ml) and the remainder to a 150-mm-diameter plate without drug selection. Foci were counted after staining the plates 3 weeks posttransfection. Puromycin-resistant colonies were also counted to ensure equivalent transfection efficiencies for all of the DNAs. Independent colonies or foci were also picked and grown in DMEM plus puromycin for later clonal analysis. COS-7 cells were maintained in DMEM supplemented with 10% calf serum in a 10% CO_2 atmosphere. These cells were transfected by electroporation (7) with a Gene Pulser (Bio-Rad Corp.).

Immunoblotting. The expression levels of Myc-tagged Raf proteins in different cell lines were determined by lysing cells in buffer A (20 mM Tris-Cl [pH 8.0], 2 mM EDTA, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 1% [vol/vol] Triton X-100, 10% [vol/vol] glycerol, 1 mM phenylmethylsulfonyl fluoride, 1-μg/ml pepstatin A, 1-μg/ml leupeptin, 2.2-μg/ml aprotinin) (23). The lysate was cleared by centrifugation at 8,800 × *g* for 10 min at 4°C. Protein concentrations were determined by bicinchoninic acid assay (Pierce Chemical Company), and equal amounts of lysate were electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Blots were probed with the 9E10 anti-Myc antibody (13) (a gift from Robert Deschenes) and then with ECL (Amersham Life Science, Inc.). Films were scanned and analyzed with Bio Image IQ software to allow estimation of relative expression levels.

Peptide antisera. Antibodies were raised against a multiple antigenic peptide (synthesized by Research Genetics) containing Raf-1 residues 337 to 354 (DSSYYWEIEASEVMLSTR) in two New Zealand White rabbits. Following prebleeding, each animal was injected intradermally with 1 mg of peptide resuspended in 2 ml of 50% complete Freund's adjuvant. Three and six weeks later, each animal was inoculated subcutaneously with 1 mg of peptide in a 2-ml volume of 50% incomplete Freund's adjuvant. At 9 weeks, a final inoculation was administered subcutaneously with 0.5 mg of peptide in 2 ml of 50% incomplete Freund's adjuvant. Animals were bled at 10 and 12 weeks. Immune serum from each rabbit was determined to recognize Raf-1 by immunoprecipitation. Preimmune sera had no reactivity towards Raf-1.

Raf kinase assays. Raf kinase levels were determined by a previously described assay (38), with modifications. COS-7 cells (3×10^6 per transfection) were electroporated with 10 μg of plasmid DNAs 2 days prior to the assay. These cells were lysed in a hypotonic lysis buffer (38), and protein concentrations in the cleared lysates were determined by Bradford assay (Bio-Rad Corp.). Rat-1 cell lines were lysed as described in the section on immunoblotting. Prior to the start of a set of assays, all lysates were normalized with respect to protein concentration. Assays were performed as previously described (38). Radioactivity incorporated into the myelin basic protein substrate was corrected to reflect the actual amount of Myc-tagged Raf as determined by immunoblotting of the lysate.

Phosphopeptide mapping. Phosphopeptide mapping was performed as described by Boyle et al. (5), with modifications (1). Clonally selected Rat-1 cell lines expressing Raf-CX and Raf-CX[A338A339] or COS-7 cells transfected with various Raf-1 and Ras[V12] constructs were grown on 150-mm-diameter plates to approximately 80% confluence. Cells were starved for 2 h in phosphate-deficient medium and then labelled for 2 to 2.5 h with 2.25 mCi of [^{32}P]orthophosphate (ICN Pharmaceuticals Inc.). Cleared lysates were prepared as described in the section on immunoblotting. Myc-tagged Raf proteins were immunoprecipitated by incubation with the 9E10 antibody for 4 to 8 h, and then immune complexes were collected with GammaBind Plus Sepharose beads (Pharmacia Biotech Inc.). Immune complexes bound to these beads were washed four times with buffer A, solubilized in SDS sample buffer, and resolved on an SDS–7.5% polyacrylamide gel. Following electrophoresis, the gel was frozen and labelled Raf protein was detected by autoradiography. The Raf band was excised, and the protein was eluted into 0.5% SDS at 37°C overnight. The eluate was lyophilized and resuspended in 100 μl of water. RNase A (150 μg) was added as a carrier, and the protein was precipitated by sequential addition and mixing of 600 μl of methanol, 250 μl of chloroform, and 300 μl of water. Following microcentrifugation for 10 min, the organic phase was removed and 500 μl of methanol was added to precipitate the protein. The pellet was washed twice with 95% methanol and air dried. Performic acid oxidation and trypsin digestion were performed exactly as previously described (5). After trypsin cleavage, the samples were lyophilized four to six times from distilled water and then dissolved in 300 μl of pH 4.72 buffer (5% *n*-butanol, 2.5% pyridine, 2.5% acetic acid) and centrifuged to remove insoluble material. The supernatants were then lyophilized, and Cerenkov radiation was counted. The samples were dissolved in distilled water, and 800 to 1,500 counts were spotted onto cellulose plates (20 by 20 cm; EM Science 5716). Samples were analyzed on an HTLE-7000 thin-layer electrophoresis (TLE) apparatus (C.B.S. Scientific Corp.) by following the manufacturer's instructions. TLE was performed in 1% $(NH_4)_2CO_3$ buffer (pH 8.9) at 1,000 V for 25 min. Second-dimension chromatography was carried out in phosphochromatography buffer (37.5% *n*-butanol, 25% pyridine, 7.5% acetic acid) for 7.5 h at room temperature.

Immunoprecipitation of the Raf-1 337-354 phosphopeptide. A procedure based on the method described by Boyle et al. (5) was developed for immunoprecipitation of the phosphopeptide containing serines 338 and 339. Antibodies directed against the 337-354 peptide were bound to protein A-Sepharose. A 20-μl volume of preimmune or immune serum was incubated with 20 μl of beads (Pharmacia Biotech Inc.) in 100 mM NH_4HCO_3 (pH 7.2). After 2 h, the beads were washed twice with 100 mM NH_4HCO_3 (pH 7.2) to remove unbound material. Prior to addition to the antibody-bead complex, the pH of the tryptic digests was adjusted with dilute acetic acid to pH 7.2. Immunoprecipitations were set up in 100-μl final volumes containing 100 mM NH_4HCO_3 (pH 7.2), the tryptic digest, 1 mM phenylmethylsulfonyl fluoride, and soybean trypsin inhibitor (Worthington Biochemical Corp.) equal in weight to the amount of trypsin present. Immunoprecipitations were carried out for 3 h at 4°C. Beads were pelleted by centrifugation, and approximately 80 μl of unbound material was transferred to a fresh tube and frozen. This material was processed for mapping in the same way as the total digest. The remainder of the unbound material was removed, and the beads were washed four times with 100 μl of NH_4HCO_3 (pH 7.2). Bound peptides were eluted from the beads in 60 μl of pH 1.9 buffer (7.8% glacial acetic acid, 2.2% formic acid). The eluate was transferred to a fresh tube, and the elution was repeated. The combined eluates were frozen and lyophilized. Immunoprecipitated peptides were washed by repeated lyophilization from water, dissolved in 200 μl of pH 4.72 buffer, vortexed well, and centrifuged at 15,000 × *g* for 5 min. The supernatant was transferred to a 600-μl microcentrifuge tube, leaving behind about 10 μl containing particulate material, and lyophilized again. The sample was brought with water or 40% acetonitrile to a concentration which allowed spotting of at least 10 cpm. We found that addition of trypsinized RNase A (15 to 30 μg) to the sample just prior to spotting reduced streaking in the first dimension. This amount of RNase A was similar to that which was present in mapping samples from the original tryptic digest. TLE and thin-layer chromatography (TLC) were carried out as described above. Autoradiography was performed with BioMax MS film and screens (Eastman Kodak Company) to provide maximum sensitivity.

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Human Raf-1        331-RPRGQRDSSYYWEIEASEVM-350
Rat A-Raf          290-knlGyRDSgYYWEvppSEVq-309
Human B-Raf        323-ktlGrRDSSddWEIpdgqit-342
Drosophila Raf     338-llRdaksSegnWnIlAeEil-357
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FIG. 1. Alignment of sequences of human, rat, and *Drosophila* Raf proteins surrounding amino acids 340 and 341. The conserved serine at position 338 is indicated in boldface. Tyrosines or acidic residues at positions 340 and 341 are underlined.

Phosphoamino acid analysis of tryptic phosphopeptides. The phosphoamino acid composition of tryptic peptides was analyzed by the method of Boyle et al. (5), with modifications. Tryptic peptides were recovered from TLC plates, following autoradiography, by scraping the matrix that corresponded to spots and collecting it with a zone recovery pipette. Peptides were eluted from the material gathered on the pipette frit with 1.3 ml of 50% pyridine. Insoluble material was removed by a 5-min spin at maximum speed in a microcentrifuge, and the phosphopeptide solution was removed and lyophilized. Tryptic peptides were recovered from protein A-Sepharose beads containing antibody-peptide immune complexes by elution with pH 1.9 buffer as described above. The eluate was frozen and lyophilized. Recovered tryptic peptides were taken up in 100 μl of 6 N hydrochloric acid and incubated at 110°C for 2.5 h. After cooling to room temperature, the samples were lyophilized, redissolved in 1 ml of water, and lyophilized again. The digests were taken up in 10 μl of a solution containing 1 mg each of cold phosphoserine, phosphothreonine, and phosphotyrosine per ml, 100-μg/ml xylene cyanole, and 500-μg/ml 2,4-dinitrophenol–lysine. Finally, digests were spotted on cellulose plates (20 by 20 cm; EM Science 5716) and phosphoamino acids were resolved by one-dimensional TLE in pH 3.5 buffer (5% glacial acetic acid, 0.5% pyridine) at 1,000 V for 90 min. Nonradiolabelled phosphoamino acid standards were visualized by spraying the plates with ninhydrin stain (0.2% [wt/vol] in ethanol) and then incubating them at 80°C for 20 min. Radiolabelled phosphoamino acids were visualized by autoradiography with Hyperfilm-MP (Amersham Life Science, Inc.).

RESULTS

Serines 338 and 339 are essential for Raf-CX transforming function. Alignment of the Raf-1, A-Raf, B-Raf, and Dm-Raf polypeptide sequences revealed a conserved serine at position 338 and a semiconserved serine at position 339 in Raf-1 (Fig. 1). These two serines were considered candidates for potential regulatory phosphorylation due to their proximity to the known regulatory phosphotyrosine site at positions 340 and 341. S338 and S339 were replaced with alanines in a mutant form of Raf-1 designated Raf-CX. Raf-CX is the human Raf-1 cDNA which has been modified to encode the C-terminal 17-amino-acid plasma membrane localization signal of Ki-Ras. This sequence is isoprenylated and results in activation of the Raf-1 protein kinase by Ras-independent plasma membrane localization. The Raf-CX[A338A339] mutant was transfected into Rat-1 fibroblasts as were vector, Ras[V12], Raf-CX, and kinase-deficient Raf-CX[M375] controls. While Ras[V12] and Raf-CX transformed fibroblasts readily, the Raf-CX[A338A339] mutant was as ineffective at transforming cells as the kinase-deficient Raf-CX mutant (Table 1). To determine which of the two serines is required for Raf-CX transforming activity, residues 338 and 339 were individually replaced with alanine and the mutant polypeptides were examined for biological potency (Table 1). The Raf-CX[A339] mutant was fully transforming, while the Raf-CX[A338] mutant was defective, identifying S338 as a residue critical for Raf-CX function and as a possible phosphorylation site. The weak transforming activity observed with the Raf-CX[A338] mutant above the null background of the Raf-CX[A338A339] mutant may be a consequence of functional redundancy provided by S339.

In many cases, protein kinases regulated by phosphorylation can be constitutively activated by replacing phosphoamino acids with acidic amino acids. To see if S338 could be replaced functionally by a negatively charged residue, both glutamic acid and aspartic acid were inserted at position 338 of Raf-CX. As shown in Table 1, the resulting acidic residue substitution mutant proteins were more active in the focus formation assay than was the Raf-CX[A338] mutant. A double acidic substitution mutant Raf-CX[D338E339] was significantly more active than either Raf-CX[A338] or Raf-CX[D338]. These results support the hypothesis that S338 and, to a lesser extent, S339 are sites of regulatory phosphorylation.

Serines 338 and 339 are required for the MEK kinase activity of Raf-CX. Stable Rat-1 fibroblast lines were cloned which expressed comparable levels of either the wild-type Raf-CX or the mutant Raf-CX[A338A339] protein. These clonal lines were analyzed for cellular morphology and Raf protein kinase activity. The wild-type Raf-CX-expressing cell lines appeared highly transformed and displayed a loss of contact inhibition similar to that shown by Ras-transformed Rat-1 cells (data not shown). The Raf-CX[A338A339]-expressing lines were indistinguishable morphologically from normal or vector-transfected Rat-1 fibroblasts. Measurement of total Raf protein kinase activity in each cell line demonstrated a large increase of activity in the Raf-CX-expressing lines but no significant increase above basal levels in the Raf-CX[A338A339]-expressing lines (Fig. 2). As determined by Western blotting, the Raf-CX-1 and Raf-CX[A338A339]-7 lines expressed similar levels of Myc-tagged protein, allowing direct comparison of catalytic activity between the wild-type Raf-CX and mutant Raf-CX[A338A339] proteins (data not shown). The highly transformed Raf-CX-5 line expressed a much higher level of catalytically active Raf protein, which was reflected by high kinase activity. A weakly transformed subline of Raf-CX[A338A339]-7 was obtained by extended passage and selection for clones which overgrew the monolayer. This subline, Raf-CX[A338A339]-7A, expressed the Raf-CX[A338A339] protein at a level comparable to that of the Raf-CX protein expressed by the highly transformed Raf-CX-5 clone. A 2.5-fold increase in Raf activity was measured in this subline, which was significantly less than was observed in the Raf-CX-5 line (Fig. 2).

These observations were confirmed by using Raf-CX proteins transiently expressed in COS-7 cells (Fig. 2). The protein kinase activity of the Raf-CX[A338A339] protein was found to

TABLE 1. Focus-forming ability of wild-type and mutant RAF-CX genes[a]

Construct	Focus formation (% of Raf-CX)[b]	Expression in Rat-1 cells
Vector	0	−
Ras[V12]	170	+
Raf	0	+
Raf-CX	100	+
Raf-CX[M375]	0	+
Raf-CX[A338A339]	0	+
Raf-CX[A338]	1.9 ± 1.6	+
Raf-CX[A339]	115 ± 23	+
Raf-CX[D338]	11.8 ± 6.8	+
Raf-CX[E338]	9.2 ± 4.3	+
Raf-CX[D338E339]	17 ± 3	ND
Raf[D338E339]	0	+

[a] Mutated genes for Raf-1 and Raf-CX cloned into the pBabepuro vector were transfected into Rat-1 cells by calcium phosphate precipitation. For focus formation, transfections were plated without drug selection and foci were counted following 2 weeks of culture incubation. Experiments were standardized to the wild-type Raf-CX gene. Typically, 10 μg of Raf-CX plasmid DNA would result in the formation of 700 foci. The expression of each protein was confirmed by Western blotting of extracts from transiently transfected Rat-1 cells by using the Myc tag antibody.

[b] The values shown are means and standard errors of the means of five experiments.

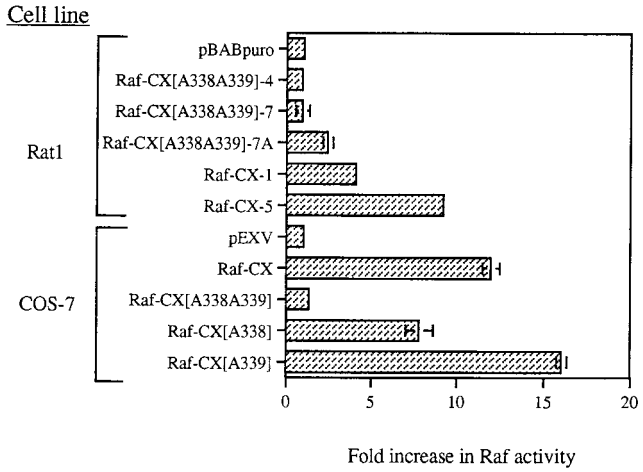

FIG. 2. Catalytic impairment of Raf-CX[A338A339] protein in Rat-1 and COS-7 cells. Rat-1 cell lines transfected with Raf-CX and Raf-CX[A338A339] were clonally selected, and expression of the recombinant protein was confirmed. Total lysates were prepared from 80% confluent cultures of each cell line and tested for Raf activity by using a coupled MEK-ERK-myelin basic protein assay. The Raf-CX[A338A339]-7 and Raf-CX-1 lines expressed similar quantities of Raf-CX protein. The Raf-CX-5 and Raf-CX[A338A339]-7A lines overexpressed each respective protein at similar levels. Raf activity is expressed as the fold increase over endogenous Raf activity alone. COS-7 cells transfected with Raf-CX, Raf-CX[A338A339], Raf-CX[A338], and Raf-CX[A339] were harvested after 3 days, and soluble extracts were tested for Raf activity by using the coupled assay. Activity is expressed as the fold increase over endogenous Raf activity following normalization to protein expression levels. These experiments are representative of three separate assays performed in duplicate.

be greatly impaired compared with that of wild-type Raf-CX. When Raf-CX lacking either S338 or S339 alone was assayed for catalytic activity in COS-7 cells, only Raf-CX[A338] had reduced activity relative to Raf-CX. However, unlike the double alanine substitution mutant protein, which was almost completely defective, Raf-CX[A338] was only 40% less active than the wild-type protein. This experiment provides additional evidence that the presence of S339 can compensate to some degree for the loss of S338.

Serines 338 and 339 are phosphorylated in Raf-CX. To directly confirm the presence of phosphoserine at position 338 or 339 in activated Raf-1, phosphopeptide maps were generated from the wild-type Raf-CX and mutant Raf-CX [A338A339] proteins immunoprecipitated from growing Rat-1 cells. Predicted mobilities (5) were calculated for the tryptic peptide containing S338-S339, as well as for phosphopeptides identified in earlier studies (33, 35). Due to its hydrophobicity, the S338-S339-containing tryptic peptide was predicted to migrate faster in the second dimension than the phosphopeptides containing the S43, S259, and S621 phosphorylation sites. Two such hydrophobic phosphopeptides were observed in maps of wild-type Raf-CX but were absent from the Raf-CX [A338A339] mutant protein (Fig. 3). Multiple experiments demonstrated that peptide 1 was reproducibly present in active Raf-CX and absent from the mutant protein. The second phosphopeptide may result from inefficient cleavage by trypsin at R336 (5) or from multiple phosphorylations. We conclude that phosphopeptide 1 is dependent upon the presence of serine at positions 338 and 339. Phosphoamino acid analysis of peptide 1 identified only phosphoserine, consistent with the proposed identity of that peptide (Fig. 4). The level of incorporation of radioactive phosphate into this peptide was much lower than that of the radioactive phosphate incorporated into the major phosphopeptides.

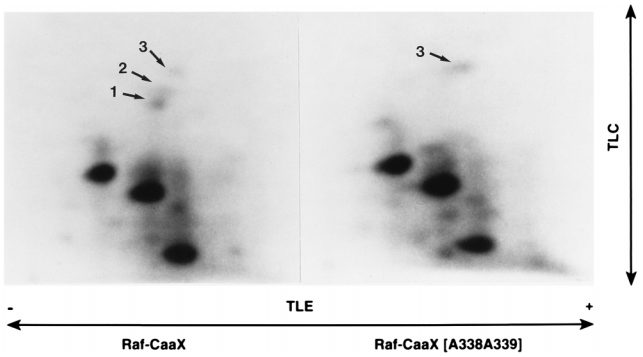

FIG. 3. Phosphopeptide mapping of Raf-CX and Raf-CX[A338A339] proteins. Clonal Rat-1 cell lines expressing Raf-CX and Raf-CX[A338A339] were starved for phosphate for 2.5 h prior to growth in medium containing [^{32}P]orthophosphate. The cells were lysed, and the Raf-CX proteins were immunoprecipitated by an anti-Myc tag antibody. The Raf-CX proteins were gel purified and digested with trypsin. Aliquots of each digest containing 1,200 cpm were applied to TLC plates and analyzed by TLE followed by TLC. Autoradiography was done for 5 days. Phosphopeptide 1 was reproducibly present in the active Raf-CX protein but absent from Raf-CX[A338A339].

Insufficient Raf protein was expressed to allow direct sequencing of the peptide implicated as containing phosphoserine at positions 338 and 339. To develop a probe specific for the S338-S339 tryptic fragment, antisera were raised in two rabbits against a synthetic peptide containing Raf-1 amino acids 337 to 354. Immune, but not preimmune, sera from both rabbits were able to immunoprecipitate Raf-1 from Rat-1 fibroblasts (data not shown). These antisera were then used to specifically immunoprecipitate the 337-354 tryptic peptide from a trypsin digest of Raf-CX protein purified from ^{32}P-labelled Rat-1 cells. Two-dimensional chromatography of the immunoprecipitate identified one predominant phosphopeptide with mobility similar to that of phosphopeptide 1, already demonstrated to be dependent upon S338-S339 (Fig. 5, Immune IP). Preimmune sera failed to precipitate this phosphopeptide (Fig. 5, Preimmune IP). The fact that phosphopeptide 1 contained phosphoserine 338-339 was confirmed by immunodepletion of this phosphopeptide from the total Raf-CX trypsin digest (Fig. 5, Preimmune UNB and Immune UNB). The immune serum also precipitated three other phos-

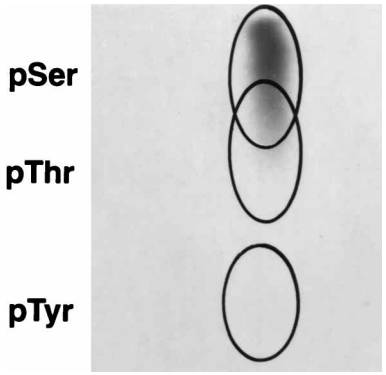

FIG. 4. Phosphoamino acid analysis of the S338-S339-dependent peptide. The phosphopeptide designated 1 in Fig. 3 was recovered from the ^{32}P-labelled tryptic fragments of the Raf-CX protein resolved on multiple two-dimensional cellulose chromatography plates. The purified phosphopeptide was acid hydrolyzed, and the products were separated by one-dimensional TLC with phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) as standards.

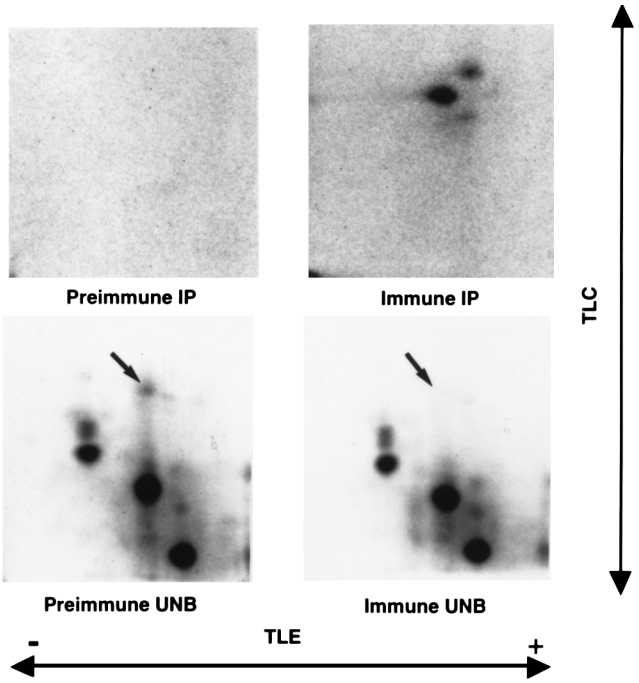

FIG. 5. Immunoprecipitation of the S338-S339-containing phosphopeptide from the trypsin-digested Raf-CX protein. The Raf-CX protein was ^{32}P labelled, purified, and trypsin digested as described in the legend to Fig. 3. The digest was divided into two aliquots containing 25,000 cpm and immunoprecipitated with either preimmune serum or immune serum raised against the 337-354 peptide. The upper panels show two-dimensional phosphopeptide maps of the immunoprecipitated material (Preimmune IP and Immune IP), while the lower panels show maps of the unbound or immunodepleted Raf-CX digest (Preimmune UNB and Immune UNB; 1,500 cpm of each). The position of the 337-354 peptide is shown by the diagonal arrows.

phopeptides that were similar in mobility and possibly representative of incomplete digestion or multiple phosphorylations. Phosphoamino acid analysis of the phosphopeptides specifically precipitated with the antipeptide serum showed only the presence of phosphoserine (Fig. 6).

Raf-1 serines 338 and 339 are required for induction by oncogenic Ras. Although replacement of S338 and S339 with alanine impaired both the catalytic activity and biological function of isoprenylated Raf-1, a role in the activation of Raf-1 by oncogenic Ras remained to be demonstrated. To approach this question, combinations of Ras[V12] with the wild-type and mutant (without the Ki-Ras C-terminal sequence) Raf-1 proteins were transiently expressed in COS-7 cells (Fig. 7). Expression of Raf-1[A338A339] or wild-type Raf-1 alone resulted in only a small increase in total Raf activity. Expression of Ras[V12] alone resulted in the induction of endogenous Raf activity and in a larger activation when cotransfected with wild-type Raf-1. Significantly, no kinase activation was observed when Ras[V12] was coexpressed with Raf-1[A338A339], demonstrating the importance of S338-S339 for induction by Ras. Replacement of both S338 and S339 with aspartic acid and glutamic acid restored Ras inducibility to Raf-1 but did not result in constitutive Raf-1 activation, as was also observed in Rat-1 fibroblasts (Table 1).

inetic data obtained from the COS cell experiments strongly suggest that phosphorylation of S338-S339 is required for activation of both the wild-type and the prenylated forms of Raf-1. One mechanism for Raf-1 activation may be that translocation to the plasma membrane results in phosphorylation of S338-S339. To test this hypothesis, Myc-tagged Raf-1 was expressed in COS-7 cells either alone or together with Ras[V12]. Following in vivo labelling with [^{32}P]orthophosphate, the Myc-tagged Raf-1 was immunoprecipitated and digested with trypsin. The resulting phosphopeptides were mapped by two-dimensional chromatography. A phosphopeptide with mobility similar to that of the 337-354 tryptic peptide from Raf-CX increased significantly in quantity when Raf-1 was coexpressed with Ras[V12] (Fig. 8A, top). Evidence for the identity of the hydrophobic peptide seen in the upper right part of Fig. 8A was provided by two separate experiments. In the first, Raf-1 [A338A339] was coexpressed with Ras[V12] (Fig. 8A, lower left). In the second, the 337-354 peptide antiserum was used to immunodeplete the 337-354 peptide from the tryptic digest of Raf-1 coexpressed with Ras[V12] (Fig. 8A, lower right). In both cases, the hydrophobic peptide was no longer present. Analysis of the immunoprecipitate from the Raf-1-Ras[V12] tryptic digest revealed the presence of the phosphorylated form of the 337-354 peptide (Fig. 8B). No phosphopeptide could be immunoprecipitated from tryptic digests of Raf-1 [A338A339] which had been coexpressed with Ras[V12]. Hence, the 337-354 peptide is phosphorylated in activated Raf-

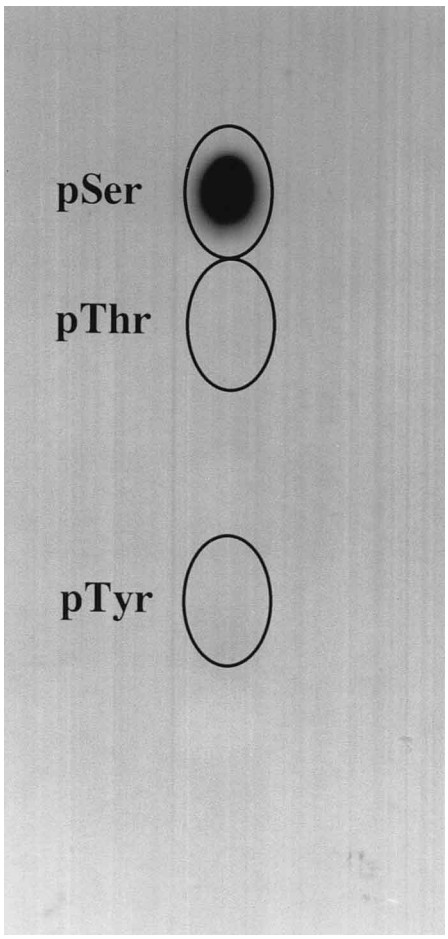

FIG. 6. Phosphoamino acid analysis of peptides precipitated from trypsin-digested Raf-CX protein by immune serum raised against the 337-354 peptide. The purified phosphopeptides shown in Fig. 5, Immune IP, were acid hydrolyzed, and the products were identified by autoradiography following one-dimensional TLC along with standards containing phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr). Shown is a scan of an autoradiogram made with a Bio-Rad 670 Imaging Densitometer with Molecular Analyst software.

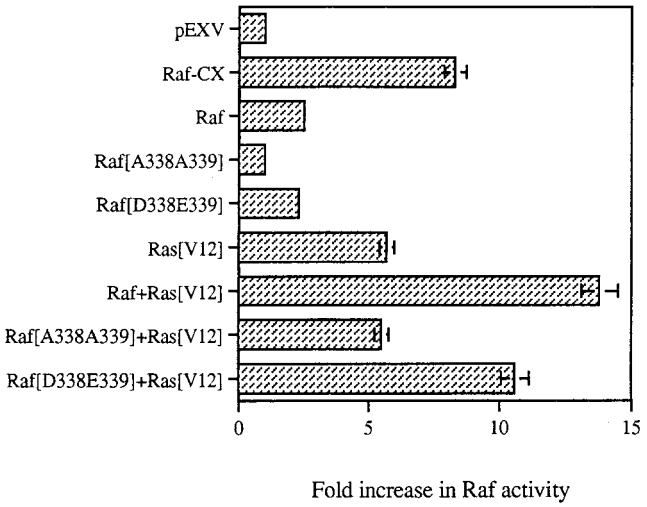

FIG. 7. Effects of replacement of Raf-1 amino acid residues S338 and S339 on kinase inducibility by oncogenic Ras[V12]. COS-7 cells were transfected with Raf-1, Raf-1[A338A339], or Raf-1[D338E339] with and without Ras[V12]. After 3 days, the cells were harvested and soluble extracts were tested for Raf activity by using the coupled assay. Activity is expressed as the fold increase over endogenous Raf activity following normalization to protein expression levels. These experiments are representative of three separate assays, each performed in duplicate.

1 in an S338-S339-dependent manner. We have not been able to immunoprecipitate a sufficient quantity of the 337-354 phosphopeptide from tryptic digests of Raf-1 coexpressed with Ras [V12] to permit phosphoamino acid analysis.

Serines 338 and 339 and tyrosines 340 and 341 are functionally distinct. Experiments with Sf9 and NIH 3T3 cells have shown that Raf-1 can be readily activated by coexpression with v-Src. Phosphorylation of Y340 or Y341 has been implicated in this process. In NIH 3T3 cells, mutations which block the binding of Raf-1 to Ras prevent Raf-1 activation by v-Src (27). Our observation that the A338A339 mutation blocks the Ras-dependent activation of Raf-1 led us to ask if this mutation would interfere with activation by v-Src. Wild-type Raf-1 and mutant Raf-1[A338A339] genes were coexpressed in COS-7 cells with v-Src, and the resulting Raf activity was measured (Fig. 9). Wild-type Raf-1 showed a large induction of activity by v-Src, while the Raf-1[A338A339] mutant was not stimulated. Double replacement of S338 and S339 with aspartic acid and glutamic acid partially restored v-Src inducibility to Raf-1. The inability of the Raf-1[A338A339] mutant to be activated by v-Src might simply be a result of altering the tyrosine kinase recognition site. Replacement of Raf-1 Y340 and Y341 with aspartic acid residues has been shown to mimic activation by v-Src (14, 27). This substitution should bypass a defect in tyrosine kinase recognition potentially introduced in the Raf-1 [A338A339] mutant. We tested this by introducing the alanine 338 mutation into the constitutively activated Raf-1 [D340D341] mutant. The kinase activity of each mutant protein was determined by transient expression in COS-7 cells (Fig. 9). As has been previously reported, the Raf-1[D340D341] mutant was highly active in the absence of both oncogenic Ras and v-Src. Replacement of S338 with alanine in Raf-1 [D340D341] reduced its activity by 65%, suggesting that the role of S338 is more complex than simply providing a recognition site for tyrosine kinases.

DISCUSSION

Regulation of the Raf protein kinases is dependent upon complicated modifications and interactions occurring primarily at the plasma membrane. Isoprenylated Ras, 14.3.3, heat shock proteins, serine/threonine and tyrosine phosphorylation, and

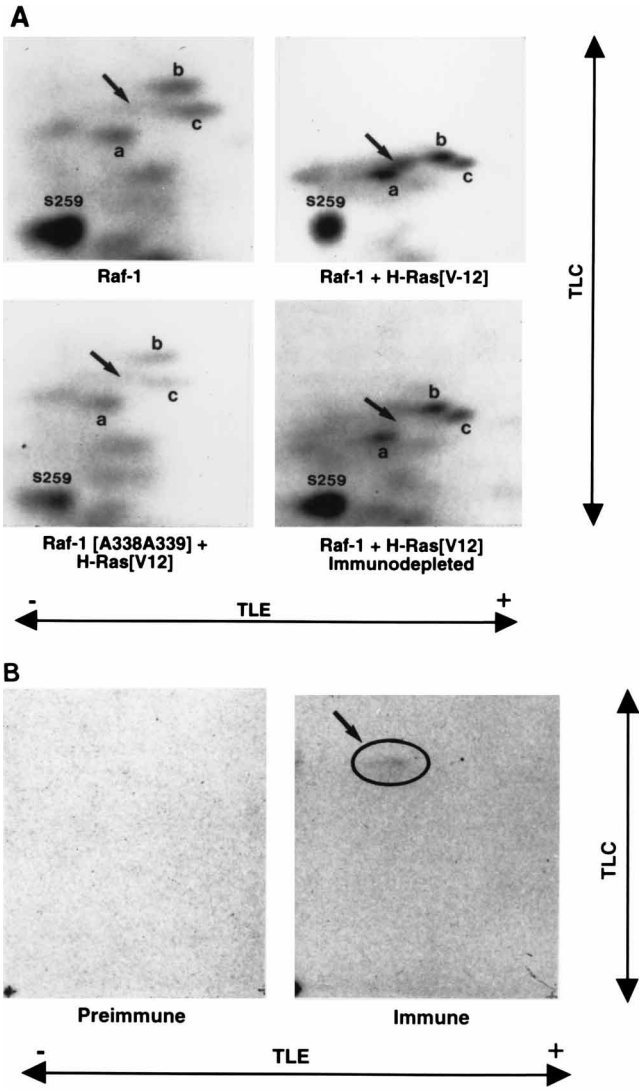

FIG. 8. (A) Phosphopeptide mapping of Raf-1 proteins coexpressed with Ras[V12] in COS-7 cells. Raf-1 or Raf-1[A338A339] was transfected into COS-7 cells alone or with Ras[V12]. Three days later, the Raf-1 protein was ^{32}P labelled, purified, and trypsin digested as described in the legend to Fig. 3. Equivalent sections of tryptic phosphopeptide maps are shown; identification of the serine 259 peptide was based on the work of Schramm et al. (35), which mapped major Raf-1 phosphorylation sites by using similar chromatography conditions. All of the samples shown are from a single experiment. Twelve hundred counts per minute were spotted on the TLC plates on the left; the plates were exposed for 15.7 days. Eight hundred counts per minute were spotted on the TLC plates on right; these plates received a 29-day exposure, which compensated for the fewer counts per minute present. The Ras[V12]-activated Raf-1 digest (upper right) was depleted of the 337-354 peptide by using the antipeptide serum to generate the lower right map. The position of the 337-354 peptide is indicated by a diagonal arrow. (B) Immunoprecipitation of the S338-S339-containing phosphopeptide from trypsin-digested Raf-1 protein activated by Ras[V12]. Tryptic digests of Ras[V12]-activated, ^{32}P-labelled Raf-1 (7,600 cpm) were immunoprecipitated with either preimmune serum or an antiserum raised against the 337-354 peptide. Immunoprecipitated phosphopeptides were detected by two-dimensional phosphopeptide mapping. Equal fractions of each immunoprecipitation were analyzed, and plates were exposed for 15.5 days. The position of the immunoprecipitated material is circled.

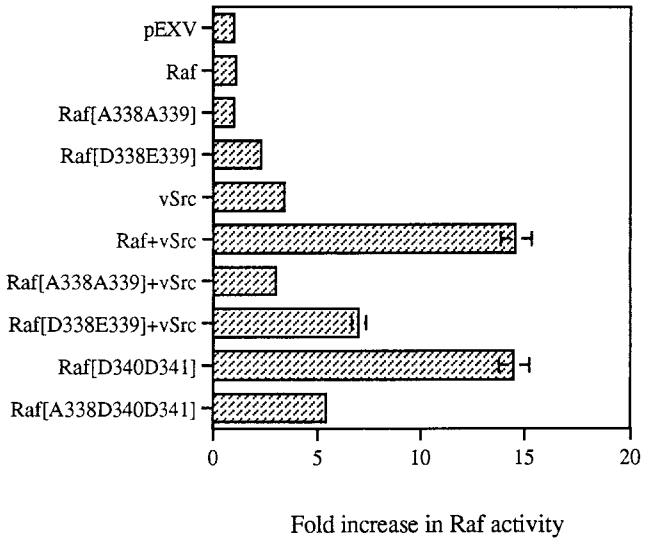

FIG. 9. Effects of replacement of Raf-1 amino acids S338 and S339 on kinase inducibility by oncogenic v-Src. COS-7 cells were transfected with Raf-1, Raf-1 [A338A339], or Raf-1[D338E339] in the presence or absence of cotransfected v-Src. The v-Src-independent mutants Raf[D340D341] and Raf[A338D340D341] were transfected alone. After 3 days, the cells were harvested and soluble extracts were tested for Raf activity by using the coupled assay. Activity is expressed as the fold increase over endogenous Raf activity following normalization to protein expression levels. These experiments are representative of multiple assays performed in duplicate.

phospholipids have all been found to play a role in the positive regulation of Raf (19, 32, 36). To simplify our investigation of Raf-1 regulation by phosphorylation, we selected isoprenylated Raf-CX as a model system for studying Ras-independent requirements for Raf activation at the plasma membrane. By introducing mutations into the Raf-CX gene, we were able to characterize the biological importance of residue-specific Raf-1 phosphorylation. By using both biological and kinetic assays to measure Raf activity, it was possible to determine the degree of kinase activation sufficient to promote cellular transformation in vitro and which regulatory phosphorylations are essential for that activation.

Protein sequence analysis of Raf family kinases revealed the presence of two highly conserved serines located adjacent to the regulatory phosphorylation site defined by Y340 and Y341. Our initial hypothesis was that phosphorylation of either S338 or S339 would result in activation of full-length Raf-1, as occurs with the v-Src-dependent phosphorylation of residues 340 and 341. According to this hypothesis, multiple protein kinases would be able to regulate Raf-1 activity through phosphorylation of this region. Our analysis of mutations in serines 338 and 339 did, indeed, demonstrate that these residues are essential for Raf-1 activation. Multiple lines of evidence support the conclusion that these are phosphorylation sites. First of all, the presence of acidic residues at positions 338 and 339 partially restored transforming function to Raf-CX while the A338A339 mutant was completely nontransforming. Similarly, nonprenylated Raf-1 protein with acidic amino acid replacement of residues 338 and 339 retained Ras and v-Src inducibility, which was lost from the Raf-1[A338A339] mutant. These results are consistent with a requirement for phosphoserine at one of these positions. An alternative explanation is that acidic amino acid substitutions at this location are less disruptive structurally than are alanine substitutions. However, alanine is normally considered the most conservative substitution for serine in contexts where phosphorylation is not occurring.

Confirmation of S338-S339 phosphorylation came through phosphopeptide mapping of Raf-CX and Raf-CX[A338A339]. An appropriately sized, hydrophobic, phosphoserine-containing peptide was observed in the Raf-CX protein but not in the Raf-CX[A338A339] mutant protein. The identity of the S338-containing phosphopeptide was confirmed by using a peptide antibody specific for the tryptic peptide containing S338-S339. Immune peptide antisera, but not preimmune sera, precipitated the phosphoserine-containing peptide and depleted the total tryptic digest of that peptide. Finally, we demonstrated that this peptide was also phosphorylated in nonprenylated Raf-1 protein which had been coexpressed with Ras[V12] in COS-7 cells. This specific peptide was not phosphorylated when the Raf-1[A338A339] mutant was coexpressed with Ras [V12]. Although we have not been able to perform phosphoamino acid analysis of the peptide from nonprenylated Raf-1, our results obtained with Raf-CX support the conclusion that S338 or S339 is inducibly phosphorylated in the process of Raf activation. Our data also suggest that although phosphorylation of S338 is most critical for Raf-1 activation, some functional redundancy is provided by phosphorylation of S339. It is interesting that the Raf-1 sequence 334-GQRDSS-339 is similar to a region of known Raf-dependent phosphorylation in MEK (213-GQLIDS-218), suggesting the possibility of auto- or transphosphorylation of S338 by Raf itself. The importance of Raf-1 oligomerization in its activation has recently been demonstrated (25).

We noted that the level of incorporation of radioactive phosphate into the 337-354 peptide was much lower than that of the radioactive phosphate incorporated into the major phosphopeptides. Strict determination of stoichiometry is probably beyond the technical limits of the mapping technique used. We consistently observed that a significant quantity of radioactivity (30 to 40%) was irreversibly bound to the walls of the microcentrifuge tubes following trypsinization. As the 337-354 peptide is very hydrophobic, it may be differentially depleted from the digest by this process. An alternative explanation requires that we accept the stoichiometric data at face value and assume that only a minor portion of the Raf-1 molecules in the cell are phosphorylated on S338 and S339. In Ras-transformed fibroblasts, only about 20% of the Raf-1 protein is associated with the plasma membrane and active and the remaining cytoplasmic pool is inactive (20). Additionally, as a key regulatory phosphorylation, phosphoserines at 338 and 339 may be rapidly turned over or required at only one specific point in the activation process.

We conclude that phosphorylation of serines 338 and 339 is an obligate but intermediate step in Raf-1 activation. Two observations support this view. First, the A338A339 substitution blocks activation of Raf-1 either by prenylation or by coexpression with oncogenic Ras or activated Src. Second, acidic replacements of S338 and S339 do not result in constitutive activation but rather promote inducibility of Raf-1 by Ras or Src. Furthermore, deletion analysis of v-Raf has previously shown that a minimal CR3 fragment of residues 336 to 648 is fully transforming whereas a fragment consisting of residues 340 to 648 is completely defective (17). Surprisingly, phosphorylation of S338 and S339 appears to be functionally distinct from phosphorylation of Y340 and Y341, despite the contiguity of these four residues. Phosphorylation or acidic amino acid replacement of Y340 and Y341 results in a powerful activation of both full-length and truncated Raf-1, although phosphorylation of Y340 and Y341 is not required for the transforming activity of a Raf-1 catalytic fragment (27, 32).

One possible role for phosphoserine at positions 338 and 339 is to function as part of a tyrosine kinase recognition sequence. Phosphorylation of either of these serines would provide a negative charge in the −2 position from Y340 or Y341, potentially enhancing substrate recognition by tyrosine kinases (12, 37). Alternatively, phosphotyrosine at positions 340 and 341 could enhance recognition of S338 and S339 by a specific protein serine kinase. We noted that replacement of S338 with alanine in the constitutively active Raf[D340D341] protein significantly reduced its activity. This observation supports a model in which phosphorylation of S338 remains functionally distinct from the adjacent tyrosine phosphorylation. Conservation of serine 338 in B-Raf and Dm-Raf in the absence of tyrosine conservation at residues 340 and 341 supports this conclusion.

The regulation of the Raf serine/threonine protein kinases is complex and involves interactions with many accessory proteins and protein kinases. Phosphorylation of Raf S338-S339 adds one more step to the known processes involved in the physiological regulation of Raf by Ras and protein tyrosine kinases. The apparent induction of S338-S339 phosphorylation by Ras suggests that this is one of several key events which occur only at the plasma membrane. It is now important to determine the actual mechanism through which phosphorylation of the clustered serines and tyrosines at positions 338 to 341 acts to regulate the activity of the Raf protein kinases.

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