Sequential Modification of Serines 621 and 624 in the Raf-1 Carboxyl Terminus Produces Alterations in Its Electrophoretic Mobility

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Alma F. Ferrier‡, Michael Lee‡, Wayne B. Anderson‡, Giovanna Benvenuto‡, Deborah K. Morrison§, Douglas R. Lowy‡, and Jeffrey E. DeClue‡‡

From the §Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, Maryland 20892-4040 and ¶Molecular Mechanisms of Carcinogenesis Laboratory, NCI-Frederick Cancer Research and Development Center, ABL-Basic Research Program, National Cancer Institute, Frederick, Maryland 21702-1201

The Raf-1 serine/threonine protein kinase plays a central role in many of the mitogenic signaling pathways regulating cell growth and differentiation. The regulation of Raf-1 is complex, and involves protein-protein interactions as well as changes in the phosphorylation state of Raf-1 that are accompanied by alterations in its electrophoretic mobility. We have previously shown that a 33-kDa COOH-terminal, kinase-inactive fragment of Raf-1 underwent a mobility shift in response to the stimulation of cells with serum or phorbol esters. Here we demonstrate that treatment of NIH 3T3 cells or SF9 cells with hydrogen peroxide (H₂O₂) also induces the stimulation of cells with serum or phorbol esters. Hereof of Raf-1 underwent a mobility shift in response to the electrophoretic mobility. We have previously shown state of Raf-1 that are accompanied by alterations in its interactions as well as changes in the phosphorylation of Raf-1 is complex, and involves protein-protein regulating cell growth and differentiation. The regulatory role in many of the mitogenic signaling pathways

Fax: 301-480-5322.

indicate this fact.

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1 The abbreviations used are: MAP, mitogen-activated protein; MEK, MAP kinase kinase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; PAG, polyacrylamide gel electrophoresis; MAPK, MAP kinase.

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munoprecipitation, as well as immunoblotting of the proteins encoded by these mutants, revealed that the altered electrophoretic mobility of the Raf-1 COOH-terminal fragments occurs following a two-step process that involves the addition of phosphate to two neighboring serine residues.

MATERIALS AND METHODS

Plasmids—Deletion mutants of Raf-1 were constructed to include a COOH-terminal epitope-tagging sequence encoding the last 12 amino acids of the protein kinase C epsilon (e) gene to provide rapid detection of the expressed protein. Deletion mutants were obtained by polymerase chain reaction amplification with the Pfu DNA polymerase (Stratagene), using a COOH-terminal e-tagged fragment of Raf-1 (RIII-e) encoding amino acids 381–643 (30), as the template. NH2-terminal sense primers containing a BamHI restriction site were created at regular intervals originating at amino acid 381, 412, 443, 474, 505, 536, or 566 of Raf-1. A COOH-terminal antisense primer was generated containing the e-tag, along with EcoRI and BamHI restriction sites. Using RIII-e as template and the COOH-terminal e-epitope tagging sequence as primer with various NH2-terminal primers, six additional NH2-terminally truncated epitope-tagged Raf-1 constructs were created. Several additional COOH-terminal e-epitope tagged constructs were generated using COOH-terminal primers, which originated at amino acids 591, 612, or 590 along with the e 590 and an amino acid 381 e-tag, along with amino acid 381 e-tag, along with the NH2-terminal primer originating at amino acid 381. The full-length Raf-1 mutant plasmid pKSRafS621A was described previously (31). The plasmid encoding activated MEK-1 and a linked hygromycin resistance gene was provided by James C. Stone, University of Alberta, Edmonton (Canada) (32).

The RII-e single and double point mutants were generated by sequential PCR amplifications (33). The point mutations other than S621A were created using the RII-e construct as template, and the following sense primers (for S621D, 5’CGCGAGGCTGCAGAGCGCATC3’; for S621D and S624A, 5’GGCGTACGAGCCAACGCATCGC3’) along with a COOH-terminal antisense primer containing the e-tag and EcoRI and BamHI restriction sites. RII-e S621A was generated using the pKSRafS621A plasmid as template, the sense primer 5’CCGGAGGCCTGCAGAGCGCATC3’ and the same antisense primer as above. The PCR product from each of these reactions was then used as the antisense primer to generate RII-e mutant fragments, which were then cloned into baculovirus expression vectors for expression in Sf9 cells. The presence of point mutations was verified by direct DNA sequence analysis.

Transfection in Graded-induced Overexpression of Recombinant Proteins in NIH 3T3 Cells—NIH 3T3 cells were maintained at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, and glutamine. The isolation of NIH 3T3 cell lines expressing epitope-tagged Raf-1 mutants is described elsewhere (30). To induce maximal expression of the recombinant proteins, the transfected cells were changed to serum-free medium supplemented with 20 μM zinc acetate (to up-regulate expression from the metallothionine promoter-linked genes) for 48 h prior to stimulation with 10% serum, 100 μM PMA, or 500 μM of H2O2. Stable transfectants expressing both RII-e and activated MEK-1 were isolated following cotransfection of cells with the expression plasmids and selec- tion in neomycin and hygromycin. The Sf9 insect cell line was maintained in spinner flasks in Grace’s supplemented medium containing 10% fetal bovine serum and 0.1% pluronics F-68 (Life Technologies, Inc.). To express the Raf-1 deletion mutants, Sf9 cells were co-transfected with the baculovirus transfer vector pEVMOD-Raf-e-tag and BaculGoI baculovirus DNA (Pharmingen) using the Lipofoetin reagent (Life Technologies, Inc.). Recombinant baculoviruses encoding the various Raf-1 proteins were isolated and enriched according to the manufacturer’s protocol (Pharmingen). For Raf-1 point mutants, the Bac-To-Bac Baculovirus expression system for Sf9 cells was used (Life Technologies, Inc.). For experimental purposes, Sf9 cells (2.5 × 10^6) were infected with virus and harvested after 48 h.

Preparation of Cell Lysates and Cell Fractionation—For the experiments shown in Figs. 1 and 2, cells were washed twice with ice-cold phosphate-buffered saline (PBS), harvested from a monolayer in suspension, resuspended in the cell pellet buffer B (0.05% SDS, 20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.5% sodium deoxycholate, 2 mM EDTA, containing 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 10 μg/ml leupeptin, and 5 mM sodium orthovanadate), and disrupted by Dounce homogenization (100 strokes). Lysates were adjusted to 1 × Laemmli sample buffer concentration and stored at −70°C. For the experiments shown in Table I and Figs. 3–6, cytosolic extracts of insect cells and mammalian cells were prepared as follows. The cells were washed twice with ice-cold phosphate-buffered saline, harvested by resuspension of the cell pellet in buffer B (0.05% SDS, 20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.5% sodium deoxycholate, 2 mM EDTA, containing 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 10 μg/ml leupeptin, and 5 mM sodium orthovanadate) for insect cells and buffer C (same as buffer B with 0.1% SDS for 0.1% SDS for 10 minutes at 4°C). Lysates for use in immunoblotting were adjusted to 1 × Laemmli sample buffer concentration and stored at −70°C.

Immunoprecipitation and Immunoblot Analysis—Immunoprecipitations of Raf-1 were performed by incubation of 4–24 h at 4°C, immunoprecipitated proteins were washed as described previously (33) and processed for SDS-PAGE. Immunoprecipitates were prepared for electrophoresis by the addition of 2 × Laemmli sample buffer with β-mercaptoethanol and boiled prior to electrophoresis on an SDS-polyacrylamide gel (12%). The proteins were transferred to nitrocellulose, and immunoblot analysis was performed using 10 μg/ml anti-PKC-e antibody as described previously (30).

Membrane-Labeling and Immunoprecipitation of the e-tagged Recombinant Proteins—Cell labeling experiments were performed by first rinsing infected cells once with either methionine- or phosphate-deficient medium. The cells were then incubated either in methionine-deficient medium containing 10% dialyzed fetal calf serum and and 1 mM ATP, and 5 μCi of [γ-32P]ATP. After incubation for 20 min at 30°C, assays were terminated by the addition of MEK from the reactions (35). Briefly, the washed immunoprecipitates were incubated in 40 μl of kinase buffer containing 100 ng of kinase-negative MEK, 10 μM ATP, and 5 μCi of [γ-32P]ATP at 30°C for 30 min. The reactions were terminated by addition of Laemmli sample buffer and the samples were separated by SDS-PAGE. The immunoprecipitated kinase-negative MEK, and the autophosphorylation of Raf-1 was assayed using the MEK activity was assayed by the phosphorylation of kinase-negative MEK, and the autophosphorylation of Raf-1 was assayed using the MEK assay as determined by autoradiography. Alternatively, Raf-1 protein kinase activity was assayed by using a coupled assay of extracellular-regulated kinase and MAP kinase activation (21), and the activity was determined by the phosphorylation of myelin basic protein by the activated MAP kinase. Briefly, Raf-1 immunoprecipitates were incubated with 1 μg of recombinant MAP kinase kinase (Santa Cruz Biotechnology) in reaction mixtures (40 μl) of kinase buffer containing 200 μM ATP along with 1 μg of recombinant kinase buffer containing 20 μl of kinase buffer containing 100 μM Na3VO4. Ten microliters of the diluted reaction mixture was combined with 30 μl of kinase buffer containing 20 μg of myelin basic protein and 5 μCi of [γ-32P]ATP. After incubation for 20 min at 30°C, assays were terminated by the addition of Sf9 cell sample buffer, the samples were resolved by SDS-PAGE, and phospho- proteins were visualized by autoradiography.

RESULTS

Mobility Shift of a COOH-terminal Fragment in Mammalian and Insect Cells—Previously, we identified a 33-kDa catalytically inactive COOH-terminal fragment of Raf-1 (amino acids 381–649; designated RIII-e), which exhibited a Ras-independent serum- and phorbol ester-induced shift in gel mobility that mimicked the shift observed with full-length Raf-1 (29). Recent interest has focused on the possible roles of these oxygen free radicals, including hydrogen peroxide (H2O2), in the regulation of various signal transduction processes. Oxygen free radicals have been suggested to play a role in regulating several enzymes involved in transmembrane signaling pathways, including protein kinase C, tyrosine-specific protein kinases, and MAPK in certain cell systems (36–38). Moreover, in one
we examined whether treatment of Src, Ras, or PKC with Raf-1 led to its activation (9, 10). Experiments performed using the Sf9 insect cell system to analyze the activation of Raf-1 have revealed that co-expression of Src, Ras, or PKC with Raf-1 led to the activation of Raf-1 COOH-terminal modifications, we examined whether treatment of Sf9 cells expressing only RIII-epitope-tagged COOH-terminal Raf-1 fragment were incubated at 37 °C in serum-free medium for 48 h prior to stimulation with the indicated agents. Cell extracts were isolated from untreated cells (lane 1), cells treated with 10% serum for 30 min (lane 2), cells treated with 100 nM PMA for 30 min, or cells treated with 500 μM H2O2 for 60 min. The electrophoretic mobility of exogenously truncated Raf-1 was investigated by immunoblot analysis as described. The molecular mass standards are indicated to the left (46, 30, and 21.5 kDa). Two bands are recognized by the PKCε antibody corresponding to the exogenously expressed e-epitope-tagged Raf-1 protein.

Since Raf-1 functions as an upstream activator in the MAPK kinase cascade, we investigated whether Raf-1 might also be affected by treatment of cells with H2O2. To examine the effect of various agents on Raf-1 mobility, quiescent NIH 3T3 cells expressing RIII-ε were treated with 10% serum, 100 nM PMA, or 500 μM H2O2. Cytosolic extracts were isolated and separated by SDS-PAGE, then subjected to immunoblot analysis (Fig. 1). In quiescent cells, only a single electrophoretic form of the RIII-ε protein was observed (Fig. 1, lane 1). In contrast, two forms of RIII-ε were observed following treatment with serum or phorbol ester, as shown previously, or hydrogen peroxide (Fig. 1, lanes 2–4). The similar migration rates of the upper band induced by each treatment suggested that this form of RIII-ε resulted from a similar modification(s) in each instance.

Experiments performed using the Sf9 insect cell system to analyze the activation of Raf-1 have revealed that co-expression of Src, Ras, or PKC with Raf-1 led to its activation (9, 10, 20, 31). To develop a simplified system for the analysis of Raf-1 COOH-terminal modifications, we examined whether treatment of Sf9 cells expressing only RIII-ε with H2O2 would lead to a shift in electrophoretic mobility similar to that observed in mammalian cells. After 48 h of infection with an RIII-ε expressing baculovirus, readily detectable levels of RIII-ε protein were expressed (Fig. 2). Unexpectedly, a doublet corresponding to RIII-ε was observed in the untreated cells (Fig. 2, lane 1). The doublet is probably a result of the presence of serum in the infected Sf9 culture. However, the more rapidly migrating form, corresponding to the unmodified form of RIII-ε, was found in greater amount in the untreated cells. As early as 30 min after H2O2 treatment, a greater amount of the more slowly migrating form of Raf-1 was observed (Fig. 2, lane 2), and after 60 min, almost all of the RIII-ε was found in the modified form (Fig. 2, lane 3). Thus, a similar shift to a more slowly migrating form of RIII-ε was noted both in insect cells and in mammalian cells following H2O2 treatment.

Deletion Analysis of the Raf-1 COOH Terminus in Sf9 Cells—To further localize the region within the Raf-1 COOH terminus required for modification(s) to occur, which result in a shift in gel mobility, a series of deletion mutants of the RIII-ε (amino acids 381–643) protein were created and expressed in Sf9 cells. Six NH2-terminal and three COOH-terminal deletion mutants were constructed in 31 amino acid intervals encompassing residues 381–643 of Raf-1 (Table I). After 48 h of infection with the various virus stocks, Sf9 cells were treated with H2O2 for 60 min to fully induce the characteristic shift in electrophoretic mobility. All of the NH2-terminal deletion mutants exhibited a mobility shift after treatment (Table I), including the smallest polypeptide (RIX-ε), which encompasses only amino acids 566–643 of Raf-1. In contrast, all deletions from the COOH terminus resulted in proteins that exhibited only a single band in the absence or presence of H2O2. Further efforts to define the minimal fragment (RIX-ε), which still exhibited a band shift, were unsuccessful, probably as a result of instability, since no expression of the smaller proteins was detected (data not shown). We conclude that a region of Raf-1 comprising only amino acids 566–643 is capable of undergoing modification(s) resulting in a shift in gel mobility upon stimulation of cells with H2O2.

Phosphorylation State of RIII-ε and RIX-ε—A change in the phosphorylation state of Raf-1 after mitogenic stimulation has been suggested as the explanation for the shift in migration rate of Raf-1 on SDS-polyacrylamide gels (26). To test this possibility, in vivo labeling experiments were performed with Sf9 cells expressing RIII-ε and RIX-ε, to examine the phosphorylation state of these COOH-terminal Raf-1 fragments. After 48 h of viral infection, cells were labeled continuously with [35S]methionine or [32P]orthophosphate for 4 h, with 500 μM H2O2 treatment for the final 60 min. Immunoprecipitation analysis of [35S]methionine-labeled cell extracts revealed that RIII-ε is expressed as a doublet under these conditions (Fig. 3, lane 2). Following [32P] labeling, however, the major band ob-
observed was the upper band of the RIII-ε doublet (Fig. 3, lane 5). These results link the modification responsible for the migration shift of RIII-ε to the presence of phosphate in the protein. Only a low level of 35S label was incorporated into RIX-ε, likely due to the low number of methionine and cysteine residues present in this fragment (Fig. 3, lane 3). However, a doublet was visible with much longer exposures of the gel (data not shown). Nonetheless, RIX-ε did exhibit significant 32P incorporation (Fig. 3, lane 6), and once again, only a single band was observed in the 32P-labeled lysates. These results suggested that the change in the rate of migration of the Raf-1 COOH-terminal fragments may be associated with a phosphorylation event occurring between residues 566 and 643.

Identification of Possible Sites of Modification Affecting the Electrophoretic Mobility of RIII-ε—One site of modification that lies within the 566–643 region is serine 621, which was previously shown to be constitutively phosphorylated in fibroblast cells (31). Additionally, mutation of this site to an alanine resulted in the absence of phosphorylation of Raf-1, and these mutants exhibited a dominant-negative phenotype (7, 40). To determine what role serine 621 might play in the mobility shift observed in RIII-ε upon mitogenic stimulation, point mutations resulting in a change of this residue to either an alanine (RIII-ε S621A) or aspartate (RIII-ε S621D) were constructed and expressed in Sf9 cells. Although RIX-ε is the smallest fragment that exhibits a mobility shift, the resolution between the two forms is more evident using RIII-ε. The S621A mutation prevents phosphorylation (Fig. 3, lanes 1, 3, and 4) and the negatively charged residue in S621D should mimic the presence of a phosphorylated serine. As expected, mutation of serine 621 to alanine prevented further modification of RIII-ε, resulting in the presence of only the faster migrating form of the protein (Fig. 4, lane 2). Surprisingly, however, mutation of serine 621 to aspartate resulted in the presence of a doublet (Fig. 4, lane 3), comparable to the doublet observed for wild-type RIII-ε (Fig. 4, lanes 1 and 5). If phosphorylation of this site alone was responsible for the induced mobility shift, we would have expected to observe the presence of a single, slowly migrating band. Therefore, the modification at Ser-621 appears to be necessary, but not sufficient, for the induced mobility shift of RIII-ε.

To identify a possible second site of modification, the sequences of different Raf proteins in the region from amino acids 566–643 were examined for the most evolutionarily conserved residues. One site, serine 624, was of particular interest because of its conservation between the three isoforms of Raf and its proximity to serine 621. To examine what effect this residue might have on the mobility shift of the Raf-1 COOH terminus, we constructed a double point mutant in the RIII-ε construct, which changed serine 624 to alanine and serine 621 to aspartate (RIII-ε S621D/S624A). Immunoblot analysis of cytosolic extracts from Sf9 cells expressing this double mutant after H2O2 treatment revealed a band pattern that differed from the one that was observed with the single mutant (RIII-ε S621D). Only the more rapidly migrating band was observed in lysates containing the RIII-ε S621D/S624A double mutant (Fig. 4, lane 4). These data suggest that one modification occurs in the COOH terminus of Raf-1 to result in the electrophoretic mobility shift of RIII-ε. Apparently, an initial modification occurs at serine 621, and this is required to allow a second modification to occur at serine 624.

Phosphorylation State of RIII-ε Proteins Bearing Point Mutations—To determine whether the electrophoretic mobility changes reflected alterations in the phosphate content of Raf-1 proteins, in vivo labeling experiments were performed using various Raf point mutants. RIII-ε S621A and RIII-ε S621D. Infected Sf9 cells were labeled with either 35Smethionine or 32Porthophosphate, exposed to H2O2 for 60 min, then lysed, and the lysates were analyzed as for Fig. 3. Metabolic labeling experiments with 35Smethionine showed that adequate levels of protein were expressed for wild-type RIII-ε and the two point mutants of RIII-ε (Fig. 5, lanes 1–3). A doublet was evident for both wild-type RIII-ε and RIII-ε S621D, while only a single, more rapidly migrating band was observed for RIII-ε S621A (consistent with the immunoblot analysis in Fig. 4). In contrast, when cells were labeled with 32P, phosphate was incorporated into both the faster and more slowly migrating forms of wild-type RIII-ε protein (Fig. 5, lane 4), while no phosphate was incorporated into the RIII-ε S621A protein (Fig. 5, lane 5). Although the RIII-ε S621D mutant expressed both forms when 35Smethionine-labeled lysates were analyzed (Fig. 5, lane 6), only the upper band was 32P-labeled (Fig. 5, lane 6). This indicates that an alteration requiring a negative
introduced an activated form of MEK-1 (32), which has been comparable to the doublet observed in exponentially growing RIII-2 autoradiography.

Lane 3 contains proteins isolated from cells co-transfected with RIII-α and activated MEK-1. Lane 2 contains proteins isolated from RIII-α cells grown in the absence of serum for 48 h. Lane 1 contains proteins isolated from RIII-α alone or cotransfected with RIII-α and activated MEK-1. Since the activation of Raf-1 appears to involve phosphorylation of serine 621, is required for the electrophoretic mobility of activated Raf-1 on SDS-polyacrylamide gels (48–50), the identity of specific site(s) responsible for this shift in gel mobility has remained elusive. Here we present evidence that modification of serine 624, along with the same modifications we have described here in the context of RIII-α, is required to cause a shift in electrophoretic mobility, was also found to be kinase-inactive. In all cases, the wild-type and mutant Raf-1 proteins were expressed and were present in the immunoprecipitates, as detected by immunoblotting (Fig. 7D). We conclude that the presence of a negative charge at amino acid 621 does not yield an activated, or even a kinase-competent Raf-1 protein. Furthermore, preventing the modification of serine 624 by replacing it with alanine does not restore kinase activity to the protein in which serine 621 has been changed to aspartate.

**DISCUSSION**

Several laboratories have presented data suggesting a correlation between the phosphorylation state of Raf-1 and activation of its kinase activity (31, 46, 47). While hyperphosphorylation also has been suggested to play a role in the shift in electrophoretic mobility of activated Raf-1 on SDS-polyacrylamide gels (48–50), the identity of specific site(s) responsible for this shift in gel mobility has remained elusive. Here we present evidence that modification of serine 624, along with phosphorylation of serine 621, is required for the electrophoretic mobility shift of a COOH-terminal fragment (RIII-α) comprising residues 566–643 of Raf-1. The band shift noted with the RIII-α fragment resembles the serum- and PMA-induced shift observed with full-length Raf-1. It is likely that the same modifications we have described here in the context of Raf-1 COOH-terminal fragments also occur with full-length Raf-1, since this protein displays an analogous electrophoretic mobility shift in response to treatment of cells with the same kinase, and the activation of MAP kinase was determined by the phosphorylation of myelin basic protein (Fig. 7C, MBP).

In each type of assay, the activity of wild-type Raf-1 was readily detected when the protein was isolated from transfected cells (lane 2), while only a low level of activity was observed in precipitates from cells infected singly with Raf-1 (lane 7). As previously reported (31), mutation of serine 621 to alanine (S621A) abolished the in vitro kinase activity of Raf-1 (lanes 3 and 8). Unexpectedly, Raf-1 protein with serine 621 changed to aspartate (S621D; lanes 4 and 9) was also defective in its kinase activity. Thus, replacement of the normal serine with a neutral amino acid (alanine) or a negatively charged amino acid (aspartate) at this site has a similar effect on Raf-1 activity. As the modification of serine 624 requires a negative charge at site 621 (see above), we also analyzed the double point mutant in which the S621D mutation was combined with a serine-to-alanine change at amino acid 624 (S621D/S624A; lanes 5 and 6 and lanes 10 and 11). The double mutant protein, which in the RIII-α form is unable to undergo the modifications required to cause a shift in electrophoretic mobility, was also found to be kinase-inactive. In all cases, the wild-type and mutant Raf-1 proteins were expressed and were present in the immunoprecipitates, as detected by immunoblotting (Fig. 7D). We conclude that the presence of a negative charge at amino acid 621 does not yield an activated, or even a kinase-competent Raf-1 protein. Furthermore, preventing the modification of serine 624 by replacing it with alanine does not restore kinase activity to the protein in which serine 621 has been changed to aspartate.

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**FIG. 5. In vivo analysis of the phosphorylation state of RIII-α point mutants.** Sf9 cells expressing either wild-type or mutant RIII-α protein were labeled with [35S]methionine (lanes 1–3) or [32P]orthophosphate (lanes 4–6) as described. Epitope-tagged Raf-1 protein was immunoprecipitated from cell lysates and samples resolved by electrophoresis on SDS-polyacrylamide gels (12%). Proteins were visualized by autoradiography. Lanes 1 and 4, RIII-α lanes 2 and 5, RIII-α S621A; lanes 3 and 6, RIIIE S621D. Molecular mass standards are indicated to the left (46, 30, and 21.5 kDa). Recombinant RIII-α mutant proteins are indicated by arrows.

**FIG. 6. The presence of activated MEK-1 induces the mobility shift of RIII-α expressed in mammalian cells.** NIH 3T3 cells were transfected with RIII-α alone or cotransfected with RIII-α and activated MEK-1. Lane 1 contains proteins isolated from exponentially growing RIII-α cells under normal growth conditions. Lane 2 contains proteins isolated from RIII-α cells grown in the absence of serum for 48 h. Proteins were separated by electrophoresis on SDS-polyacrylamide gels (12%). The electrophoretic mobility of RIII-α was examined by immunoblot analysis using the anti-PKCε antibody. Molecular mass standards are indicated to the left (46, 30, and 21.5 kDa). Arrows indicate the two forms of expressed ε-tagged RIII.
occurs only after the modification of serine 621. It is required for the mobility shift, and that this change results in the S621D protein, but not into the S621A mutant. These results suggest that a second modification, involving serine 624, affects the regulation of Raf-1 in a successive manner.

Serine 621 of Raf-1 has previously been shown to be phosphorylated in quiescent as well as in platelet-derived growth factor-stimulated fibroblasts (31). The importance of this residue for Raf-1 function is suggested by the lack of kinase activity exhibited by Raf-1(S621A) in vitro and in vivo (31, 40). Here we have made the unexpected observation that substitution of serine 621 with aspartate (S621D) also yields an inactive kinase. Using a constitutively activated COOH-terminal fragment of Raf-1, other investigators have independently reached the same conclusion (59). The results suggest that either Raf-1 proteins with a negative charge (e.g., a phosphorylated serine) at this site are inactive, or that the aspartate substitution does not completely mimic the phosphorylated serine. Our finding that mutation of serine 621 to aspartate does permit the electrophoretic mobility shift to occur, while the alanine 621 mutation does not, suggests that aspartate at this residue does substitute for at least this aspect of serine 621 function.

Our results raise several questions regarding the regulation of Raf-1 function. One is the nature of the modifications themselves. While the metabolic labeling experiments clearly demonstrated the incorporation of phosphate into RHI-e as a correlate of the mobility shift, the phosphate group could comprise part of a more complex modification rather than phosphorylation per se. Indeed, some investigators have found no effect by either phosphoserine- or phosphothreonine-specific phosphatases on Raf-1 enzymatic activity (17, 29, 60). In contrast, more recent studies have shown that treatment of Raf-1 with membrane-associated protein phosphatases can inactivate its kinase activity (47). Thus, while the simplest explanation is that both serine 621 and serine 624 undergo phosphorylation, we cannot exclude the possibility that other modifications occur at these sites. A second and related question is the identity of the enzymes responsible for the modifications of the Raf-1 COOH terminus. There could be a single enzyme that modulates both events, or there might be two distinct enzymes involved. The occurrence of phosphorylation at serine 621 in both quiescent and stimulated cells makes it difficult to speculate about the identity of the kinase(s) responsible. However, the ability of MEK-1, which has a very strict substrate specificity for MAPK (45, 61), to induce the Raf-1 shift implies that MAPK, or another kinase activated by MAPK, may be responsible for the modification of serine 624.

A final critical issue is the role of these residues and their modifications in the regulation of Raf-1 activity. Initially, it appeared that the mobility shift of Raf-1 correlated with increased kinase activity of the protein, especially since agents that induced the shift (e.g., serum, PMA) also activated Raf-1 activity. Later reports, however, suggested that Raf-1 activa-

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2 A. F. Ferrier, unpublished data.
tion might actually occur prior to the mobility shift, raising the possibility that the mobility shift may in fact reflect a feedback mechanism to turn off Raf-1 (42, 43). Thus, it also is possible that the more highly modified (slower migrating) form of Raf-1 may actually be inactive. The experiments described here do not definitively distinguish between these possibilities. Indeed, the lack of kinase activity by Raf-1(S621A) would seem to support the former model, while the lack of activity of Raf-1(S621D) would seem to support the latter. However, the fact that MEK-1 can induce the band shift in the RIII-fragment when co-expressed in intact NIH 3T3 cells is most easily interpreted in light of the feedback model. Further experiments are required to determine the precise manner in which Raf-1 enzymatic activity is affected by these modifications.

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