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Critical Tyrosine Residues Regulate the Enzymatic and Biological Activity of Raf-1 Kinase

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The serine/threonine kinase activity of the Raf-1 proto-oncogene product is stimulated by the activation of many tyrosine kinases, including growth factor receptors and pp60$^{v\text{-}src}$. Recent studies of growth factor signal transduction pathways demonstrate that Raf-1 functions downstream of activated tyrosine kinases and p21^{ras} and upstream of mitogen-activated protein kinase. However, coexpression of both activated tyrosine kinases and p21^{ras} is required for maximal activation of Raf-1 in the baculovirus-Sf9 expression system. In this study, we investigated the role of tyrosine kinases and tyrosine phosphorylation in the regulation of Raf-1 activity. Using the baculovirus-Sf9 expression system, we identified Tyr-340 and Tyr-341 as the major tyrosine phosphorylation sites of Raf-1 when coexpressed with activated tyrosine kinases. Introduction of a negatively charged residue that may mimic the effect of phosphorylation at these sites activated the catalytic activity of Raf-1 and generated proteins that could transform BALB/3T3 cells and induce the meiotic maturation of *Xenopus* oocytes. In contrast, substitution of noncharged residues that were unable to be phosphorylated produced a protein that could not be enzymatically activated by tyrosine kinases and that could block the meiotic maturation of oocytes induced by components of the receptor tyrosine kinase pathway. These findings demonstrate that mutation of the tyrosine phosphorylation sites can dramatically alter the function of Raf-1. In addition, this is the first report that a transforming Raf-1 protein can be generated by a single amino acid substitution.

One of the key pathways involved in the transmission of proliferative, developmental, and oncogenic signals from receptor tyrosine kinases to the nucleus involves the activation of p21^{ras} and the serine/threonine kinases Raf-1 and mitogen-activated protein kinase (MAPK, also known as ERK [reviewed in references 28 and 34]). Biochemical and genetic studies have revealed that this pathway functions in many cell types in organisms as diverse as *Caenorhabditis elegans*, *Drosophila melanogaster*, *Xenopus laevis*, and mammals (8–10, 20, 42). In mammalian cells, activation of p21^{ras} and Raf-1 by receptors that stimulate cellular tyrosine phosphorylation results in the sequential activation of the serine/threonine kinase activities of MEK (5) (also known as MKK1 [50]), MAPK, and RSK (28, 34). The activation of Raf-1 in many cases is dependent upon the activity of p21^{ras} and can be induced by expression of oncogenic p21$^{v\text{-}ras}$, indicating that Raf-1 may function downstream of p21^{ras} (27, 41, 49). In addition, p21^{ras} enhances the kinase activity of Raf-1 when these proteins are coexpressed in the baculovirus-Sf9 system (48). However, for maximal activation of Raf-1 in this system, the presence of activated tyrosine kinases is also required. Because oncogenic forms of Raf-1 can phosphorylate and activate MEK, thereby activating MAPK, and RSK, these kinases are thought to function downstream of Raf-1 (7, 18). In addition, immunoprecipitates of mitogen-stimulated Raf-1 are capable of phosphorylating and activating MEK in vitro (13, 20). Raf-1 thus plays a central role in p21^{ras}-dependent signal transduction pathways.

The importance of Raf-1 in mitogenic signalling events has been demonstrated in three types of studies. First, Raf-1 that is activated by mutation can induce gene transcription, DNA synthesis, and morphological transformation (22, 24, 31). Second, expression of a dominant inhibitory *raf-1* gene can block the transforming ability of p21$^{v\text{-}ras}$ and growth factor-mediated proliferative responses (17). Third, the phosphorylation state and kinase activity of Raf-1 are enhanced by the activation of many growth factor receptors and proto-oncogene products (12, 19, 24).

The *raf-1* gene was first identified as the normal cellular counterpart of v-*raf*, the transforming gene of murine sarcoma virus 3611 (32), and is ubiquitously expressed in all cell types examined (38). Subsequently, two related mammalian *raf* genes, A-*raf* and B-*raf*, and *Xenopus*, *Drosophila*, and *C. elegans raf* genes have been identified (1, 10, 15, 21, 29). A unique characteristic of the Raf family of proteins is the presence of three conserved domains (CR1, CR2, and CR3) (33). CR1 is a region rich in cysteine residues and contains a putative zinc binding region, CR2 is a region rich in serine and threonine residues, and CR3 contains the protein kinase domain. Both CR1 and CR2 are located in the amino-terminal half of the protein, and it is this region that appears to regulate the catalytic activity of the carboxyl-terminal kinase domain, because deletion or mutation of the amino terminus activates the oncogenic potential of Raf-1 (11, 36).

In contrast to oncogenic constitutive activation, Raf-1 activation under mitogenic conditions would be expected to be mediated by reversible modifications. In mammalian cells, phosphorylation on serine, threonine, and tyrosine residues is a common mechanism used to regulate the function of proteins (14). Phosphorylation also appears to be involved in the regulation of Raf-1 activity. Treatment of cells with various growth factors and mitogens results in the rapid hyperphosphorylation and activation of the Raf-1

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kinase. While the enhancement of Raf-1 phosphorylation in mitogen-stimulated cells occurs predominantly on serine residues, increases in threonine and tyrosine phosphorylation have been observed (12, 24). We have recently identified that Ser-43, Ser-259, and Ser-621 are the major in vivo sites of Raf-1 serine phosphorylation (25). The Ser-259 site is located in the serine/threonine-rich CR2 region and is phosphorylated in response to platelet-derived growth factor (PDGF) treatment of cells. Mutation of Ser-259 results in a Raf-1 protein that is hyperactivated. In contrast, mutation of Ser-621 located in the CR3 domain inactivates Raf-1.

Phosphorylation of Raf-1 on tyrosine residues has been detected in several systems, although the importance of this modification in vivo has not yet been established. Tyrosine phosphorylation of Raf-1 has been detected in several systems, including growth factor-stimulated myeloid cells (4, 16), T cells (40, 43), fibroblasts (26), $pp60^{v\text{-}src}$-transformed fibroblasts (27), and Sf9 cells coexpressing Raf-1 and activated tyrosine kinases (48). However, the extent of Raf-1 tyrosine phosphorylation observed in growth factor-treated cells varies greatly. For example, approximately 1% of the phosphate incorporated in Raf-1 in PDGF-treated fibroblasts is phosphotyrosine (23a), while 20 to 50% of Raf-1 molecules are phosphorylated on tyrosine in interleukin-2-treated T cells (43). To determine the significance of tyrosine phosphorylation in Raf-1 function requires that the sites of phosphorylation be identified and mutational analysis be performed.

In this report, we identify, using the baculovirus-Sf9 expression system, in vivo sites of Raf-1 tyrosine phosphorylation. In addition, we examine the biochemical activity of Raf-1 proteins that contain mutations at these phosphorylation sites. Finally, we demonstrate the importance of the tyrosine sites in regulating the biological activity of Raf-1 in mammalian cells and in *Xenopus* oocytes.

MATERIALS AND METHODS

Phosphorylation site mapping and phosphoamino acid analysis. ^{32}P-labeled Raf-1 protein was isolated from Sf9 cells coexpressing Raf-1, $p21^{ras}$, and activated $pp60^{src}$ as previously described (25). ^{32}P-labeled Raf-1 was then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), eluted from the gel matrix, precipitated with trichloroacetic acid, and subjected to enzymatic digestion with trypsin or Lys-C proteases. Labeled peptides were separated and isolated by reversed-phase high-performance liquid chromatography (HPLC) with a C-18 column (25). To identify the residue phosphorylated within the isolated peptides, semiautomated amino-terminal sequence analysis was performed as previously described (25). For phosphoamino acid analysis, labeled protein and peptides were hydrolyzed with 5.7 N HCl for 70 min at 110°C. The recovered phosphoamino acids were analyzed at pH 1.9 and 3.5 (2).

Construction of Raf-1 mutants and expression constructs. The full-length pKS:cRaf and truncated pKS:Δ-N′Raf human Raf-1 constructs were used for mutagenesis (9). Site-directed mutagenesis was performed according to the procedure described by the vendor (Bio-Rad Mutagenesis Kit; Bio-Rad, Richmond, Calif.), with custom oligonucleotide primers to introduce the desired base changes. The resulting mutant constructs were subsequently sequenced to confirm the specific base changes. Sequences encoding the entire full-length wild-type (WT) and mutant Raf-1 proteins (amino acids 1 to 648) were inserted into the pVL941 baculoviral transfer vector, the pLJ retroviral vector, and the pSP64T transcription vector. Sequences encoding the truncated WT and mutant Δ-N′Raf proteins (amino acids 305 to 648) were inserted into the $pAcC_4$ baculoviral transfer vector, the pLNC retroviral vector, and the pSP64T transcription vector.

Expression of Raf Proteins in Sf9 and BALB/3T3 cells. To generate recombinant baculoviruses expressing the various Raf proteins, WT *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA and each of the recombinant vectors were cotransfected into Sf9 cells, and recombinant baculoviruses were isolated by plaque purification as previously described (39). Routinely, for recombinant protein production, 2×10^6 Sf9 cells were infected with the desired virus(es) at a multiplicity of infection of 10 and were lysed at 48 h postinfection. Sf9 cells were assayed for the expression of the recombinant proteins by Western blot (immunoblot) analysis. Recombinant baculoviruses encoding PDGF receptors (PDGFr) (26), $pp60^{src}$ (supplied by D. Morgan, University of California—San Francisco), $p21^{ras}$ (provided by N. Williams, Dana-Farber Cancer Institute), $p56^{lck}$ and NGF/TrkA receptor (provided by D. Kaplan and B. Stephans, National Cancer Institute-Frederick Cancer Research and Development Center, respectively) were also used.

To generate cell lines expressing the various Raf proteins, the pLJ/Raf and pLNC/Δ-N′Raf constructs were transfected in Ψ2 cells and virus stocks were derived. Expression of the *raf* alleles in the pLJ and the pLNC vectors is promoted by the murine sarcoma virus long terminal repeat and cytomegalovirus promoter, respectively, and both vectors encode resistance to G418 ($G418^r$). For focus-forming assays, BALB/3T3 cells were infected with virus stocks encoding the various *raf* alleles. The cells were trypsinized 24 h after infection, and 50% of the cells were grown in the absence of any drug selection and 50% were grown in the presence of 400 μg of G418 per ml. After 3 weeks, cells were fixed and stained with 0.2% methylene blue, and the foci and $G418^r$ colonies were counted.

Immunoprecipitation and in vitro protein kinase assays. Infected Sf9 cells were lysed on ice in radioimmunoprecipitation assay (RIPA) buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 20 μM leupeptin, and 5 mM sodium vanadate. Insoluble material was removed by centrifugation, and cell lysates were normalized for Raf protein expression. Raf-1 proteins were immunoprecipitated with a specific anti-Raf-1 antibody (35), and the antigen-antibody complexes were collected with protein A-Sepharose beads. The Raf-1 immunoprecipitates were washed three times with lysis buffer and once with kinase buffer (30 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4], 1 mM dithiothreitol, 7 mM $MnCl_2$, 5 mM $MgCl_2$, 15 μM ATP) and then were incubated in 40 μl of kinase buffer containing 20 μCi of [γ-^{32}P]ATP for 20 min at 25°C in the presence or absence of 5′-*p*-fluorosulfonyl-bezoyladenosine-treated MKK1. The assays were terminated by the addition of gel loading buffer (4% SDS, 80 mM dithiothreitol, 10% glycerol), the samples were resolved by SDS-PAGE, and the phosphoproteins were visualized by autoradiography.

Phosphatase assays. Phosphatase assays were performed by incubating washed Raf-1 immunoprecipitates with 5 μg of placental tyrosine phosphatase per ml (kindly provided by N. Tonks, Cold Spring Harbor Laboratories) in 50 μl of reaction buffer containing 25 mM HEPES (pH 7.2), 2 mM dithiothreitol, 0.05% Triton X-100, 5 mM EDTA, 5% glyc-

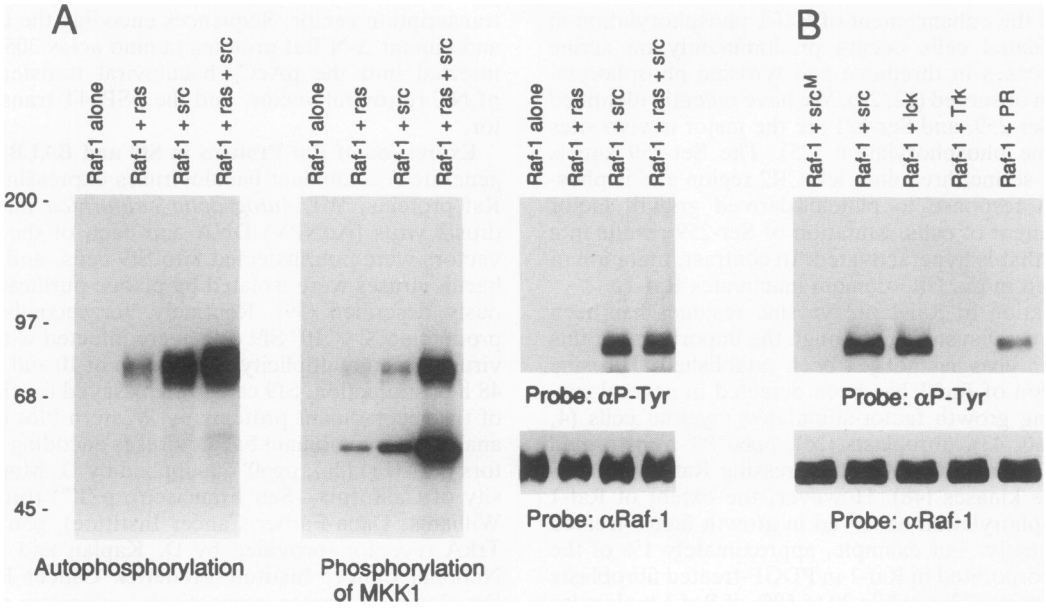

FIG. 1. Tyrosine phosphorylation of Raf-1 by activated tyrosine kinases. (A) In vitro autokinase assay (autophosphorylation), in vitro kinase assays with MKK1 as an exogenous substrate (phosphorylation of MKK1), anti-P-Tyr (αP-Tyr) and anti-Raf-1 (αRaf-1) Western blot analysis with Raf-1 immunoprecipitated from Sf9 cells infected with baculoviruses expressing Raf-1 proteins (Raf-1 alone), Raf-1 and $p21^{ras}$ proteins (Raf-1 + ras), Raf-1 and activated $pp60^{src}$ proteins (Raf-1 + src), or Raf-1, $p21^{ras}$, and activated $pp60^{src}$ (Raf-1 + ras + src). (B) Anti-P-Tyr and anti-Raf-1 Western blot analysis of Raf-1 immunoprecipitated from Sf9 cells infected with baculoviruses expressing Raf-1 and kinase-inactive $pp60^{src}$ proteins (Raf-1 + src^M), Raf-1 and activated $pp60^{src}$ proteins (Raf-1 + src), Raf-1 and $p56^{lck}$ proteins (Raf-1 + lck), Raf-1 and activated NGF/Trk receptors (Raf-1 + Trk), or Raf-1 and activated PDGF receptors (Raf-1 + PR). All samples were resolved by electrophoresis on SDS–7.5% polyacrylamide gels, and the phosphoproteins were visualized by autoradiography. Migration of molecular mass standards (in kilodaltons) is shown on the left. For Western blot analysis, antigen-antibody reactions were visualized with secondary antibodies coupled to horseradish peroxidase and the ECL detection system.

erol, and 1 mg of bovine serum albumin per ml for 1 h at 30°C. For some experiments, 1 mM sodium vanadate was added to inhibit the tyrosine phosphatase activity. Prior to kinase assays, the treated immunoprecipitates were washed three times with RIPA buffer and once with kinase buffer.

Oocyte injection and analysis. *X. laevis* females were purchased from Xenopus I (Ann Arbor, Mich.). Oocytes were removed and defolliculated by incubation in modified Barth solution (88 mM NaCl, 1 mM KCl, 2.5 mM $NaHCO_3$, 10 mM HEPES [pH 7.5], 0.82 mM $MgSO_4$, 0.33 mM $[CaNO_3]_2$, 0.4 mM $CaCl_2$) containing collagenase A (1.5 mg/ml) for 2 h. Oocytes were then washed several times in modified Barth solution and cultured overnight in 50% Leibovitz-15 medium. Eighteen hours after isolation, oocytes were injected with 30 ng of in vitro-transcribed RNA encoding the various Raf-1 proteins. Oocytes were scored for germinal vesicle breakdown (GVBD), as evidenced by the appearance of a white spot at the animal pole. This observation was verified by manual dissection of oocytes after fixation in 8% trichloroacetic acid.

To examine protein expression, oocytes were homogenized in lysis buffer containing 137 mM NaCl, 20 mM Tris (pH 8.0), 2 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.15 U/ml), 20 μM leupeptin, and 0.5 μM sodium vanadate (10 μl per oocyte). Insoluble material was removed by centrifugation at 14,000 × *g* for 10 min at 4°C. Lysates (1.5 oocyte equivalents per lane) were resolved by SDS-PAGE, transferred to nitrocellulose filters, and examined by Western blot analysis with anti-Raf-1 or anti-phosphotyrosine antibodies. Antigen-antibody reactions were visualized with secondary antibodies coupled to horseradish peroxidase and the enhanced chemiluminescence (ECL) detection system. Histone H1 kinase assays were performed on extracts prepared from 10 oocytes as previously described (6).

RESULTS

Identification of in vivo tyrosine phosphorylation site(s) of Raf-1. When the Raf-1 protein is coexpressed with $pp60^{src}$ and $p21^{ras}$ in the baculovirus-Sf9 system, it is tyrosine phosphorylated and highly active (48). As reported by Williams et al. (48), we found that maximal activation of Raf-1 autophosphorylation in this system required the presence of both activated tyrosine kinases and $p21^{ras}$ (Fig. 1A). Similar results were obtained when MKK1 was used as an exogenous substrate to measure Raf-1 activity (Fig. 1A). The tyrosine phosphorylation of Raf-1, though, was not enhanced by the presence of $p21^{ras}$ in Sf9 cells coexpressing Raf-1 and $pp60^{src}$ (Fig. 1A [48]). In addition, a functional tyrosine kinase was required for Raf-1 tyrosine phosphorylation in Sf9 cells, because kinase-inactive $pp60^{src}$ (src^M) could not mediate this effect (Fig. 1B). Coexpression of Raf-1 with $p56^{lck}$ and activated PDGFr also resulted in the tyrosine phosphorylation of Raf-1 (Fig. 1B), and both tyrosine kinases were able to cooperate with $p21^{ras}$ in inducing Raf-1 activity (data not shown). However, the tyrosine phosphorylation mediated by the PDGFr was significantly less than that induced by the *src* family tyrosine kinases, perhaps because of the low specific activity of the baculovirus-expressed PDGFr (Fig. 1B and data not shown). In mammalian cells, activation of these tyrosine kinases also

induces the activity and tyrosine phosphorylation (to various extents) of Raf-1. In contrast, coexpression of Raf-1 with activated nerve growth factor (NGF)/TrkA receptors in Sf9 cells did not result in the tyrosine phosphorylation of Raf-1 (Fig. 1B), indicating that the effect on Raf-1 is not simply due to the overexpression of an activated tyrosine kinase in this system. Similarly, in PC12 cells expressing TrkA, NGF does not stimulate the tyrosine phosphorylation or kinase activity of Raf-1 (37).

To determine the significance of tyrosine phosphorylation in regulating Raf-1 activity, we initiated experiments to identify the site of tyrosine phosphorylation. For this analysis, ^{32}P-labeled Raf-1 proteins were isolated from Sf9 cells coexpressing Raf-1, activated pp60^{src}, and p21^{ras}. This system was chosen because it is amenable to the production of quantities of Raf-1 protein sufficient to allow determination of the phosphorylated residues. The amount of Raf-1 expressed in mammalian cells, in which this protein is tyrosine phosphorylated, such as T cells and 3T3 cells, has not been adequate for the identification of the in vivo tyrosine phosphorylation sites (data not shown). Raf-1 protein produced in Sf9 cells coexpressing Raf-1, activated pp60^{src}, and p21^{ras} was phosphorylated on serine, threonine, and tyrosine residues (Fig. 2A). The isolated Raf-1 protein was digested with trypsin, and the tryptic phosphopeptides were separated and eluted from a reverse-phase HPLC-C-18 column. The profile of the radioactivity released from the C-18 column revealed the presence of six distinguishable peaks (Fig. 2A). We have previously identified the peptides present within fractions 5, 21, and 36 and have found that they contain the serine-phosphorylated residues Ser-43, Ser-621, and Ser-259, respectively (25). By phosphoamino acid analysis, the peptide found in fraction 32 was determined to be phosphorylated exclusively on tyrosine (Fig. 2B). To identify which tyrosine residue was labeled, the phosphopeptide isolated in fraction 32 was subjected to automated Edman degradation in a spinning cup sequenator. This analysis revealed that the phosphate group was released on cycle four (Fig. 2B). When ^{32}P-labeled Raf-1 protein was digested with the proteolytic enzyme Lys-C, a tyrosine-phosphorylated peptide was identified in which the label was released on cycle 11 (Fig. 2C). From these data, we determined that Tyr-340 was the major site of phosphorylation because this was the only tyrosine residue in the Raf-1 protein that was 4 and 11 residues from a trypsin and Lys-C cleavage site, respectively (Fig. 2D). However, because of the technical limitations of this analysis, we could not distinguish whether the adjacent tyrosine, Tyr-341, was a minor site of phosphorylation. By protein sequencing analysis, Tyr-340 was also determined to be the predominant tyrosine residue phosphorylated when Raf-1 was coexpressed with p21^{ras} and p56^{lck} or PDGFr (data not shown). Examination of the residues surrounding Tyr-340 (RDSSY) indicate that XD/EXXY may be a consensus motif for tyrosine phosphorylation at this site (30).

Expression and characterization of Raf-1 tyrosine phosphorylation site mutant proteins in the baculovirus-Sf9 system. By site-directed mutagenesis, Tyr-340 and Tyr-341 were either changed to noncharged phenylalanine residues or were mutated to negatively charged aspartic acid residues. The mutant Raf-1 proteins were expressed in the baculovirus-Sf9 cell system in the absence or presence of activated pp60^{src} and p21^{ras} proteins, and their phosphorylation state in vivo and enzymatic activity in vitro were examined (Fig. 3). By Western blot analysis with anti-phosphotyrosine (anti-P-Tyr) antibodies, the WT/Raf-1, kinase-inactive Raf-1 (ATP^{M}), and Raf-1 with an aspartic acid residue at the 340 (Y340D/

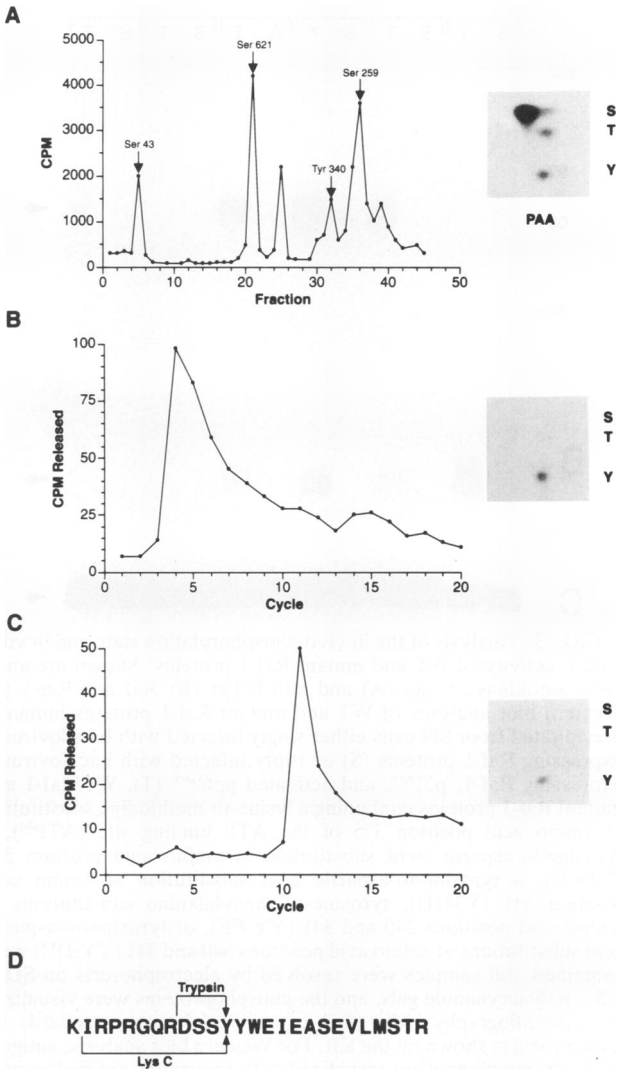

FIG. 2. Identification of a Raf-1 peptide containing phosphotyrosine. (A) Reverse-phase HPLC analysis and phosphoamino acid analysis of in vivo ^{32}P-labeled Raf-1 phosphopeptides. In vivo-phosphorylated Raf-1 isolated from Sf9 cells coexpressing Raf-1, p21^{ras}, and activated pp60^{src} was either subjected to phosphoamino acid analysis (PAA) or was digested with trypsin. The tryptic phosphopeptides were then separated and eluted from a reverse-phase HPLC-C-18 column. The amount of ^{32}P radioactivity collected in each column fraction is shown. Phosphorylated residues found in fractions 5, 21, and 36 have been previously identified (25) and are indicated. (B) Edman degradation (left panel) and phosphoamino acid analysis (right panel) of the tryptic phosphopeptide isolated in HPLC fraction 32 (shown in panel A). The amount of ^{32}P radioactivity released during each cycle of Edman degradation is shown. (C) Edman degradation and phosphoamino acid analysis of a Lys-C-digested Raf-1 phosphopeptide. In vivo-phosphorylated Raf-1 was isolated and digested with the Lys-C proteolytic enzyme. Shown are the Edman degradation profile (left panel) and the phosphoamino acid analysis (right panel) for the HPLC-isolated Lys-C peptide containing phosphotyrosine. The amount of ^{32}P radioactivity released during each cycle of degradation is depicted. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. (D) Amino acid sequence of the residues surrounding the identified Tyr-340 phosphorylation site. The predicted trypsin and Lys-C proteolytic cleavage sites are indicated.

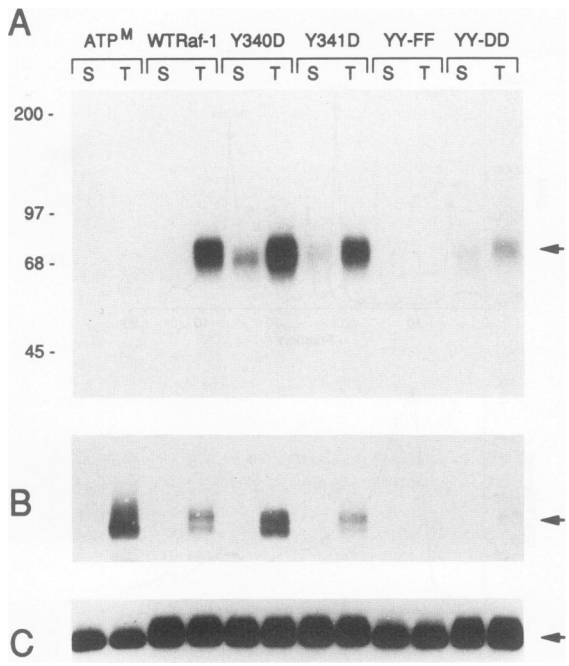

FIG. 3. Analysis of the in vivo phosphorylation state and in vitro kinase activity of WT and mutant Raf-1 proteins. Shown are an in vitro autokinase assay (A) and anti-P-Tyr (B) and anti-Raf-1 (C) Western blot analyses of WT and mutant Raf-1 proteins immunoprecipitated from Sf9 cells either singly infected with baculoviruses expressing Raf-1 proteins (S) or triply infected with baculoviruses expressing Raf-1, p21^{ras}, and activated pp60^{src} (T). WT/Raf-1 and mutant Raf-1 proteins containing a lysine-to-methionine substitution at amino acid position 375 of the ATP binding site (ATP^{M}), a tyrosine-to-aspartic acid substitution at amino acid position 340 (Y340D), a tyrosine-to-aspartic acid substitution at amino acid position 341 (Y341D), tyrosine-to-phenylalanine substitutions at amino acid positions 340 and 341 (YY-FF), or tyrosine-to-aspartic acid substitutions at amino acid positions 340 and 341 (YY-DD) were examined. All samples were resolved by electrophoresis on SDS–7.5% polyacrylamide gels, and the phosphoproteins were visualized by autoradiography. Migration of molecular mass standards (in kilodaltons) is shown on the left. For Western blot analysis, antigen-antibody reactions were visualized with secondary antibodies coupled to horseradish peroxidase and the ECL detection system.

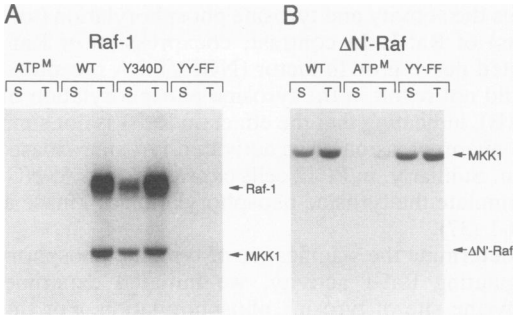

FIG. 4. In vitro phosphorylation of MKK1 by WT and mutant Raf-1 and Δ-N′Raf proteins. The in vitro kinase assay was performed with WT and mutant Raf-1 (A) and Δ-N′Raf (B) proteins immunoprecipitated from Sf9 cells either singly infected with baculoviruses expressing Raf-1 proteins (S) or triply infected with baculoviruses expressing Raf-1, p21^{ras}, and activated pp60^{src} (T). Purified recombinant MKK1 (0.1 μg) that had been treated with 5′-*p*-fluorosulfonyl-benzoadenosine (to inactivate MKK1 autokinase activity) was added as an exogenous substrate. All samples were resolved by electrophoresis on SDS–7.5% polyacrylamide gels, and the phosphoproteins were visualized by autoradiography. The migration of MKK1, Raf-1, and Δ-N′Raf is indicated.

Raf-1) or 341 (Y341D/Raf-1) site were tyrosine phosphorylated only when coexpressed with activated pp60^{src} (Fig. 3B). Interestingly, the Y340D/Raf-1 was highly tyrosine phosphorylated. Protein sequencing analysis of this mutant revealed that Tyr-341 was the site of phosphorylation (data not shown). This phosphorylation presumably occurs because the introduction of a negative charge at residue 340 generates an XD/EYX consensus motif for tyrosine phosphorylation at Tyr-341 (30). However, Raf-1 proteins containing a noncharged phenylalanine residue at the 340 site were only weakly tyrosine phosphorylated when coexpressed with activated pp60^{src} (data not shown). Mutation of both the 340 and 341 sites to either phenylalanine (YY-FF/Raf-1) or aspartic acid residues (YY-DD/Raf-1) produced proteins that were unable to be tyrosine phosphorylated in this system (Fig. 3B), confirming the direct sequencing data showing that Tyr-340 was the major tyrosine phosphorylation site of Raf-1.

To measure the catalytic activity of the Raf-1 mutant proteins, in vitro autokinase assays were performed. To ensure that the kinase activity was due to Raf-1, the kinase-inactive ATP^{M}/Raf-1 was included as a control. Compared with WT/Raf-1, all mutants containing aspartic acid substitutions displayed enhanced basal kinase activity (activity of Raf-1 proteins expressed alone) (Fig. 3A), with Y340D/Raf-1 being most active (Y340D incorporated 15- to 30-fold more label than the WT, Y341D incorporated 5-fold more, and YY-DD incorporated 4-fold more). The induced activity (activity of Raf-1 proteins coexpressed with activated pp60^{src} and p21^{ras}) of Y340D/Raf-1 was also enhanced, while the activity of Y341D/Raf-1 was equivalent to that of the WT (Fig. 3A). In contrast, the activity of the YY-DD/Raf-1 did not significantly increase above the already elevated basal levels, despite the presence of p21^{ras} and pp60^{src} (Fig. 3A). Further analysis revealed that the autophosphorylation of the YY-DD mutant could be increased 1.5-fold by coexpression with p21^{ras} but could not be further activated by coexpression with activated pp60^{src} (data not shown). Raf-1 containing phenylalanine residues at the 340 and 341 sites (YY-FF/Raf-1) had a low level of basal kinase activity that could not be induced by coexpression with p21^{ras} and pp60^{src} (Fig. 3A).

To determine whether substitution of phenylalanine residues at the 340 and 341 sites had inactivated the Raf-1 kinase domain, these mutations were incorporated into an amino-terminal-truncated protein (Δ-N′Raf). The proteins were then expressed in Sf9 cells in the absence or presence of activated pp60^{src} and p21^{ras} proteins, and their ability to phosphorylate purified recombinant MKK1 was determined. MKK1 was chosen as the exogenous substrate because it has been reported to be a physiological substrate of Raf-1 and because the major site of Raf-1 autophosphorylation (Thr-268) is deleted in Δ-N′Raf (25). In these assays, phosphorylation of MKK1 paralleled Raf-1 autophosphorylation for the full-length Raf-1 proteins (Fig. 4A) and again demonstrated that the YY-FF/Raf-1 was not activatable in this system. Furthermore, the addition of MKK1 did not inhibit Raf-1 autophosphorylation. For the truncated Δ-N′Raf proteins, the kinase-inactive ATP^{M}/Δ-N′Raf did not phosphorylate MKK1. In contrast, both WT/Δ-N′Raf and YY-

FF/Δ-N'Raf efficiently phosphorylated MKK1 even when expressed in the absence of pp60^{src} and p21^{ras} (Fig. 4B). These results demonstrate that the phenylalanine substitutions at residues 340 and 341 did not inactivate the catalytic domain of Raf-1.

Tyrosine-phosphorylated Raf-1 has enhanced autokinase activity. To determine the effect that tyrosine phosphorylation has on the biochemical activity of Raf-1, we measured the autokinase activity of tyrosine- and non-tyrosine-phosphorylated Raf-1 isolated from Sf9 cells coexpressing Raf-1, p21^{ras}, and pp60^{src} proteins. For these assays, tyrosine-phosphorylated Raf-1 proteins (which represent ~30 to 40% of the total Raf-1 protein produced in this system) were isolated from Sf9 lysates with a phosphotyrosine (P-Tyr) antibody column. The tyrosine-phosphorylated Raf-1 protein was then eluted from the column with phenyl phosphate and immunoprecipitated with anti-Raf-1 antibody. Non-tyrosine-phosphorylated Raf-1 protein was obtained from the Sf9 lysate that had been precleared of tyrosine-phosphorylated proteins. In autokinase assays, we found that tyrosine-phosphorylated Raf-1 incorporated 5- to 10-fold more ^{32}P than did equivalent amounts of non-tyrosine-phosphorylated Raf-1 (Fig. 5A). In addition, if tyrosine-phosphorylated Raf-1 was treated with a tyrosine-specific phosphatase prior to autokinase assays, the induced autokinase activity of Raf-1 was decreased (Fig. 5B). However, this decrease could be prevented by adding the phosphatase inhibitor, sodium vanadate, to the phosphatase reaction (Fig. 5B). These findings indicate that tyrosine-phosphorylated Raf-1 has higher autokinase activity.

To provide further evidence that phosphorylation of Tyr-340 and Tyr-341 is involved in modulating Raf-1 activity, we constructed a Raf-1 protein containing a mutation at Asp-337. This residue is part of a consensus sequence motif surrounding certain tyrosine phosphorylation sites (XD/EXXY) (30). Raf-1 encoding an alanine residue at the 337 site failed to become tyrosine phosphorylated or activated by pp60^{src} in Sf9 cells (Fig. 5C). However, this mutant was able to be activated by coexpression with p21^{ras} (data not shown).

Examination of the transforming potential of the Raf-1 tyrosine phosphorylation site mutant proteins. To assess the biological activities of the mutant Raf-1 and Δ-N'Raf proteins, we examined the ability of these proteins to transform BALB/3T3 cells (Fig. 6). As expected, infection of BALB/3T3 cells with retroviruses encoding WT/Raf-1 or kinase-inactive ATP^{M}/Raf-1 proteins did not induce focus formation in BALB/3T3 cells (0%), while infection with WT/Δ-N'Raf-encoding viruses did (98%). Expression of the Y340D/Raf-1 in BALB/3T3 cells efficiently (96%) gave rise to foci, while the Y341D/Raf-1 induced focus formation much less efficiently (22%). Further examination revealed that the foci and G418^{r} cells expressing the Y340D protein displayed a highly refractile transformed morphology (Fig. 6). When phenylalanine residues were introduced at positions 340 and 341, the transforming potential of full-length Raf-1 was not activated. However, Δ-N'Raf protein encoding these mutations (YY-FF/Δ-N'Raf) was able to transform BALB/3T3 cells (95%), indicating that mutation of these sites did not impair the transforming potential of Δ-N'Raf.

Effects of Raf-1 tyrosine phosphorylation site mutant proteins on the meiotic maturation of *Xenopus* oocytes. To further characterize the biological activities of the mutant Raf-1 and Δ-N'Raf proteins, we examined the ability of these proteins to induce the meiotic maturation of *Xenopus* oocytes (Fig. 7A [9]). Expression of WT/Raf-1, ATPM/Raf-1, YY-FF/Raf-1, and ATPM/Δ-N'Raf was unable to mediate oocyte maturation. In contrast, expression of WT/Δ-N'Raf induced M-phase promoting factor activation and the resultant GVBD in 94% of injected stage VI oocytes. Likewise, expression of the Y340D mutant induced GVBD in 85% of oocytes, while the Y341D mutant only induced GVBD in 39% of oocytes. Expression of the YY-FF/Δ-N'Raf also efficiently induced oocyte maturation (95%), indicating again that mutation of the 340 and 341 sites did not impair the biological activity of Δ-N'Raf. However, GVBD mediated by the introduction of Tpr-Met (an oncogenic activated form of hepatocyte growth factor) or H-*ras*$^{V-12}$ was blocked in oocytes expressing YY-FF/Raf-1 but not in oocytes expressing WT/Raf-1 (Fig. 8), indicating that YY-FF/Raf-1 can act in a dominant-inhibitory manner to block oocyte maturation induced by the receptor tyrosine kinase pathway.

By Western blot analysis, all Raf-1 and Δ-N'Raf proteins capable of inducing oocyte maturation exhibited a shift in their electrophoretic mobility and a complete shift in Y340D/Raf-1 was observed (Fig. 7B). In addition, all Raf-1 and

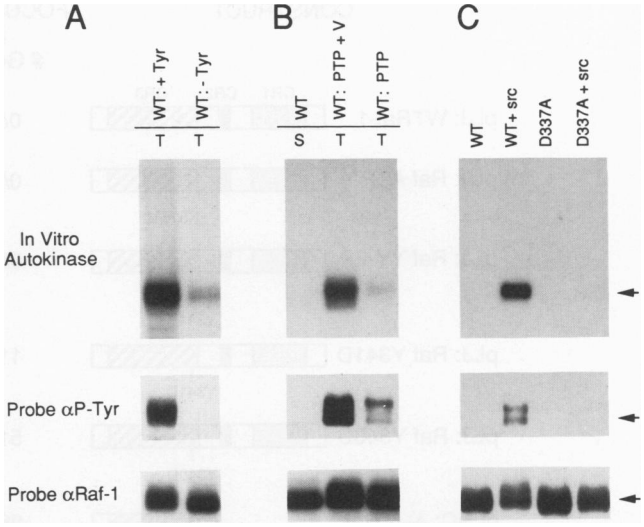

FIG. 5. Examination of the autokinase activity of tyrosine-phosphorylated Raf-1. (A) In vitro autokinase assay and anti-P-Tyr (αP-Tyr) and anti-Raf-1 (αRaf-1) Western blot analysis of tyrosine (+ Tyr) and non-tyrosine (− Tyr)-phosphorylated Raf-1 immunoprecipitated from Sf9 cells triply infected with baculoviruses expressing Raf-1, p21^{ras}, and activated pp60^{src} (T). (B) In vitro autokinase assay and anti-P-Tyr and anti-Raf-1 Western blot analysis of tyrosine phosphatase-treated Raf-1. WT/Raf-1 was immunoprecipitated from Sf9 cells singly infected with baculoviruses expressing Raf-1 proteins (S) or triply infected with baculoviruses expressing Raf-1, p21^{ras}, and activated pp60^{src} (T). The Raf-1 immunoprecipitates from triply infected cells were treated with placental tyrosine phosphatase in the presence (PTP + V) or absence (PTP) of the phosphatase inhibitor sodium vanadate and were washed extensively before analysis. (C) In vitro autokinase assay and anti-P-Tyr and anti-Raf-1 Western blot analysis of WT/Raf-1 (WT) and Raf-1 containing an aspartic acid-to-alanine substitution at amino acid position 337 (D337A) proteins immunoprecipitated from Sf9 cells either infected with baculoviruses expressing Raf-1 proteins alone or infected with baculoviruses expressing Raf-1 and activated pp60^{src} (+ src). All samples were resolved by electrophoresis on SDS–7.5% polyacrylamide gels, and the phosphoproteins were visualized by autoradiography. Migration of Raf-1 is indicated by arrows. For Western blot analysis, antigen-antibody reactions were visualized with secondary antibodies coupled to horseradish peroxidase and the ECL detection system.

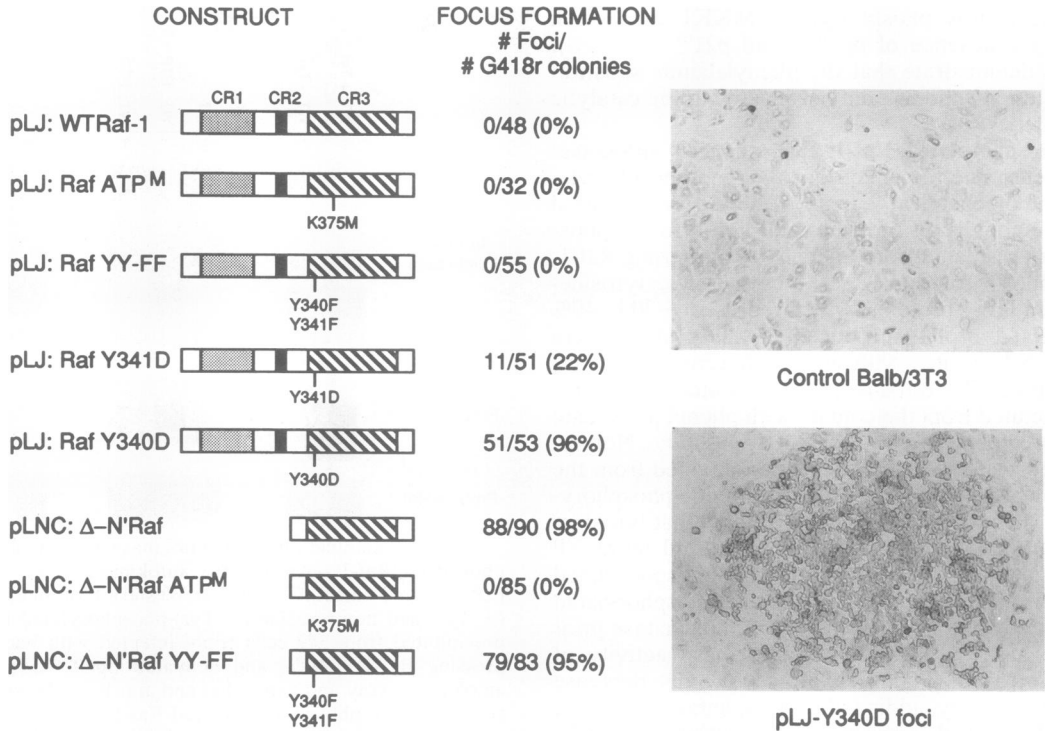

FIG. 6. Examination of the transforming potential of WT and mutant Raf-1 and Δ-N′raf proteins. BALB/3T3 cells were infected with retroviruses expressing WT/Raf-1 or full-length mutant Raf-1 proteins containing a lysine-to-methionine substitution at amino acid position 375 of the ATP binding site (Raf ATP^M), a tyrosine-to-aspartic acid substitution at amino acid position 340 (Raf Y340D), a tyrosine-to-aspartic acid substitution at amino acid position 341 (Raf Y341D), or tyrosine-to-phenylalanine substitutions at amino acid positions 340 and 341 (Raf YY-FF) or Δ-N′Raf and Δ-N′Raf mutant proteins containing a lysine-to-methionine substitution at amino acid position 375 of the ATP binding site (Δ-N′Raf ATP^M) or tyrosine-to-phenylalanine substitutions at amino acid positions 340 and 341 (Δ-N′Raf YY-FF). The transforming potential of the proteins was determined in focus formation assays. (The numbers listed represent the results of two experiments.) A schematic depiction of the Raf-1 protein is shown. The shaded areas depict the cysteine-rich CR1 region; the solid areas depict the serine/threonine-rich CR2 region; and the hatched areas depict the CR3 kinase domain. Photographs of control BALB/3T3 cells and typical foci induced by expression of the Y340D mutant Raf-1 protein (pLJ-Y340D) are also shown.

Δ-N′Raf proteins capable of mediating oocyte maturation were able to induce the tyrosine phosphorylation associated with the activation of MAPK (Fig. 7C).

DISCUSSION

In mammalian signalling pathways, the activity of Raf-1 may be regulated by multiple mechanisms, including phosphorylation or alteration of the amino-terminal regulatory domain through truncation or binding to a putative activator molecule (3, 11, 12, 23, 36, 45–47, 51). The involvement of serine phosphorylation in the regulation of Raf-1 is supported by experiments showing that, in response to many mitogens, the enhancement of Raf-1 activity correlates with increases in serine phosphorylation. Furthermore, mutation of two in vivo serine phosphorylation sites (Ser-259 and Ser-621) can alter the activity of Raf-1 (25). In this study, we demonstrate that the function of the Raf-1 kinase can be modified by mutation of critical tyrosine phosphorylation sites, suggesting that tyrosine phosphorylation can also regulate the activity of Raf-1. The sites of Raf-1 tyrosine phosphorylation were identified in Sf9 cells coexpressing tyrosine kinases and p21^{ras}. In this system, both tyrosine kinases and p21^{ras} are required for full activation of Raf-1. Similarly, in mitogen-treated T cells and 3T3 cells, both tyrosine kinases and p21^{ras} may contribute to the efficient induction of Raf-1 kinase activity. Unfortunately, because of the level of Raf-1 expression, we have been unable to obtain definitive information about the location of the tyrosine phosphorylation sites in mammalian cells. However, mutation of the sites identified in the baculovirus-Sf9 system had dramatic effects on the activity and function of Raf-1 when these mutant proteins were expressed in mammalian cells and in *Xenopus* oocytes.

The sites of Raf-1 tyrosine phosphorylation identified with the baculovirus-Sf9 expression system were two adjacent tyrosine residues, Tyr-340 and Tyr-341, located in the kinase domain of Raf-1. From protein sequencing data, Tyr-340 was the major site of tyrosine phosphorylation and Tyr-341 was a minor phosphorylation site. However, if Tyr-340 was changed to a negatively charged aspartic acid residue, Tyr-341 became a major site of phosphorylation. To elucidate the significance of Raf-1 tyrosine phosphorylation, the sites of tyrosine phosphorylation were mutated and the biological and enzymatic activities of the mutants were examined. Our findings indicate that substitution of a negatively charged amino acid for the Tyr-340 or Tyr-341 residue activated the in vitro and in vivo activity of Raf-1, perhaps by mimicking constitutive phosphorylation of these sites. In BALB/3T3 cells, expression of either Y340D/Raf-1 or Y341D/Raf-1 mutant proteins resulted in morphological transformation. The extent of transformation, as measured by focus formation, induced by the Y340D mutant was equivalent to that of amino-terminally truncated Raf-1 (Δ-N′Raf). Interestingly,

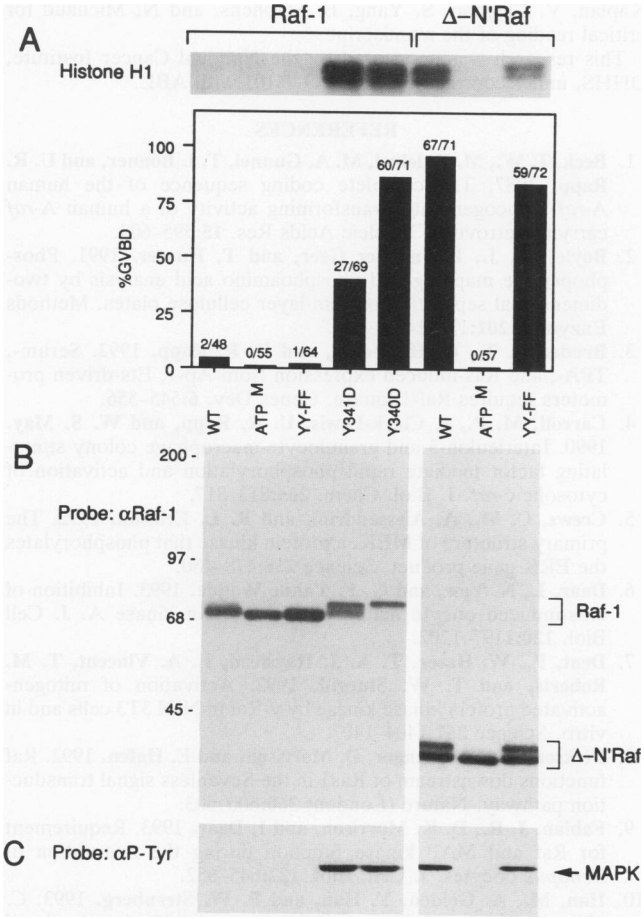

FIG. 7. Expression of WT and mutant Raf-1 and Δ-N'Raf proteins in *Xenopus* oocytes. (A) Induction of meiotic maturation by expression of WT and mutant Raf-1 and Δ-N'Raf proteins in *Xenopus* stage VI oocytes. Eighteen to 24 h after injection with RNA encoding Raf-1 and Δ-N'Raf proteins, oocytes were scored for GVBD, as evidenced by the appearance of a white spot at the animal pole. The ratio of the number of oocytes with GVBD to the total number injected is displayed above each bar (each number listed represents the results of three experiments). Histone H1 kinase assays were performed with extracts from five injected oocytes, and the autoradiograph is displayed above each bar. (B) Anti-Raf-1 (αRaf-1) Western blot analysis of Raf-1 and Δ-N'Raf proteins produced in *Xenopus* oocytes. (C) Anti-P-Tyr (αP-Tyr) Western blot analysis of MAPK from *Xenopus* oocytes expressing Raf-1 and Δ-N'Raf proteins. Oocytes examined in this analysis were injected with RNA encoding the full-length Raf-1 proteins (WT, ATP^{M}, Y340D, Y341D, and YY-FF) and the truncated Δ-N'Raf proteins (WT, ATPM, and YY-FF). A schematic depiction of the *raf* alleles is shown in Fig. 3.

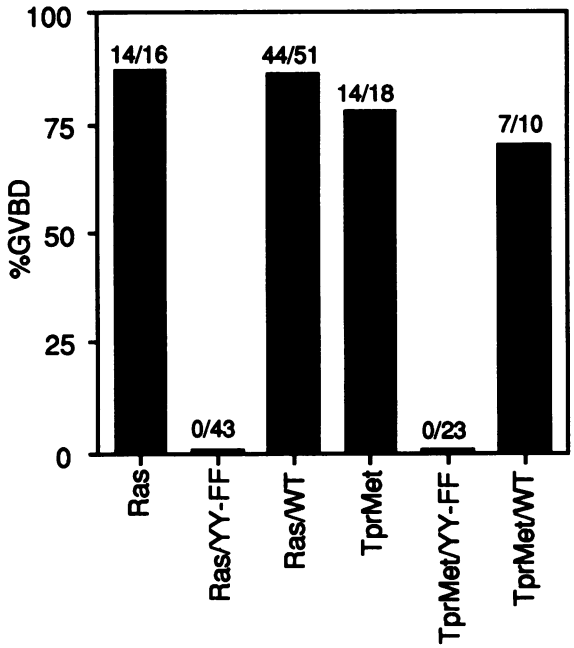

FIG. 8. YY-FF/Raf-1 blocks oocyte maturation induced by the receptor tyrosine kinase pathway. Uninjected oocytes or oocytes preinjected with capped transcripts encoding WT/Raf-1 or YY-FF/Raf-1 were microinjected with the capped transcripts (30 ng per oocyte) encoding H-*ras*$^{V-12}$ or Tpr-Met. GVBD was examined 12 to 18 h later. The ratio of the number of oocytes with GVBD to the total number injected is displayed above each bar.

Y340D/Raf-1 is the first demonstration that a highly transforming Raf-1 protein can be generated by a single amino acid substitution. All other transforming Raf-1 proteins have been generated by more extensive mutations, such as amino-terminal truncation, linker insertion, and fusion to other protein sequences (11, 36). Expression of the Y340D/Raf-1 was able to induce GVBD, H1 kinase activity, and the activation of MAPK in *Xenopus* oocytes. In both systems, Y340D/Raf-1 was considerably more active than Y341D/Raf-1, reflecting the higher relative enzymatic activity of the Y340D mutant. In contrast, replacing the Tyr-340 and Tyr-341 residues with noncharged amino acids that were unable to be phosphorylated generated a Raf-1 protein that could not be enzymatically activated by tyrosine kinases or p21^{ras}. Furthermore, expression of the YY-FF/Raf-1 in *Xenopus* oocytes prevented the meiotic maturation induced by signalling components of the receptor tyrosine kinase pathway.

The phenotypic effects of mutation of Tyr-340 and Tyr-341 suggest that the tyrosine residues themselves may perform a critical role in Raf-1 function. Therefore, mutation of these residues would result in the inactivation or deregulation of Raf-1 kinase activity. Alternatively, it could be that phosphorylation of the tyrosine sites regulates the activity of Raf-1. Our experiments suggest that in the baculovirus-Sf9 expression system, tyrosine phosphorylation can alter Raf-1 activity. First, the autokinase activity of tyrosine-phosphorylated Raf-1 was 5- to 10-fold higher than that of Raf-1 lacking this phosphorylation. Second, dephosphorylation of Raf-1 by a tyrosine-specific phosphatase decreased the elevated levels of Raf-1 autokinase activity induced by tyrosine kinases and p21^{ras}. Third, mutation of Asp-337, which is part of a consensus sequence motif for certain substrates of tyrosine kinases (XD/EXXY), prevents the tyrosine phosphorylation and activation of Raf-1 by tyrosine kinases.

In quiescent mammalian cells, the activity of the kinase domain of Raf-1 appears to be suppressed by the amino-terminal regulatory domain. Therefore, the function of Raf-1 tyrosine phosphorylation may be to alter the conformation of the protein such that the kinase domain is released from the suppression exerted by the amino-terminal domain. This model is supported by the observation that if tyrosine phosphorylation was prevented, the enzymatic activity of full-length YY-FF/Raf-1 failed to be induced by tyrosine kinases. However, the catalytic activity of truncated YY-

FF/Δ-N'Raf was constitutively activated and was unaffected by mutation at the tyrosine residues, demonstrating that the tyrosine-to-phenylalanine mutations did not impair kinase activity. Furthermore, expression of YY-FF/Raf-1 in oocytes inhibited the meiotic maturation induced by the Tpr-Met tyrosine kinase and H-*ras*$^{V-12}$, while truncated YY-FF/Δ-N'Raf stimulated meiotic maturation. These results indicate that mutation of Tyr-340 and Tyr-341 to phenylalanine residues does not prevent the interaction of the kinase domain of Δ-N'Raf with downstream targets. Rather, the functional defect of the YY-FF/Raf-1 appears to be caused by constitutive suppression of the kinase domain by the amino-terminal regulatory domain, thereby inhibiting the interactions of this protein with its substrates. As a result, the dominant inhibitory effect observed for YY-FF/Raf-1 may be caused by the binding of upstream Raf activators, preventing the activation of the endogenous *Xenopus raf*. These activators may include p21^{ras}, which has recently been shown to bind to Raf-1 in vitro (23, 45–47, 51).

In mammalian cells, the tyrosine phosphorylation of Raf-1 has been observed in four types of cell systems: PDGF-treated fibroblasts, v-*src*-transformed cells, interleukin-3 and granulocyte macrophage-colony-stimulating factor-treated myeloid cells, T cells cross-linked with anti-CD4 antibodies, and interleukin-2-treated T cells. The amount of tyrosine phosphorylation of Raf-1 seen in these cells varies from approximately 1 to 50% of total incorporated phosphate. In systems in which Raf-1 tyrosine phosphorylation is substantial, such as interleukin-2-treated T cells, this event may play an important role in regulating Raf-1 activity, as has been suggested by in vitro tyrosine phosphatase experiments (44). In systems in which only small amounts of Raf-1 tyrosine phosphorylation are detected, the effect of tyrosine phosphorylation may be obscured by other mechanisms of Raf-1 regulation. Alternatively, the tyrosine phosphorylation may induce the kinase activity of a few Raf-1 molecules, which then catalytically activate the remainder of the Raf-1 population by serine phosphorylation events. This model would be similar to the activation of calmodulin-dependent kinases, in which the binding of Ca^{2+}-calmodulin to a small number of molecules stimulates the autophosphorylation of the protein which activates the kinase and renders it independent of Ca^{2+}-calmodulin.

Sequence alignment of the catalytic domains for Raf proteins reveals that all Raf-1 and A-Raf proteins have tyrosine residues at the positions analogous to Tyr-340 and Tyr-341, while B-Raf, *Drosophila* Raf, and *C. elegans* Raf have negatively charged residues at these positions. Because substitution of a charged amino acid at Tyr-340 or Tyr-341 activates Raf-1 catalytic activity, the Raf proteins encoding charged residues at these sites may be partially activated. Alternatively, the mechanism of the regulation of B-Raf, *Drosophila* Raf, and *C. elegans* Raf activity may differ from that of Raf-1 and A-Raf. Interestingly, the amino-terminal domains of B-Raf, *Drosophila* Raf, and *C. elegans* Raf are much larger than those of Raf-1 and A-Raf. The additional sequences may encode a novel regulatory domain.

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REFERENCES

1. **Beck, T. W., M. Huleihel, M. A. Gunnel, T. I. Bonner, and U. R. Rapp.** 1987. The complete coding sequence of the human A-*raf*-1 oncogene and transforming activity of a human A-*raf* carrying retrovirus. Nucleic Acids Res. **15**:595–609.
2. **Boyle, W. J., P. van der Geer, and T. Hunter.** 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Methods Enzymol. **201**:110–149.
3. **Bruder, J. T., G. Heidecker, and U. R. Rapp.** 1992. Serum-, TPA-, and Ras-induced expression from Ap-1, Ets-driven promoters requires Raf-1 kinase. Genes Dev. **6**:545–556.
4. **Carroll, M. P., I. Clark-Lewis, U. R. Rapp, and W. S. May.** 1990. Interleukin-3 and granulocyte-macrophage colony stimulating factor mediate rapid phosphorylation and activation of cytosolic c-*raf*. J. Biol. Chem. **265**:812–817.
5. **Crews, C. M., A. Alessandrini, and R. L. Erikson.** 1992. The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. Science **258**:478–480.
6. **Daar, I., N. Yew, and G. F. Vande Woude.** 1993. Inhibition of mos-induced oocyte maturation by protein kinase A. J. Cell Biol. **120**:1197–1202.
7. **Dent, P., W. Haser, T. A. J. Haystead, L. A. Vincent, T. M. Roberts, and T. W. Sturgill.** 1992. Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro. Science **257**:1404–1407.
8. **Dickson, B., F. Sprenger, D. Morrison, and E. Hafen.** 1992. Raf functions downstream of Ras1 in the Sevenless signal transduction pathway. Nature (London) **360**:600–603.
9. **Fabian, J. R., D. K. Morrison, and I. Daar.** 1993. Requirement for Raf and MAP kinase function during the maturation of *Xenopus* oocytes. J. Cell. Biol. **122**:645–652.
10. **Han, M., A. Golden, Y. Han, and P. W. Sternberg.** 1993. *C. elegans lin-45 raf* gene participates in *let-60 ras*-stimulated vulval differentiation. Nature (London) **363**:137–140.
11. **Heidecker, G., M. Huleihel, J. L. Cleveland, W. Kolch, T. W. Beck, P. Lloyd, T. Pawson, and U. R. Rapp.** 1990. Mutational activation of c-*raf*-1 and definition of the minimal transforming sequence. Mol. Cell. Biol. **10**:2503–2512.
12. **Heidecker, G., W. Kolch, D. K. Morrison, and U. R. Rapp.** 1991. The role of Raf-1 phosphorylation in signal transduction. Adv. Cancer Res. **58**:53–73.
13. **Howe, L. R., S. J. Leevers, N. Gomez, S. Nakielny, P. Cohen, and C. J. Marshall.** 1992. Activation of the MAP kinase pathway by the protein kinase raf. Cell **71**:335–342.
14. **Hunter, T.** 1991. Protein kinase classification. Methods Enzymol. **200**:3–37.
15. **Ikawa, S., M. Fukui, Y. Ueyama, N. Tamaoki, T. Yamamoto, and K. Toyoshima.** 1988. B-*raf*, a new member of the *raf* family, is activated by DNA rearrangement. Mol. Cell. Biol. **8**:2651–2654.
16. **Kanakura, Y., B. Druker, K. W. Wood, H. J. Mamon, K. Okuda, T. M. Roberts, and J. D. Griffin.** 1991. Granulocyte-macrophage colony-stimulating factor and interleukin-3 induce rapid phosphorylation and activation of the proto-oncogene Raf-1 in a human factor-dependent myeloid cell line. Blood **77**:243–248.
17. **Kolch, W., G. Heidecker, P. Lloyd, and U. R. Rapp.** 1991. Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. Nature (London) **349**:426–428.
18. **Kyriakis, J. M., H. App, X.-F. Zhang, P. Banerjee, D. L. Brautigan, U. R. Rapp, and J. Avruch.** 1992. Raf-1 activates MAP kinase-kinase. Nature (London) **358**:417–421.
19. **Kyriakis, J. M., T. L. Force, U. R. Rapp, J. V. Bonventre, and J. Avruch.** 1993. Mitogen regulation of c-Raf-1 protein kinase activity toward mitogen-activated protein kinase-kinase. J. Biol. Chem. **268**:16009–16019.
20. **Lange-Carter, C., C. M. Pleiman, A. M. Gardner, K. J. Blumer,**

and G. L. Johnson. 1993. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science **260**:315–319.

21. **Le Guellec, R., A. Couturier, K. Le Guellec, J. Paris, N. Le Fur, and M. Philippe.** 1991. *Xenopus* c-*raf* proto-oncogene: cloning and expression during oogenesis and early development. Biol. Cell **72**:39–45.
22. **Li, P., K. Wood, H. Mamon, W. Haser, and T. Roberts.** 1991. Raf-1: a kinase currently without a cause but not lacking in effects. Cell **64**:479–482.
23. **Moodie, S. A., B. M. Willumsen, M. J. Weber, and A. Wolfman.** 1993. Complexes of Ras-GTP with Raf-1 and mitogen-activated protein kinase kinase. Science **260**:1658–1661.

23a. **Morrison, D. K.** Unpublished data.

24. **Morrison, D. K.** 1990. The Raf-1 kinase as a transducer of mitogenic signals. Cancer Cells **2**:377–382.
25. **Morrison, D. K., G. Heidecker, U. R. Rapp, and T. D. Copeland.** 1993. Identification of the major phosphorylation sites of the Raf-1 kinase. J. Biol. Chem. **268**:17309–17316.
26. **Morrison, D. K., D. R. Kaplan, J. A. Escobedo, U. R. Rapp, T. M. Roberts, and L. T. Williams.** 1989. Direct activation of serine-threonine kinase activity of the protooncogene Raf-1 through tyrosine phosphorylation by the PDGF-beta receptor. Cell **58**:649–657.
27. **Morrison, D. K., D. R. Kaplan, U. Rapp, and T. M. Roberts.** 1988. Signal transduction from membrane to cytoplasm: growth factors and membrane-bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity. Proc. Natl. Acad. Sci. USA **85**:8855–8859.
28. **Nishida, E., and Y. Gotoh.** 1993. The MAP kinase cascade is essential for diverse signal transduction pathways. Trends Biochem. Sci. **18**:128–131.
29. **Nishida, Y., M. Hata, T. Ayaki, H. Ryo, M. Yamagata, K. Shimizu, and Y. Nishizuka.** 1988. Proliferation of both somatic and germ cells is affected in the Drosophila mutants of *raf* proto-oncogene. EMBO J. **7**:775–781.
30. **Pearson, R. B., and B. E. Kemp.** 1991. Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. Methods Enzymol. **200**:62–81.
31. **Rapp, U. R.** 1991. Role of Raf-1 serine/threonine protein kinase in growth factor signal transduction. Oncogene **6**:495–500.
32. **Rapp, U. R., M. D. Goldsborough, G. E. Mark, T. I. Bonner, J. Groffen, J. F. H. Reynolds, and J. R. Stephenson.** 1983. Structure and biological activity of v-*raf*, a unique oncogene transduced by a retrovirus. Proc. Natl. Acad. Sci. USA **80**:4218–4222.
33. **Rapp, U. R., G. Heidecker, M. Huleihel, J. L. Cleveland, W. C. Choi, T. Pawson, J. N. Ihle, and W. B. Anderson.** 1988. *raf* family serine/threonine protein kinase in mitogen signal transduction. Cold Spring Harbor Symp. Quant. Biol. **53**:173–184.
34. **Roberts, T. M.** 1992. A signal chain of events. Nature (London) **360**:534–535.
35. **Schultz, A. M., T. D. Copeland, G. E. Mark, U. R. Rapp, and S. Oroszlan.** 1985. Detection of the myristylated *gag-raf* transforming protein with *raf*-specific antipeptide sera. Virology **146**:78–89.
36. **Stanton, V. P., Jr., D. W. Nichols, A. P. Laudano, and G. M. Cooper.** 1989. Definition of the human *raf* amino-terminal regulatory region by deletion mutagenesis. Mol. Cell. Biol. **9**:639–647.
37. **Stephens, R. M., G. Sithanandam, T. D. Copeland, D. R. Kaplan, U. R. Rapp, and D. K. Morrison.** 1992. 95-Kilodalton B-raf serine/threonine kinase: identification of the protein and its major autophosphorylation site. Mol. Cell. Biol. **12**:3733–3742.
38. **Storm, S. M., J. L. Cleveland, and U. R. Rapp.** 1990. Expression of *raf* family proto-oncogenes in normal mouse tissues. Oncogene **5**:345–351.
39. **Summers, M. D., and G. Smith.** 1987. A manual of methods for baculovirus vector and insect cell culture procedures. Texas Agricultural Experiment Station, College Station, Tex.
40. **Thompson, P. A., J. A. Ledbetter, U. R. Rapp, and J. Bolen.** 1991. The Raf-1 serine-threonine kinase is a substrate for the p56lck protein kinase in human T-cells. Cell Growth Differ. **2**:609–618.
41. **Troppmair, J., J. T. Bruder, H. App, H. Cai, L. Liptak, J. Szeberenyi, G. M. Cooper, and U. R. Rapp.** 1992. Ras controls coupling of growth factor receptors and protein kinase C in the membrane to Raf-1 and B-Raf protein serine kinases in the cytosol. Oncogene **7**:1867–1873.
42. **Tsuda, L., Y. H. Inoue, M.-A. Yoo, M. Mizuno, M. Hata, Y.-M. Lim, T. Adachi-Yamada, H. Ryo, Y. Masamune, and Y. Nishida.** 1993. A protein kinase similar to MAP kinase activator acts downstream of the Raf kinase in Drosophila. Cell **72**:407–414.
43. **Turner, B. C., U. R. Rapp, H. App, M. I. Greene, K. Dobashi, and J. C. Reed.** 1991. Interleukin 2 induces tyrosine phosphorylation and activation of p72-74 Raf-1 kinase in a T-cell line. Proc. Natl. Acad. Sci. USA **88**:1227–1231.
44. **Turner, B. C., N. K. Tonks, U. R. Rapp, and J. C. Reed.** 1993. Interleukin 2 regulates Raf-1 kinase activity through a tyrosine phosphorylation-dependent mechanism in a T-cell line. Proc. Natl. Acad. Sci. USA **90**:5544–5548.
45. **Van Aelst, L., M. Barr, S. Marcus, A. Polverino, and M. Wigler.** 1993. Complex formation between Ras and Raf and other protein kinases. Proc. Natl. Acad. Sci. USA **90**:6213–6217.
46. **Vojtek, A. B., S. M. Hollenberg, and J. A. Cooper.** 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74**:205–214.
47. **Wasylyk, C., B. Wasylyk, G. Heidecker, M. Huleihel, and U. R. Rapp.** 1989. Expression of *raf* oncogenes activates the PEA1 transcription factor motif. Mol. Cell. Biol. **9**:2247–2250.
48. **Williams, N. G., T. M. Roberts, and P. Li.** 1992. Both $p21^{ras}$ and $pp60^{v\text{-}src}$ are required, but neither alone is sufficient, to activate the Raf-1 kinase. Proc. Natl. Acad. Sci. USA **89**:2922–2926.
49. **Wood, K. W., C. Sarnecki, T. M. Roberts, and J. Blenis.** 1992. *ras* mediates nerve growth factor receptor modulation and three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. Cell **68**:1041–1050.
50. **Wu, J., J. K. Harrison, L. A. Vincent, C. Haystead, T. A. Haystead, H. Michel, D. F. Hunt, K. R. Lynch, and T. W. Sturgill.** 1993. Molecular structure of a protein-tyrosine/threonine kinase activating p42 mitogen-activated protein (MAP) kinase: MAP kinase kinase. Proc. Natl. Acad. Sci. USA **90**:173–177.
51. **Zhang, X. F., J. Settleman, J. M. Kyriakis, E. Takeuchi-Suzuki, S. J. Elledge, M. S. Marshall, J. T. Bruder, U. R. Rapp, and J. Avruch.** 1993. Normal and oncogenic $p21^{ras}$ proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature (London) **364**:308–313.