Raf-1 and p21ras cooperate in the activation of mitogen-activated protein kinase

(baculovirus/SF9 insect cells/ERK1 protein kinase)

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ABSTRACT Mitogen-activated protein (MAP) kinases Raf-1, p60rep5, and p21ras all play important roles in the transition of signals from the cell surface to the nucleus. We have used the baculovirus/SF9 insect cell system to elucidate the regulatory relationships between p60rep5, p21ras, MAP kinase (p44erk1/mapk), and Raf-1. In SF9 cells, p44erk1/mapk and Raf-1. In SF9 cells, p44erk1/mapk and Raf-1 are activated by coexpression with either v-Raf or a constitutively activated form of Raf-1 (Raf22W). In contrast, p44erk1/mapk is activated to only a limited extent by coexpression with either Raf-1 or p21ras alone. This activation of p44erk1/mapk is greatly enhanced by coexpression with both p21ras and Raf-1. Since we have previously shown that p21ras stimulates Raf-1 activity, the activation of p44erk1/mapk by p21ras may occur exclusively via a Raf-1-dependent pathway. However, a dominant-inhibitory mutant of Raf-1 (Raf301) does not block the activation of p44erk1/mapk by p21ras alone. Further, p60rep5, which activates Raf-1 at least as effectively as p21ras fails to enhance p44erk1/mapk activity greatly when coexpressed with Raf-1. These data suggest that activation of p44erk1/mapk by p21ras 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, p44erk1/mapk and p42erk2/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 p60rep5 (9) or activated p21ras (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-ras-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 p44erk1/mapk and p42erk2/mapk is clearly of interest. In response to extracellular stimuli, p44erk1/mapk and p42erk2/mapk undergo rapid phosphorylation on threonine and tyrosine residues and retardation in gel mobility. Activation of p44erk1/mapk and p42erk2/mapk requires phosphorylation of these proteins on both tyrosine and threonine residues (21, 22). These tyrosine and threonine residues, Thr183 and Tyr185 in p42erk2/mapk, are conserved in both isoforms, p44erk1/mapk and p42erk2/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-ras can activate partially purified preparations of MAP kinase activator (14–16).

Raf-1 and p44erk1/mapk both appear to be regulated by p21ras and by membrane tyrosine kinases (10, 11, 28, 29). Stimulation of PC12 cells by nerve growth factor or epidermal growth factor activates p44erk1/mapk in these cells. In fact, expression of activated p21ras in PC12 cells is sufficient to activate p44erk1/mapk partially and to cause hyperphosphorylation and retardation in gel mobility of Raf-1 (10, 11). Moreover, expression of a dominant interfering allele of p21ras is sufficient to block nerve growth factor-induced p44erk1/mapk activation and hyperphosphorylation of Raf-1 (10, 11). Since both Raf-1 and p44erk1/mapk are regulated by p21ras and by membrane tyrosine kinases, we have used baculovirus-encoded p21ras, p60rep5, p44erk1/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, p44erk1/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 p44erk1/mapk is seen when coexpression with either Raf-1 or with p21ras. The kinase activity of p44erk1/mapk is greatly enhanced by coex-

Abbreviations: MAP, mitogen-activated protein; MBP, myelin basic protein.
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pression with Raf-1 and p21V-ras. We have shown previously that coexpression with either pp60src or p21V-ras increases the kinase activity of Raf-1 (30). Yet the kinase activity of p44erkl is greatly enhanced by coexpression with p21V-ras and Raf-1 (31). Consistent with predicted sizes, recombinant baculovirus encoding v-Raf produces two proteins, one of predicted size 90 kDa and the other of smaller size, 58 kDa (possibly a posttranslationally related product of the full-length protein or an altered phosphoform). Production of recombinant baculovirus encoding Raf-1 (35), p21V-ras, or kinaseinactive Raf-1 (Raf-301) (30) has been described previously. Recombinant baculovirus encoding pp60src was obtained from Ray Erikson (Harvard University). High-titer viral supernatants of each of the recombinant baculoviruses encoding Raf-1, Raf-301 (Raf*), pp60src, p21V-ras, v-Raf, Raf22W, or p44erkl 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, p44erkl, and p21V-ras proteins were assayed by Western blot analysis for use as 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 bead-bound 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 immuno 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 Coomasie-blue staining. Raf-1 autophosphorylation after kinase reaction in vitro was quantitated on a PhosphorImager (Molecular Dynamics). Reactions using myelin basic protein (MBP, 4 μg) 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).

**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 350-1605A) supplemented with 10% fetal calf serum. All protocols for passage, infection, and transfection of SF9 cells were as described (11).

Monoclonal antibody 327 was a gift from Josef Brugge (University of Pennsylvania, Philadelphia). Anti-phosphotyrosine monoclonal antibody 4G10 (32) was a gift from Dr. Brian Drucker (Dana–Farber Cancer Institute, Boston). Anti-p21 monoclonal antibody YA6-259 was produced as a supernatant from a rat hybridoma cell line kindly supplied by Larry Feig (Tufts University, Medford, MA). Rabbit polyclonal antisera was raised against a peptide corresponding to the 12 C-terminal residues of Raf-1 (CTLTTSRPLVFS (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 p44erkl (8) (Upstate Biotechnology, Lake Placid, NY). Rabbit polyclonal anti-MAP kinase C2 antibody, used for immunoprecipitations, was 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 polyethylene glycolase) 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 (Raf22W) was constructed by subcloning of the EcoRI/Xba I fragment from the truncated clone designated 22W (19) in the BamHI/Xba I site of the baculovirus transfer vector pVL1393 by using BamHI/EcoRI adapters (New England Biolabs nos. 1105 and 1106). The baculovirus transfer vectors encoding human p44ERK1/MApk (D.L.C. and S.L.P., unpublished work) and kinase-inactive human p44ERK1 (lysine-to-methionine mutation) were constructed by subcloning of the BamHI/ EcoRI fragment encoding the full-length proteins in the BamHI/EcoRI sites of the baculovirus transfer vector pVL1393. Human p44ERK1/MApk shares 96% identity with rat and mouse p44ERK1/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 p44ERK1/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-
RESULTS
To assess the effects of p21v-ras, pp60v-src, Raf-1, and Raf-1 mutants on p44erk1/mapk, a series of baculovirus coinfections were performed, using different combinations of recombinant baculoviruses expressing each of these proteins. Cells were lysed and p44erk1/mapk and/or Raf-1 proteins were immunoprecipitated from the lysates. The same number of cells was used for each infection. Levels of p44erk1/mapk, Raf-1, pp60v-src, and p21v-ras proteins in the different lysates were normalized by immunoblotting.

Activation of p44erk1/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 coinfected with a baculovirus encoding p44erk1/mapk and the activity of p44erk1/mapk was assessed after each of the infections. Since activation of p44erk1/mapk requires phosphorylation of p44erk1/mapk on tyrosine, anti-phosphotyrosine antibody immunoblots were first used to compare the levels of tyrosine phosphorylated p44erk1/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 p44erk1/mapk is most highly tyrosine phosphorylated in cells coexpressing either p44erk1/mapk and Raf22W (lane 4) or p44erk1/mapk and v-Raf (lane 5). An immunoblot showing the relative amounts of p44erk1/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 p44erk1/mapk in those lysates. To see if changes in anti-phosphotyrosine immunoblot with changes in kinase activity, immune-complex kinase assays were performed to directly assess the phosphotransferase activity of p44erk1/mapk in each of the lysates. Immuno precipitates of p44erk1/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 kinase activity of p44erk1/mapk is greatly enhanced by coexpression with either v-Raf or activated Raf-1 (Raf22W) (lanes 4 and 5). In contrast, coexpression of p44erk1/mapk with p21v-ras resulted in only limited activation of p44erk1/mapk (Fig. 2, lane 6). Activation of p44erk1/mapk by v-Raf or Raf22W is not blocked by coexpression with a dominant inhibitory mutant of p21v-ras (data not shown; description of the mutant is in ref. 30), suggesting that the v-Raf-induced activation of p44erk1/mapk is independent of endogenous p21v-ras.

Activation of p44erk1/mapk by Raf-1, pp60v-src, and p21v-ras. Since constitutively activated Raf-1 greatly stimulated p44erk1/mapk activity, we decided to assess the effect of Raf-1 on p44erk1/mapk and the effect of p21v-ras and pp60v-src on Raf-1 and p44erk1/mapk activities. We have previously shown that Raf-1 kinase activity is stimulated by coexpression with either pp60v-src or p21v-ras (30). p44erk1/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 p44erk1/mapk immune complexes from cells infected with p44erk1/mapk alone is low (Fig. 3A, lane 3). Either p21v-ras or Raf-1 caused only a limited increase in the kinase activity of p44erk1/mapk (Fig. 3A, lanes 5 and 6). However, kinase activity of p44erk1/mapk from cells coinfected with both p21v-ras and Raf-1 is greatly enhanced (Fig. 3A, lane 9). Since activation of p44erk1/mapk by Raf-1 is greatly enhanced by coexpression with p21v-ras and since p21v-ras has been shown to activate Raf-1, this suggested that p21v-ras may act exclusively via Raf-1 in activation of p44erk1/mapk. However pp60v-src, which activates Raf-1 at least as effectively as p21v-ras (see below), fails to greatly enhance p44erk1/mapk activity when coinfected with Raf-1 (Fig. 3A, lane 14). The relatively small increase in activation of p44erk1/mapk by coinfection with Raf-1 and pp60v-src is consistent with limited activation of Raf-1 by pp60v-src (30). Clearly, the large enhancement of p44erk1/mapk activity seen upon coexpression with p21v-ras and Raf-1 is not seen upon coexpression of pp60v-src with Raf-1. Further, a dominant-inhibitory mutant of Raf-1 (Raf031, which is also the kinase-inactive mutant of Raf-1 (35J) does not block the activation of p44erk1/mapk by p21v-ras (Fig. 3A), compare lanes 5 and 10. These data suggest that activation of p44erk1/mapk by p21v-ras may occur via both Raf-1-dependent and Raf-1-independent pathways.

To examine Raf-1 activity from lysates of similarly coinfected 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. Autorphosphorylation of Raf-1 is accompanied by a relatively large retardation in its gel mobility resulting in a form labeled
Fig. 3. Activation of p44erk1/mapk kinase activity by pp60v-src, p21v-ras, and Raf-1. Equal numbers of S9 cells were infected by baculoviruses encoding Raf-1 alone (lane 1), kinase-inactive mutant of Raf-1 (Raf* alone (lane 2), p44erk1/mapk alone (lane 3), p21v-ras alone (lane 4), p21v-ras and p44erk1/mapk (lane 5), Raf-1 and p44erk1/mapk (lane 6), Raf* and p44erk1/mapk (lane 7), Raf-1 and p21v-ras (lane 8), Raf-1, p44erk1/mapk, and p21v-ras (lane 9), Raf*, p44erk1/mapk, and p21v-ras (lane 10), pp60v-src alone (lane 11), pp60v-src and p44erk1/mapk (lane 12), Raf-1 and pp60v-src (lane 13), Raf-1, p44erk1/mapk, and pp60v-src (lane 14), or Raf*, p44erk1/mapk, and pp60v-src (lane 15). The 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 p44erk1/mapk toward added MBP. p44erk1/mapk was immunoprecipitated from lysates of cells infected as indicated. Immune-complex kinase assays of pp44erk1/mapk immunoprecipitates were performed to measure phosphotransferase activity toward exogenously added MBP. Products were separated by SDS/PAGE and detected by autoradiography. (B) Immuneblots 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 autokinease assays. This autokinase activity is increased to a limited extent by coinfection with p21v-ras (Fig. 4; compare lanes 4 and 10) or by pp60v-src (compare lanes 4 and 11), while coinfection with pp44erk1/mapk produces no increase (compare lanes 4, 8, and 9). Coexpression with pp44erk1/mapk also does not enhance the increase in autokinase activity of Raf-1 caused by p21v-ras (Fig. 4; compare lanes 10 and 12) or by pp60v-src (compare lanes 11 and 13). Thus, though pp60v-src has a larger effect than p21v-ras on Raf-1 kinase activity, it is p21v-ras and not pp60v-src that cooperates with Raf-1 in pp44erk1/mapk activation.

Effect of p44erk1/mapk on Raf-1. Although coinfection with pp44erk1/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 pp44erk1/mapk protein kinase, since the retardation of Raf-1 in gel mobility increases as the level of activation of pp44erk1/mapk increases (Fig. 3B, compare lanes 1, 6, and 9). Coinfection of pp44erk1/mapk either with activated Raf (Raf22W) or with Raf-1 and p21v-ras causes activation of pp44erk1/mapk, manifested by retardation in gel mobility of pp44erk1/mapk (data not shown) and an increase in tyrosine phosphorylation of pp44erk1/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 pp44erk1/mapk either with activated Raf (Raf22W) or with Raf-1 and p21v-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 S9 cells were infected by baculoviruses encoding p21v-ras alone (lane 1), pp60v-src alone (lane 2), pp44erk1/mapk alone (lane 3), Raf-1 alone (lane 4), kinase-inactive mutant of Raf-1 (Raf*) alone (lane 5), Raf-1, pp44erk1/mapk, and p21v-ras (lane 6), Raf* and pp44erk1/mapk (lane 7), Raf-1 and pp44erk1/mapk (lane 8), Raf* and pp44erk1/mapk (lane 9), Raf-1 and p21v-ras (lane 10), Raf-1 and pp60v-src (lane 11), Raf-1, pp44erk1/mapk, and p21v-ras (lane 12), Raf-1, pp44erk1/mapk, and pp60v-src (lane 13), Raf*, pp44erk1/mapk, and p21v-ras (lane 14), or Raf*, pp44erk1/mapk, and pp60v-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.

p44erk1/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 pp44erk1/mapk is lacking in activity, all signals leading to its activation are in place. Thus this pp44erk1/mapk-dependent retardation in gel mobility of Raf-1 is not required for its ability to activate pp44erk1/mapk. These data are consistent with the presence of a feedback loop whereby pp44erk1/mapk is activated by signals generated by Raf-1 and/or p21v-ras and the activated pp44erk1/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/S9 cell system to elucidate the regulatory relationships among pp60v-src, p21v-ras, MAP kinase (pp44erk1/mapk), and Raf-1. In S9 cells, pp44erk1/mapk is activated by coexpression with either v-Raf or a constitutively activated form of Raf-1 (Raf22W). In contrast, pp44erk1/mapk is activated to only a limited extent by coexpression with Raf-1 or p21v-ras. Activation of pp44erk1/mapk is greatly enhanced by coexpression with both Raf-1 and p21v-ras. We have shown previously that p21v-ras activates Raf-1 (30). Taken together, these data suggest that p21v-ras may act exclusively via a Raf-1-dependent pathway to activate pp44erk1/mapk. However, pp60v-src, which activates Raf-1 at least as effectively as does p21v-ras, fails to enhance pp44erk1/mapk activity as effectively as does p21v-ras when coexpressed with Raf-1. Further, a dominant-inhibitory mutant of Raf-1 (Raf301) does not block the activation of pp44erk1/mapk by p21v-ras. These data show that Raf-1 and p21v-ras cooperate in the activation of pp44erk1/mapk and suggest that activation of pp44erk1/mapk by p21v-ras may occur via both Raf-1-dependent and Raf-1-independent pathways.

p44erk1/mapk is not without effect on Raf-1. Although pp44erk1/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 p44erk1. 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. p44erk1 has been shown to phosphorylate Raf-1 in vitro (36, 37). Since p44erk1 is dependent on Raf-1 for activity (data presented here), p44erk1 could be the kinase that phosphorylates Raf-1 in vivo.

If a function were to be found for the p44erk1, dependent phosphorylated form of Raf-1, it would theoretically be possible to reverse the order of activation of Raf-1 and p44erk1. We have shown that p44erk1 can be activated by p21ras independently of Raf-1. Activated p44erk1 could then phosphorylate and lead to retardation in gel mobility of Raf-1 (Fig. 3F, compare lanes 10 and 2). Thus Raf-1, in some cases, could appear to be downstream of p44erk1.

It is worth noting that changes in gel mobility are often taken as a 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 p44erk1. 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 p44erk1) distinct from Raf-1.

Several recent reports have shown cell-specific differences in activation of p44erk1 by Raf-1 and p21ras (11, 15, 38). In NIH 3T3 cells either p21ras or v-Raf is sufficient to activate p44erk1 (14, 15, 16, 38). In Raf 1a cells neither oncogenic p21ras nor oncogenic Raf is sufficient to activate p44erk1 (38). These results are in apparent contrast to the situation in PC12 cells, where p21ras is seen to activate p44erk1 partially, while v-Raf does not (11). It is difficult to explain these results by using a simple linear pathway connecting p21ras to Raf-1 to p44erk1. However, the signaling network connecting membrane tyrosine kinases, p21ras, Raf-1, and p44erk1, is not necessarily linear. We have previously demonstrated that both p21ras-dependent and p21ras-independent pathways exist for activation of Raf-1. The data presented in this report suggest the existence of both Raf-dependent and p21ras-induced Raf-independent pathways to activate p44erk1. A p21ras-independent pathway for the activation of p44erk1 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 p44erk1. The data suggest the existence of a very complex regulatory network for the activation of Raf-1 and p44erk1, involving as-yet-unidentified intermediates. It is possible that differences seen between PC12 cells, NIH 3T3 cells, and Rat 1a cells regarding activation of p44erk1 by Raf-1 and p21ras 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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