Raf-1 and p21^{v-ras} cooperate in the activation of mitogen-activated protein kinase

(baculovirus/Sf9 insect cells/ERK1 protein kinase)

NIDHI G. WILLIAMS^{†‡}, HELENE PARADIS^{†§}, SADHANA AGARWAL^{†§}, DAVID L. CHAREST[¶],
STEVEN L. PELECH[¶], AND THOMAS M. ROBERTS^{†§||}

[†]Dana–Farber Cancer Institute and Departments of [‡]Biological Chemistry and Molecular Pharmacology and [§]Pathology, Harvard Medical School, Boston, MA 02115; and [¶]Department of Medicine, Biomedical Research Centre, University of British Columbia, Vancouver, BC, Canada V6T 1Z3 and Kinetek Biotechnology Corp., 7600 No. 1 Road, Richmond, BC, Canada V7C 1T6

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ABSTRACT Mitogen-activated protein (MAP) kinases Raf-1, pp60^{src}, and p21^{ras} all play important roles in the transfer of signals from the cell surface to the nucleus. We have used the baculovirus/Sf9 insect cell system to elucidate the regulatory relationships between pp60^{v-src}, p21^{v-ras}, MAP kinase (p44^{erk1}/mapk), and Raf-1. In Sf9 cells, p44^{erk1}/mapk is activated by coexpression with either v-Raf or a constitutively activated form of Raf-1 (Raf22W). In contrast, p44^{erk1}/mapk is activated to only a limited extent by coexpression with either Raf-1 or p21^{v-ras} alone. This activation of p44^{erk1}/mapk is greatly enhanced by coexpression with both p21^{v-ras} and Raf-1. Since we have previously shown that p21^{v-ras} stimulates Raf-1 activity, the activation of p44^{erk1}/mapk by p21^{v-ras} may occur exclusively via a Raf-1-dependent pathway. However, a dominant-inhibitory mutant of Raf-1 (Raf301) does not block the activation of p44^{erk1}/mapk by p21^{v-ras}. Further, pp60^{v-src}, which activates Raf-1 at least as effectively as p21^{v-ras}, fails to enhance p44^{erk1}/mapk activity greatly when coexpressed with Raf-1. These data suggest that activation of p44^{erk1}/mapk by p21^{v-ras} may occur via both Raf-1-dependent and Raf-1-independent pathways.

The transduction of signals from the cell surface to the nucleus is mediated by several distinct families of serine–threonine protein kinases (1–4). Included among these are the mitogen-activated protein (MAP) kinases and the *raf*-encoded protein kinases. The MAP kinases, also known as extracellular signal-regulated kinases (ERKs), represent a growing family of cytoplasmic kinases (5, 6). At least two MAP kinase isoforms, p44^{erk1}/mapk and p42^{erk2}/mapk, are activated in response to a wide variety of extracellular stimuli such as insulin, nerve growth factor (NGF), platelet derived-growth factor (PDGF), epidermal growth factor (EGF), phorbol esters, and okadaic acid (2, 7, 8). MAP kinases are also activated in cells expressing either pp60^{v-src} (9) or activated p21^{c-ras} (10–12). The activities of members of the Raf family (includes isoforms Raf-1, A-raf, and B-raf) of serine–threonine kinases also appear to be modulated by a wide variety of extracellular stimuli and oncogenes (3). Interestingly, both Raf and MAP families of kinases are ubiquitously expressed (5, 13). Further, the sets of stimuli which activate the two kinase families overlap extensively. Thus it is not surprising that recent reports place Raf and MAP kinases in the same signaling pathway (14–16).

The product of the *c-raf-1* gene, Raf-1, is a 72- to 74-kDa cytoplasmic protein with intrinsic serine–threonine kinase activity. Raf-1 is the cellular homolog of v-Raf, the product of the transforming gene of murine sarcoma virus 3611 (17). On the basis of functional analysis, the Raf-1 protein can be

divided into two domains, a C-terminal kinase domain and an N-terminal regulatory domain (18). The transforming potential of the kinase domain of Raf-1 is normally suppressed by the N-terminal regulatory domain; v-Raf is essentially an N-terminal truncation of Raf-1. Consistent with this, truncated forms of Raf-1 (including the clone designated Raf22W) containing only the kinase domain are potent transformers of NIH 3T3 cells (19). Raf-1 is hyperphosphorylated and seen to be retarded in mobility on SDS/PAGE gels in response to a wide variety of extracellular stimuli and oncogenes. In cases involving stimulation by platelet-derived growth factor and insulin (20) a concomitant increase in the autokinase activity of Raf-1 from mammalian cells has been demonstrated.

The mechanism of activation of the cytoplasmic serine–threonine kinases p44^{erk1}/mapk and p42^{erk2}/mapk is clearly of interest. In response to extracellular stimuli, p44^{erk1}/mapk and p42^{erk2}/mapk undergo rapid phosphorylation on threonine and tyrosine residues and retardation in gel mobility. Activation of p44^{erk1}/mapk and p42^{erk2}/mapk requires phosphorylation of these proteins on both tyrosine and threonine residues (21, 22). These tyrosine and threonine residues, Thr-183 and Tyr-185 in p42^{erk2}/mapk, are conserved in both isoforms, p44^{erk1}/mapk and p42^{erk2}/mapk. They are the only known cytoplasmic serine–threonine kinases known to be activated by tyrosine phosphorylation. Cytoplasmic MAP kinase activating factors have been identified (23, 24), purified to homogeneity (7, 25, 26), and recently cloned (27). *In vitro*, activated Raf-1 or v-raf can activate partially purified preparations of MAP kinase activator (14–16).

Raf-1 and p44^{erk1}/mapk both appear to be regulated by p21^{ras} and by membrane tyrosine kinases (10, 11, 28, 29). Stimulation of PC12 cells by nerve growth factor or epidermal growth factor activates p44^{erk1}/mapk in these cells. In fact, expression of activated p21^{ras} in PC12 cells is sufficient to activate p44^{erk1}/mapk partially and to cause hyperphosphorylation and retardation in gel mobility of Raf-1 (10, 11). Moreover, expression of a dominant interfering allele of p21^{c-ras} is sufficient to block nerve growth factor-induced p44^{erk1}/mapk activation and hyperphosphorylation of Raf-1 (10, 11).

Since both Raf-1 and p44^{erk1}/mapk are regulated by p21^{v-ras} and by membrane tyrosine kinases, we have used baculovirus-encoded p21^{v-ras}, pp60^{v-src}, p44^{erk1}/mapk, and Raf-1 to examine the effects of each of the activated kinases on the activity of the other. As reported here and by others, p44^{erk1}/mapk is activated by coexpression with either v-Raf or a constitutively activated form of Raf-1 (Raf22W). We report, however, that only a limited increase in the kinase activity of p44^{erk1}/mapk is seen after coexpression with either Raf-1 or with p21^{v-ras}. The kinase activity of p44^{erk1}/mapk is greatly enhanced by coex-

pression with Raf-1 and p21^{v-ras}. We have shown previously that coexpression with either pp60^{v-src} or p21^{v-ras} increases the kinase activity of Raf-1 (30). Yet the kinase activity of p44^{erk1/mapk} is greatly enhanced by coexpression with p21^{v-ras} and Raf-1 and not by coexpression with pp60^{v-src} and Raf-1. Further, a dominant-inhibitory mutant of Raf-1 (Raf301) does not block the activation of p44^{erk1/mapk} by p21^{v-ras}. These data show that p21^{v-ras} and Raf-1 cooperate to activate p44^{erk1/mapk}, and they suggest that activation of p44^{erk1/mapk} by p21^{v-ras} occurs in one pathway via Raf-1 and in another pathway independent of Raf-1. Our data further show that pp60^{v-src} does not activate p44^{erk1/mapk} directly but requires intermediates such as Raf-1 or others that are missing or limiting in Sf9 insect cells.

MATERIALS AND METHODS

Cell Culture and Antibodies. *Spodoptera frugiperda* (Sf9) cells, wild-type baculovirus (*Autographa californica* nuclear polyhedrosis virus), and baculovirus transfer vectors were provided by Max Summers (Texas A & M, College Station). Sf9 cells were grown either in suspension or as a monolayer culture in Grace's medium (GIBCO 350-1605AJ) supplemented with 10% fetal calf serum. All protocols for passage, infection, and transfection of Sf9 cells are as described (31).

Monoclonal anti-src antibody 327 was a gift from Joan Brugge (University of Pennsylvania, Philadelphia). Anti-phosphotyrosine monoclonal antibody (4G10) was a gift from Brian Drucker (Dana-Farber Cancer Institute, Boston). Anti-p21 monoclonal antibody YA6-259 (32) was produced as a supernatant from a rat hybridoma cell line kindly supplied by Larry Feig (Tufts University, Medford, MA). Rabbit polyclonal antiserum was raised against a peptide corresponding to the 12 C-terminal residues of Raf-1 (CTLTTSRPLPVF) (33). Rabbit polyclonal anti-MAP kinase R1 antibody, used for immunoblotting, was prepared from antisera raised against a peptide corresponding to residues 63–98 of rat p44^{erk1/mapk} (8) (Upstate Biotechnology, Lake Placid, NY). Rabbit polyclonal anti-MAP kinase C2 antibody, used for immunoprecipitations, prepared against a C-terminal peptide (34), was a gift from John Blenis (Harvard University, Cambridge, MA).

Construction and Isolation of Recombinant Baculovirus. The baculovirus transfer vector encoding v-Raf was constructed by subcloning of the *Bgl* II/*Pvu* I fragment (the *Pvu* I end was filled in by the Klenow fragment of *Escherichia coli* DNA polymerase) from p3611-MSV (17) in the *Bgl* II/*Xba* I site (the *Xba* I end was filled in by the Klenow fragment) of the baculovirus transfer vector pVL1393. The baculovirus transfer vector encoding activated Raf (R22W) was constructed by subcloning of the *Eco*RI/*Xba* I fragment from the truncated clone designated 22W (19) in the *Bam*HI/*Xba* I site of the baculovirus transfer vector pVL1393 by using *Bam*HI/*Eco*RI adapters (New England Biolabs nos. 1105 and 1106). The baculovirus transfer vectors encoding human p44^{erk1/mapk} (D.L.C. and S.L.P., unpublished work) and kinase-inactive human p44^{erk1} (lysine-to-methionine mutation) were constructed by subcloning of the *Bam*HI/*Eco*RI fragment encoding the full-length proteins in the *Bam*HI/*Eco*RI sites of the baculovirus transfer vector pVL1393. Human p44^{erk1/mapk} shares 96% identity with rat and mouse p44^{erk1/mapk}s (D.L.C., G. Mordret, F. Jirik, K. Harder, and S.L.P., unpublished results). The resulting transfer vectors, pVL.v-raf, pVL.Raf22W, and pVL.hERK1, were checked for the proper orientation of insertion before transfection into Sf9 cells.

The transfer vectors encoding v-Raf, activated Raf-1 (Raf22W), or human p44^{erk1/mapk} were cotransfected with wild-type baculoviral DNA into Sf9 cells as described by Summers and Smith (31). Each of the recombinant baculoviruses was checked for protein production by immunoblotting. Consistent with predicted sizes, recombinant baculo-

virus encoding v-Raf produces a 90-kDa protein, while that encoding activated Raf (Raf22W) produces a 38-kDa protein. As seen in the case of bacterially expressed p44^{erk1/mapk}, recombinant baculovirus encoding p44^{erk1/mapk} produces two proteins, one of predicted size 44 kDa and the other of smaller size, 42 kDa (possibly a proteolytically related product of the full-length protein or an altered phosphoform). Production of recombinant baculovirus encoding Raf-1 (35), p21^{v-ras}, or kinase-inactive Raf-1 (Raf-301) (30) has been described previously. Recombinant baculovirus encoding pp60^{v-src} was obtained from Ray Erikson (Harvard University). High-titer viral supernatants of each of the recombinant baculoviruses encoding Raf-1, Raf-301 (Raf*), pp60^{v-src}, p21^{v-ras}, v-Raf, Raf22W, or p44^{erk1/mapk} were prepared and used in various combinations to infect Sf9 cells.

Preparation of Baculovirus-Infected Sf9 Cell Lysates. Initially, Sf9 cells (3×10^6) were infected with various amounts of each of the recombinant baculoviruses to determine amounts required to attain maximal protein production. Protein production from each of the recombinant baculoviruses was assayed by immunoblotting. Sf9 cells (3×10^6) were infected with the determined amounts of each of the recombinant baculovirus or with determined amounts of each recombinant baculovirus in desired combinations. Forty hours after infection, cells were lysed and cell lysates were prepared as described previously (30). Cell lysates were assayed for recombinant protein production or total phosphotyrosine content by SDS/PAGE followed by immunoblotting as described elsewhere (11). Cell lysates containing approximately equivalent amounts of Raf-1, p44^{erk1/mapk}, and p21^{v-ras} proteins as assayed by Western blot analysis were used for kinase assays performed *in vitro*.

Raf Immune-Complex Kinase Assays. Anti-Raf-1 antibody was incubated with protein A-Sepharose beads for 1 hr at 4°C. The beads bound to anti-raf antibody were washed once with RIPA buffer (0.02 M Tris-HCl, pH 7.4/0.137 M NaCl/1% Triton X-100/0.05% sodium deoxycholate/0.1% SDS/10% glycerol) and then incubated with appropriate amounts of cell lysates at 4°C for 4 hr. Immunoprecipitation and immune complex kinase assays were conducted as described previously (30). The reaction was terminated by the addition of Laemmli buffer (10% glycerol/2% SDS/0.1 M dithiothreitol) and the samples were boiled before SDS/PAGE. Proteins were detected by autoradiography and Coomassie-blue staining. Raf-1 autophosphorylation after kinase reaction *in vitro* was quantitated on a PhosphorImager (Molecular Dynamics). Reactions using myelin basic protein (MBP, 4 μ g) as the exogenous substrate were performed by including it in the kinase buffer (25 mM Hepes, pH 7.4/1 mM dithiothreitol/10 mM MgCl₂/10 mM MnCl₂).

p44^{erk1/mapk} Immune-Complex Kinase Assays. Anti-ERK1 antibody was incubated with staphylococcal protein A-Sepharose beads for 1 hr at 4°C. The beads bound to anti-ERK1 antibody were washed once with RIPA buffer and then incubated with appropriate amounts of cell lysates at 4°C for 2 hr. The immunoprecipitates were washed once with RIPA buffer, twice with 0.5 M LiCl/0.1 M Tris base, pH 8.0, and once in kinase buffer before kinase reaction *in vitro*. For kinase reactions, washed immunoprecipitates were incubated at room temperature for 20 min in 30 μ l of kinase buffer supplemented with 15 μ M unlabeled ATP, 10 μ Ci of [γ -³²P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq), and 4 μ g of MBP. The reaction was terminated by the addition of Laemmli buffer and the samples were boiled before SDS/PAGE. Proteins were detected by Coomassie-blue staining. Phosphorylation of MBP was detected by autoradiography. Phosphorylation of MBP after kinase reaction *in vitro* was quantitated on the PhosphorImager (Molecular Dynamics).

RESULTS

To assess the effects of p21^{v-ras}, pp60^{v-src}, Raf-1, and Raf-1 mutants on p44^{erk1/mapk}, a series of baculovirus coinfections were performed, using different combinations of recombinant baculoviruses expressing each of these proteins. Cells were lysed and p44^{erk1/mapk} and/or Raf-1 proteins were immunoprecipitated from the lysates. The same number of cells was used for each infection. Levels of p44^{erk1/mapk}, Raf-1, pp60^{v-src}, and p21^{v-ras} proteins in the different lysates were normalized by immunoblotting.

Activation of p44^{erk1/mapk} by a Constitutively Activated Form of Raf-1 (Raf22W) as well as by v-Raf. Baculoviruses encoding either v-Raf or the constitutively activated form of Raf-1 (Raf22W) were coinfecting with a baculovirus encoding p44^{erk1/mapk} and the activity of p44^{erk1/mapk} was assessed after each of the infections. Since activation of p44^{erk1/mapk} requires phosphorylation of p44^{erk1/mapk} on tyrosine, anti-phosphotyrosine antibody immunoblots were first used to compare the levels of tyrosine phosphorylated p44^{erk1/mapk} in the different lysates. As seen in the anti-phosphotyrosine immunoblot of lysates of cells infected in the combinations indicated (Fig. 1A), a protein comigrating with p44^{erk1/mapk} is most highly tyrosine phosphorylated in cells coexpressing either p44^{erk1/mapk} and Raf22W (lane 4) or p44^{erk1/mapk} and v-Raf (lane 5). An immunoblot showing the relative amounts of p44^{erk1/mapk} in each of the lysates used (Fig. 1B) shows that the increase in tyrosine phosphorylation in lanes 4 and 5 cannot be due to increased amounts of p44^{erk1/mapk} in those lysates. To see if changes in anti-phosphotyrosine correlated with changes in kinase activity, immune-complex kinase assays were performed to directly assess the phosphotransferase activity of p44^{erk1/mapk} in each of the lysates. Immunoprecipitates of p44^{erk1/mapk} from the same relative amounts of lysates were assayed for immune-complex phosphotransferase activity using MBP as substrate. As seen in Fig. 2, the

FIG. 1. Activation of p44^{erk1/mapk} kinase activity by an activated form of Raf-1 (R22W) and by v-Raf. Equal numbers of Sf9 cells were infected by baculoviruses encoding v-Raf alone (lane 1), activated Raf-1 (R22W) alone (lane 2), p44^{erk1/mapk} alone (lane 3), v-Raf and p44^{erk1/mapk} (lane 4), R22W and p44^{erk1/mapk} (lane 5), or p44^{erk1/mapk} and p21^{v-ras} (lane 6). Cells were lysed in RIPA buffer and whole cell lysates were resolved by SDS/PAGE. (A) Anti-phosphotyrosine immunoblot displaying the phosphotyrosine content of each of the lysates. Sizes in kDa are on the left. (B) Immunoblot displaying the relative amounts of p44^{erk1/mapk} in the same lysates.

FIG. 2. Autoradiogram displaying the phosphotransferase activity of p44^{erk1/mapk} toward added MBP *in vitro*. p44^{erk1/mapk} was immunoprecipitated from lysates of cells infected with viruses in the combinations indicated. Relative amounts of p44^{erk1/mapk} in each of the lysates are shown in Fig. 1A. Immune-complex kinase assays of p44^{erk1/mapk} immunoprecipitates were performed to measure phosphotransferase activity toward exogenously added MBP. Products from the kinase reaction were separated by SDS/PAGE and detected by autoradiography.

kinase activity of p44^{erk1/mapk} is greatly enhanced by coexpression with either v-Raf or activated Raf-1 (Raf22W) (lanes 4 and 5). In contrast, coexpression of p44^{erk1/mapk} with p21^{v-ras} resulted in only limited activation of p44^{erk1/mapk} (Fig. 2, lane 6). Activation of p44^{erk1/mapk} by v-Raf or Raf22W is not blocked by coexpression with a dominant inhibitory mutant of p21^{v-ras} (data not shown; description of the mutant is in ref. 30), suggesting that the v-Raf-induced activation of p44^{erk1/mapk} is independent of endogenous p21^{ras}.

Activation of p44^{erk1/mapk} by Raf-1, pp60^{v-src}, and p21^{v-ras}. Since constitutively activated Raf-1 greatly stimulated p44^{erk1/mapk} activity, we decided to assess the effect of Raf-1 on p44^{erk1/mapk} and the effect of p21^{v-ras} and pp60^{v-src} on Raf-1 and p44^{erk1/mapk} activities. We have previously shown that Raf-1 kinase activity is stimulated by coexpression with either pp60^{v-src} or p21^{v-ras} (30). p44^{erk1/mapk} immune complexes from lysates of cells infected with various combinations of recombinant baculoviruses (as indicated in Fig. 3A) were tested for phosphotransferase activity towards MBP in kinase assays performed *in vitro*. As shown in Fig. 2, phosphotransferase activity of p44^{erk1/mapk} immune complexes from cells infected with p44^{erk1/mapk} alone is low (Fig. 3A, lane 3). Either p21^{v-ras} or Raf-1 caused only a limited increase in the kinase activity of p44^{erk1/mapk} (Fig. 3A, lanes 5 and 6). However, kinase activity of p44^{erk1/mapk} from cells coinfecting with both p21^{v-ras} and Raf-1 is greatly enhanced (Fig. 3A, lane 9). Since activation of p44^{erk1/mapk} by Raf-1 is greatly enhanced by coexpression with p21^{v-ras} and since p21^{v-ras} has been shown to activate Raf-1, this suggested that p21^{v-ras} may act exclusively via Raf-1 in activation of p44^{erk1/mapk}. However pp60^{v-src}, which activates Raf-1 at least as effectively as p21^{v-ras} (see below), fails to greatly enhance p44^{erk1/mapk} activity when coinfecting with Raf-1 (Fig. 3A, lane 14). The relatively small increase in activation of p44^{erk1/mapk} by coinfection with Raf-1 and pp60^{v-src} is consistent with limited activation of Raf-1 by pp60^{v-src} (30). Clearly, the large enhancement of p44^{erk1/mapk} activity seen upon coexpression with p21^{v-ras} and Raf-1 is not seen upon coexpression of pp60^{v-src} with Raf-1. Further, a dominant-inhibitory mutant of Raf-1 [Raf301, which is also the kinase-inactive mutant of Raf-1 (35)] does not block the activation of p44^{erk1/mapk} by p21^{v-ras} (Fig. 3A, compare lanes 5 and 10). These data suggest that activation of p44^{erk1/mapk} by p21^{v-ras} may occur via both Raf-1-dependent and Raf-1-independent pathways.

To examine Raf-1 activity from lysates of similarly coinfecting cells, Raf-1 immune complexes were assayed for autokinase activity. An autoradiogram showing Raf-1 autokinase activity from cells infected as indicated is shown in Fig. 4. Autophosphorylation of Raf-1 is accompanied by a relatively large retardation in its gel mobility resulting in a form labeled

FIG. 3. Activation of p44^{erk1}/mapk kinase activity by pp60^{v-src}, p21^{v-ras}, and Raf-1. Equal numbers of Sf9 cells were infected by baculoviruses encoding Raf-1 alone (lane 1), kinase-inactive mutant of Raf-1 (Raf*) alone (lane 2), p44^{erk1}/mapk alone (lane 3), p21^{v-ras} alone (lane 4), p21^{v-ras} and p44^{erk1}/mapk (lane 5), Raf-1 and p44^{erk1}/mapk (lane 6), Raf* and p44^{erk1}/mapk (lane 7), Raf-1 and p21^{v-ras} (lane 8), Raf-1, p44^{erk1}/mapk, and p21^{v-ras} (lane 9), Raf*, p44^{erk1}/mapk, and p21^{v-ras} (lane 10), pp60^{v-src} alone (lane 11), pp60^{v-src} and p44^{erk1}/mapk (lane 12), Raf-1 and pp60^{v-src} (lane 13), Raf-1, p44^{erk1}/mapk, and pp60^{v-src} (lane 14), or Raf*, p44^{erk1}/mapk, and pp60^{v-src} (lane 15). The same relative amounts of whole cell lysates were used for the experiments displayed in A and B. (A) Autoradiogram displaying the *in vitro* phosphotransferase activity of p44^{erk1}/mapk toward added MBP. p44^{erk1}/mapk was immunoprecipitated from lysates of cells infected as indicated. Immune-complex kinase assays of p44^{erk1}/mapk immunoprecipitates were performed to measure phosphotransferase activity toward exogenously added MBP. Products were separated by SDS/PAGE and detected by autoradiography. (B) Immunoblots displaying the change in gel mobility of Raf-1 from each of the lysates. The various lanes correspond to lysates from cells infected in the combinations indicated in A.

Raf-P in Fig. 4 (lanes 10–13) (30). As reported previously (30), Raf-1 from singly infected cells displays only low levels of autokinase activity in immune-complex autokinase assays. This autokinase activity is increased to a limited extent by coinfection with p21^{v-ras} (Fig. 4; compare lanes 4 and 10) or by pp60^{v-src} (compare lanes 4 and 11), while coinfection with p44^{erk1}/mapk produces no increase (compare lanes 4, 8, and 9). Coexpression with p44^{erk1}/mapk also does not enhance the increase in autokinase activity of Raf-1 caused by p21^{v-ras} (Fig. 4; compare lanes 10 and 12) or by pp60^{v-src} (compare lanes 11 and 13). Thus, though pp60^{v-src} has a larger effect than p21^{v-ras} on Raf-1 kinase activity, it is p21^{v-ras} and not pp60^{v-src} that cooperates with Raf-1 in p44^{erk1}/mapk activation.

Effect of p44^{erk1}/mapk on Raf-1. Although coinfection with p44^{erk1}/mapk does not increase Raf-1 autokinase activity, it does cause a retardation in gel mobility of Raf-1 (Fig. 3B). The resulting form of Raf-1 is intermediate in mobility compared with forms labeled Raf and Raf-P in Fig. 4. Here, the retardation in gel mobility of Raf-1 is dependent on activation of p44^{erk1}/mapk protein kinase, since the retardation of Raf-1 in gel mobility increases as the level of activation of p44^{erk1}/mapk increases (Fig. 3B, compare lanes 1, 6, and 9). Coinfection of p44^{erk1}/mapk either with activated Raf (Raf22W) or with Raf-1 and p21^{v-ras} causes activation of p44^{erk1}/mapk, manifested by retardation in gel mobility of p44^{erk1}/mapk (data not shown) and an increase in tyrosine phosphorylation of p44^{erk1}/mapk (Fig. 1A and data not shown). A concomitant retardation in gel mobility of Raf from each of the lysates is seen (data not shown and Fig. 3B). Conversely, coinfection of a kinase-inactive mutant of p44^{erk1}/mapk either with activated Raf (Raf22W) or with Raf-1 and p21^{v-ras} also results in the expected retardation in gel mobility and increase in tyrosine phosphorylation of

FIG. 4. Autoradiogram of Raf-1 autokinase assay performed *in vitro*. Equal numbers of Sf9 cells were infected by baculoviruses encoding p21^{v-ras} alone (lane 1), pp60^{v-src} alone (lane 2), p44^{erk1}/mapk alone (lane 3), Raf-1 alone (lane 4), kinase-inactive mutant of Raf-1 (Raf*) alone (lane 5), p44^{erk1}/mapk and p21^{v-ras} (lane 6), pp60^{v-src} and p44^{erk1}/mapk (lane 7), Raf-1 and p44^{erk1}/mapk (lane 8), Raf* and p44^{erk1}/mapk (lane 9), Raf-1 and p21^{v-ras} (lane 10), Raf-1 and pp60^{v-src} (lane 11), Raf-1, p44^{erk1}/mapk, and p21^{v-ras} (lane 12), Raf-1, p44^{erk1}/mapk, and pp60^{v-src} (lane 13), Raf*, p44^{erk1}/mapk, and p21^{v-ras} (lane 14), or Raf*, p44^{erk1}/mapk, and pp60^{v-src} (lane 15). Raf-1 was immunoprecipitated from lysates of cells infected with viruses in the combinations indicated. Kinase assays on Raf-1 immunoprecipitates were conducted, and products were separated by SDS/PAGE and detected by autoradiography. No appreciable phosphotransferase activity by Raf-1 toward MBP was seen (data not shown). Raf-P refers to the slowest-migrating, autophosphorylated, form of Raf-1 protein, while Raf refers to the faster-migrating forms of Raf-1 protein present before the autokinase reaction.

p44^{erk1}/mapk (data not shown), but no retardation in the gel mobility of Raf is seen (data not shown). This suggests that though the kinase-inactive mutant of p44^{erk1}/mapk is lacking in activity, all signals leading to its activation are in place. Thus this p44^{erk1}/mapk-dependent retardation in gel mobility of Raf-1 is not required for its ability to activate p44^{erk1}/mapk. These data are consistent with the presence of a feedback loop whereby p44^{erk1}/mapk is activated by signals generated by Raf-1 and/or p21^{v-ras} and the activated p44^{erk1}/mapk feeds back to generate signals required to phosphorylate and cause a retardation in gel mobility of Raf-1.

DISCUSSION

We have used the baculovirus/Sf9 cell system to elucidate the regulatory relationships among pp60^{v-src}, p21^{v-ras}, MAP kinase (p44^{erk1}/mapk), and Raf-1. In Sf9 cells, p44^{erk1}/mapk is activated by coexpression with either v-Raf or a constitutively activated form of Raf-1 (Raf22W). In contrast, p44^{erk1}/mapk is activated to only a limited extent by coexpression with Raf-1 or p21^{v-ras}. Activation of p44^{erk1}/mapk is greatly enhanced by coexpression with both Raf-1 and p21^{v-ras}. We have shown previously that p21^{v-ras} activates Raf-1 (30). Taken together, these data suggest that p21^{v-ras} may act exclusively via a Raf-1-dependent pathway to activate p44^{erk1}/mapk. However, pp60^{v-src}, which activates Raf-1 at least as effectively as does p21^{v-ras}, fails to enhance p44^{erk1}/mapk activity as effectively as does p21^{v-ras} when coexpressed with Raf-1. Further, a dominant-inhibitory mutant of Raf-1 (Raf301) does not block the activation of p44^{erk1}/mapk by p21^{v-ras}. These data show that Raf-1 and p21^{v-ras} cooperate in the activation of p44^{erk1}/mapk and suggest that activation of p44^{erk1}/mapk by p21^{v-ras} may occur via both Raf-1-dependent and Raf-1-independent pathways.

p44^{erk1}/mapk is not without effect on Raf-1. Although p44^{erk1}/mapk does not increase Raf-1 autokinase activity, it does cause a retardation in gel mobility of Raf-1. The significance of this species of Raf-1 that is retarded in gel mobility remains unclear, since its presence does not correlate either

with changes in the autokinase activity of Raf-1 or with the ability of Raf-1 to activate p44^{erk1}/mapk. The data are consistent with the presence of a feedback loop whereby Raf-1 is phosphorylated and hence retarded in gel mobility by a downstream kinase whose activity is dependent on the activity of Raf-1. p44^{erk1}/mapk has been shown to phosphorylate Raf-1 *in vitro* (36, 37). Since p44^{erk1}/mapk is dependent on Raf-1 for activity (data presented here), p44^{erk1}/mapk could be the kinase that phosphorylates Raf-1 *in vivo*.

If a function were to be found for the p44^{erk1}/mapk-dependent phosphorylated form of Raf-1, it would theoretically be possible to reverse the order of activation of Raf-1 and p44^{erk1}/mapk. We have shown that p44^{erk1}/mapk can be activated by p21^{v-ras} independently of Raf-1. Activated p44^{erk1}/mapk could then phosphorylate and lead to retardation in gel mobility of Raf-1 (Fig. 3B, compare lanes 10 and 2). Thus Raf-1, in some cases, could appear to be downstream of p44^{erk1}/mapk.

It is worth noting that changes in gel mobility are often taken as an indication of Raf-1 activation. However, the modification leading to the retardation in gel mobility of Raf-1 is not required for Raf-1-dependent activation of p44^{erk1}/mapk. The data presented here show that a shift in gel mobility of Raf-1 may, in some cases, correlate better with the activation of a kinase (in this case p44^{erk1}/mapk) distinct from Raf-1.

Several recent reports have shown cell-specific differences in activation of p44^{erk1}/mapk by Raf-1 and p21^{v-ras} (11, 15, 38). In NIH 3T3 cells either p21^{v-ras} or v-Raf is sufficient to activate p44^{erk1}/mapk (14, 15, 16, 38). In Rat 1a cells neither oncogenic p21^{ras} nor oncogenic Raf is sufficient to activate p44^{erk1}/mapk (38). These results are in apparent contrast to the situation in PC12 cells, where p21^{v-ras} is seen to activate p44^{erk1}/mapk partially, while v-Raf does not (11). It is difficult to explain these results by using a simple linear pathway connecting p21^{ras} to Raf-1 to p44^{erk1}/mapk. However, the signaling network connecting membrane tyrosine kinases, p21^{v-ras}, Raf-1, and p44^{erk1}/mapk is not necessarily linear. We have previously demonstrated that both p21^{ras}-dependent and p21^{ras}-independent pathways exist for activation of Raf-1. The data presented in this report suggest the existence of both Raf-dependent and p21^{ras}-induced Raf-independent pathways to activate p44^{erk1}/mapk. A p21^{ras}-independent pathway for the activation of p44^{erk1}/mapk has also been demonstrated (28, 29). Thus several parallel independent signals, either complementary or redundant, appear to be capable of activating Raf-1 and/or p44^{erk1}/mapk. The data suggest the existence of a very complex regulatory network for the activation of Raf-1 and p44^{erk1}/mapk, involving as-yet-unidentified intermediates. It is possible that differences seen between PC12 cells, NIH 3T3 cells, and Rat 1a cells regarding activation of p44^{erk1}/mapk by Raf-1 and p21^{ras} may reflect cell-specific variations in the availability of key intermediates, rather than a fundamental change in the basic hard wiring of the network.

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