Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 2922–2926, April 1992
Cell Biology

Both $p21^{ras}$ and $pp60^{v\text{-}src}$ are required, but neither alone is sufficient, to activate the Raf-1 kinase

(signal transduction/baculovirus/oncogenes/protein serine/threonine kinase/phosphorylation)

NIDHI GUPTA WILLIAMS*†, THOMAS M. ROBERTS*‡§, AND PING LI¶

*Dana–Farber Cancer Institute and Departments of †Biological Chemistry and Molecular Pharmacology and ‡Pathology, Harvard Medical School, Boston, MA 02115; and ¶BASF Bioresearch Corp., Cambridge, MA 02139

Communicated by Robert A. Weinberg, December 30, 1991

ABSTRACT **The *raf* genes encode a family of cytoplasmic proteins with intrinsic protein-serine/threonine kinase activity. The c-*raf* gene is the cellular homolog of v-*raf*, the transforming gene of murine sarcoma virus 3611. The constitutive kinase activity of the v-Raf protein has been implicated in transformation and mitogenesis. The activity of Raf-1, the protein product of the c-*raf* gene, is normally suppressed by a regulatory N-terminal domain. Activation of various tyrosine-kinase growth factor receptors results in activation of Raf-1 and its hyperphosphorylation. Further, Raf-1 has been shown to act either downstream or independently of the $p21^{ras}$ protein, as indicated by experiments involving microinjection of anti-Ras antibodies. To investigate the potential role of $p21^{ras}$ in the activation of Raf-1 by tyrosine kinases, we have used the baculovirus/Sf9 cell system to overproduce various wild-type and mutant forms of $pp60^{src}$, $p21^{ras}$, and Raf-1 proteins. We show that either $pp60^{v\text{-}src}$ or $p21^{c\text{-}ras}$ can independently activate the autokinase activity of Raf-1, but only to a limited extent. Surprisingly, both $pp60^{v\text{-}src}$ and $p21^{c\text{-}ras}$ are required to fully activate Raf-1. Analysis of the Raf-1 autokinase activity *in vitro* shows that Raf-1 autophosphorylation sites are distributed equally on serine and threonine residues. When Raf-1 is analyzed by immunoblotting, as previously reported for mammalian cell experiments, a marked increase in the apparent molecular weight of Raf-1 is seen only when it is coexpressed with both $pp60^{v\text{-}src}$ and $p21^{ras}$.**

Pathways used to transfer signals from protein-tyrosine kinases at the cell surface to transcription factors in the nucleus remain to be unraveled (1). Several protein-serine/threonine kinases have been implicated as intermediates in these signal transduction pathways. These kinases include mitogen-activated protein (MAP) kinase (2), ribosomal S6 kinase (rsk) (3), and Raf-1 (4). The importance of Raf-1 in transfer of mitogen-induced signals from the plasma membrane to the nucleus has been revealed by a series of recent observations. Raf-1 is activated in response to activation of a variety of tyrosine kinase receptors as well as in response to $pp60^{v\text{-}src}$ expression (5–12). Microinjection of oncogenically activated Raf-1 protein into NIH 3T3 mouse fibroblasts stimulated DNA synthesis and morphologically transformed the cells (13). Finally, transcription that was dependent on serum response element (SRE) and PEA1-motif (14–16) enhancer elements was induced by activated mutants of Raf-1. These and other observations clearly implicate Raf-1 as an intermediate in the transfer of information from the cell surface to the nucleus.

Raf-1 is a 72- to 76-kDa cytoplasmic protein with intrinsic serine/threonine kinase activity. It has been found in nearly all cell lines examined (17, 18). Raf-1 is the cellular homolog of v-Raf, the product of the transforming gene of murine sarcoma virus 3611 (19, 20). The unregulated kinase activity of the v-Raf protein has been associated with transformation and mitogenesis (21). The activity of Raf-1, on the other hand, is normally suppressed by a regulatory N-terminal domain (21–23). Raf-1 activation has been correlated with an apparent increase in molecular weight as assayed by a decrease in mobility during SDS/PAGE (4). No specific cellular substrate for the Raf-1 kinase has been identified, nor has the mechanism for its activation by tyrosine kinases been determined (1).

Several lines of evidence suggest that $p21^{c\text{-}ras}$ may play a role in activation of Raf-1 and may even play the role of messenger from membrane tyrosine kinases to Raf-1. Reversal of the transformed phenotype by microinjection of inhibitory anti-Ras antibodies into cells transformed by v-*src* and v-*fms* demonstrated that $p21^{c\text{-}ras}$ is required for v-*src*- or v-*fms*-induced transformation. The transformed phenotype of v-raf-transformed cells, on the other hand, was unaffected by the antibody injections, which suggests that Raf-1 acts either downstream from or independently of $p21^{c\text{-}ras}$ (24, 25). Very recently, experiments involving expression of a dominant inhibitory mutant of c-*raf* and of c-*raf* antisense mRNA in $p21^{ras}$-transformed cells demonstrated that Raf-1 is required for *ras*-induced transformation (26).

To investigate the potential role of $p21^{c\text{-}ras}$ in the activation of Raf-1 by tyrosine kinases, we have used the baculovirus/Sf9 cell system (27) to overproduce various wild-type and mutant forms of $pp60^{src}$, $p21^{c\text{-}ras}$, and Raf-1 proteins. We show that coexpression of $pp60^{v\text{-}src}$ and $p21^{c\text{-}ras}$ with Raf-1 activates the autokinase activity of Raf-1. To a limited extent, either $pp60^{v\text{-}src}$ or $p21^{c\text{-}ras}$ independently can activate the autokinase activity of Raf-1. However, for full activation both $pp60^{v\text{-}src}$ and $p21^{c\text{-}ras}$ are required. We observe a characteristic shift in gel mobility of Raf-1 protein from Sf9 cells only when Raf-1 is activated by both $p21^{c\text{-}ras}$ and $pp60^{v\text{-}src}$. The activation of Raf-1 in Sf9 cells requires active forms of both $p21^{c\text{-}ras}$ and $pp60^{v\text{-}src}$. Neither the dominant inhibitory mutant of $p21^{c\text{-}ras}$ ($p21^{ras\text{-}N17}$) nor a kinase-inactive mutant of $pp60^{c\text{-}src}$ is able to activate the Raf-1 kinase.

MATERIALS AND METHODS

Spodoptera frugiperda (Sf9) cells, wild-type baculovirus (*Autographa californica* nuclear polyhedrosis virus), and the transfer vector (pVL941) were provided by Max Summers (Texas A&M University). Sf9 cells were grown either in suspension or as a monolayer culture in Grace's medium (GIBCO) supplemented with 10% fetal bovine serum. All protocols for passage, infection, and transfection of Sf9 cells were as described by Summers and Smith (27). Rabbit polyclonal antiserum was raised against a peptide corresponding to the 12 C-terminal residues of Raf-1 (CTLTTSPRLPVF) (28).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: GAP, GTPase-activating protein.

§To whom reprint requests should be addressed.

Monoclonal anti-Src hybridoma EC10 was a gift from Sally Parsons (University of Virginia, Charlottesville). Monoclonal anti-Src antibody 327 was a gift from Joan Brugge (University of Pennsylvania). RAS10 monoclonal anti-Ras (pan) antibody was purchased from New England Nuclear.

Construction and Isolation of Recombinant Baculovirus. All *ras* genes used were derivatives of the c-Ha-*ras* gene allele. To construct the baculoviral transfer vectors encoding $p21^{v\text{-}ras}$, $p21^{c\text{-}ras}$, or $p21^{ras\text{-}N17}$, the *Bgl* II–*Bam*HI fragments from $pXVR^{Har}$ [0.7 kilobase (kb)], pXCR (1 kb), and $pXCR^{Asn17}$ (1 kb) (29) were ligated into *Bam*HI-digested baculoviral transfer plasmid pVL941. The resulting transfer vectors, pVL941-v-ras, pVL941-c-ras, and pVL941-ras-17, were checked for the proper orientation of insertion before transfection into Sf9 cells.

The isolation of recombinant baculoviruses encoding Raf-1 (4) and $pp60^{src\text{-}295}$ (30) has been described. Recombinant baculovirus encoding $pp60^{v\text{-}src}$ was obtained from Ray Erickson (Harvard University). The baculoviral transfer vector carrying the kinase-inactive Raf-1 mutant (pVL941-Raf301) was kindly provided by G. Heidecker and U. Rapp (National Cancer Institute, Frederick, MD). The pVL941-Raf301 vector was cotransfected with wild-type baculoviral DNA into Sf9 cells as described (27). The viruses released into the supernatant were plaqued after appropriate dilutions. Occlusion-negative plaques, containing pure recombinant baculovirus encoding kinase-inactive Raf-1 mutant (Raf-301), were isolated after one to three rounds of plaque purification. A similar procedure was used to isolate recombinant baculoviruses encoding $p21^{v\text{-}ras}$, $p21^{c\text{-}ras}$, and dominant inhibitory $p21^{c\text{-}ras}$ mutant ($p21^{ras\text{-}N17}$). High-titer viral supernatants ($\approx 10^8$ plaque-forming units/ml) of each of the baculoviruses encoding one of the proteins (Raf-1, Raf-301, $p21^{v\text{-}ras}$, $p21^{c\text{-}ras}$, $p21^{ras\text{-}N17}$, $pp60^{src\text{-}295}$, or $pp60^{v\text{-}src}$) were prepared and used in various combinations to infect Sf9 cells. Infections were always carried out at excess multiplicity of infection for each virus. For coinfections, the amounts of infecting viruses were adjusted to keep the ratio of any two proteins being studied the same when protein production in a double infection was compared with that in a triple infection. To accomplish this, preliminary infections were performed using differing ratios of the infecting viruses over a range of multiplicities of infection at each ratio. Protein production was monitored by immunoblotting.

Preparation of Lysates from Baculovirus-infected Sf9 Cells. Sf9 cells (2×10^6) were infected with the desired recombinant baculovirus or with combinations of the different recombinant baculoviruses in appropriate ratios. Forty to 48 hr postinfection, cells were washed twice in cold phosphate-buffered saline and then lysed for 10 min in RIPA buffer [20 mM Tris, pH 7.4/137 mM NaCl/10% (vol/vol) glycerol/0.1% (wt/vol) SDS/0.5% (wt/vol) sodium deoxycholate/1% (vol/vol) Triton/2 mM EDTA] supplemented with phenylmethylsulfonyl fluoride (1 mM), aprotinin (0.15 unit/ml), and sodium orthovanadate (1 mM). The lysates were centrifuged at $10,000 \times g$ for 10 min to remove insoluble material, and the supernatants were assayed for recombinant protein production by Western immunoblotting using an alkaline phosphatase detection system. Cell lysates containing equivalent amounts of Raf-1, $pp60^{src}$, and $p21^{ras}$ proteins as assayed by Western blot analysis were used for kinase assays performed *in vitro*.

Immunoprecipitation and Phospho Amino Acid Analysis of Metabolically Labeled Raf-1. Thirty-nine hours postinfection, cells were rinsed twice with cold phosphate-buffered saline and then incubated for 4 hr with labeling mix (1.5 ml of phosphate-free Grace's medium supplemented with 1.5% dialyzed fetal bovine serum, 2 mM glutamine, and 2 mCi of [^{32}P]orthophosphate) before lysis as described above. Lysates of equivalent numbers of cells were immunoprecipitated with anti-Raf polyclonal antibody as described below, and the immunoprecipitates were resolved by SDS/PAGE. After transfer to Immobilon-P membrane (Millipore) and immunoblotting, the labeled Raf-1 bands were cut out and subjected to phospho amino acid analysis by one-dimensional thin-layer electrophoresis (31).

Raf-1 Kinase Assays *in Vitro*. Anti-Raf 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 and then incubated with cell lysates at 4°C for 4 hr. The immunoprecipitates were washed once with RIPA buffer, twice with 0.5 M LiCl/0.1 M Tris, pH 8.0, and once in kinase buffer (25 mM Hepes, pH 7.4/25 mM glycerol 2-phosphate/1 mM dithiothreitol/10 mM $MgCl_2$/10 mM $MnCl_2$) before assay. For autokinase reactions, washed immunoprecipitates were incubated in 40 μl of kinase buffer supplemented with 15 μM nonradioactive ATP and 10 μCi (370 kBq) of [γ-^{32}P]ATP (3000 Ci/mmol) at room temperature for 20 min. The reaction was terminated by the addition of Laemmli sample buffer and the samples were boiled before SDS/PAGE. Proteins were detected either by autoradiography or by immunoblotting. Quantitation of Raf-1 phosphorylation following kinase reaction *in vitro* was performed on a Betascope model 603 (Betagen, Waltham, MA). When syntide-2 (amino acid sequence PLARTLSVAGLPGKK) or histone H1 was used as exogenous substrate for kinase reactions, they were included in the kinase buffer at 1 mM and 5 μg, respectively. Analysis of histone H1 phosphorylation was done by SDS/PAGE. Processing of the samples for syntide-2 was performed as described elsewhere (5).

RESULTS

Activation of Raf-1 by $p21^{c\text{-}ras}$ and $pp60^{v\text{-}src}$ as Measured in Living Cells. To assay the effects of $p21^{c\text{-}ras}$ and $pp60^{v\text{-}src}$ on the activation of Raf-1, Sf9 cells were infected either (*i*) singly with baculovirus encoding Raf-1 alone, (*ii*) doubly with baculoviruses encoding Raf-1 and $p21^{c\text{-}ras}$ or Raf-1 and $pp60^{v\text{-}src}$, or (*iii*) triply with all three baculoviruses, encoding Raf-1, $p21^{c\text{-}ras}$, and $pp60^{v\text{-}src}$. To ensure that the effects we were assaying were dependent on Raf-1 activity, cells infected triply with baculoviruses encoding a kinase-inactive mutant of Raf-1 (Raf-301), $p21^{c\text{-}ras}$, and $pp60^{v\text{-}src}$ were included as a control. Infected cells were metabolically labeled with [^{32}P]orthophosphate. Raf-1 protein was immunoprecipitated from lysates of infected, labeled cells, resolved by SDS/PAGE, and immunoblotted with anti-Raf antibody. An autoradiogram of the labeled and immunoprecipitated Raf-1 protein (Fig. 1) shows that only in the case of the triply infected cells containing wild-type Raf-1, $p21^{c\text{-}ras}$, and $pp60^{v\text{-}src}$ (lane 4) was Raf-1 hyperphosphorylated and retarded in gel mobility, as seen when Raf-1 is activated in mammalian cells. That Raf-1 from triply infected Sf9 cells is actually activated will be demonstrated below.

The ^{32}P radiolabeled Raf-1 bands from each lane were cut out and subjected to phospho amino acid analysis. Though most of the phosphate label resided on serines, a small amount of phosphotyrosine was detected in Raf-1 from all cells infected with baculovirus encoding $pp60^{v\text{-}src}$ (Fig. 2, lanes 3–5). Yet only Raf-1 from triply infected cells is activated fully (see below) and retarded in gel mobility (Fig. 1, lane 4). This indicates that if tyrosine phosphorylation is important for Raf-1 activation, it cannot be sufficient for full activation. There is clearly a higher level of threonine phosphorylation on Raf-1 from cells infected triply with baculoviruses encoding wild-type Raf-1, $pp60^{v\text{-}src}$, and $p21^{c\text{-}ras}$ as compared to Raf-1 from cells infected triply with baculoviruses encoding kinase-inactive Raf-1 (Raf-301), $pp60^{v\text{-}src}$, and $p21^{c\text{-}ras}$ (Fig. 2, compare lanes 4 and 5). It is not clear whether the increase in threonine phosphorylation results from auto-

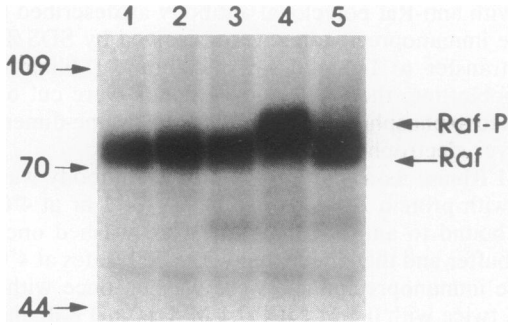

FIG. 1. Autoradiogram of metabolically [^{32}P]phosphorylated Raf-1. Raf-1 immunoprecipitates from ^{32}P-radiolabeled cells infected with baculoviruses encoding either Raf-1 alone (lane 1), Raf-1 and p21$^{c\text{-}ras}$ (lane 2), Raf-1 and pp60$^{v\text{-}src}$ (lane 3), Raf-1, p21$^{c\text{-}ras}$, and pp60$^{v\text{-}src}$ (lane 4), or a kinase-inactive mutant of Raf-1 (Raf-301), p21$^{c\text{-}ras}$, and pp60$^{v\text{-}src}$ (lane 5) were resolved by SDS/PAGE. Proteins were transferred to Immobilon-P and immunoblotted with anti-Raf antibody, and the immunoblot was exposed to film. Raf-P, hyperphosphorylated Raf-1. Molecular size markers (kDa) are indicated at left.

phosphorylation of Raf-1 or from phosphorylation of Raf-1 by a kinase-dependent activity of Raf-1.

Activation of Raf-1 by p21$^{c\text{-}ras}$ and pp60$^{v\text{-}src}$ as Measured *in Vitro*. Since Raf-1 protein is a kinase, traditional assays for measurement of changes in activity have included kinase assays performed *in vitro* with either itself as substrate or an exogenous substrate. Previously, substrates for assaying kinase activity of Raf-1 *in vitro* have included histone H1 and the synthetic peptide syntide-2. We were not able to detect a differential phosphorylation of either of these substrates when phosphorylation by Raf-1 from cells infected triply with baculoviruses encoding wild-type Raf-1, pp60$^{v\text{-}src}$, and p21$^{c\text{-}ras}$ was compared with phosphorylation by Raf-1 from cells infected triply with baculoviruses encoding kinase-inactive Raf-1 (Raf-301), pp60$^{v\text{-}src}$, and p21$^{c\text{-}ras}$ (data not shown). This leads us to believe that the phosphorylation seen on these substrates was due to the presence of a kinase other than Raf-1 in the assay mixture. Similar results have been reported by Isumi *et al.* (10). However, autokinase reactions *in vitro* provided a sensitive assay for Raf-1 activity. As described above, Sf9 cells were infected either singly, doubly, or triply with pp60$^{v\text{-}src}$, p21$^{c\text{-}ras}$, and Raf-1 baculoviruses. Raf-1 protein from lysates normalized for amounts of Raf-1, p21$^{c\text{-}ras}$, and pp60$^{v\text{-}src}$ was immunoprecipitated and subjected to autokinase reactions *in vitro*, to assay for differential activation of Raf-1. The autokinase activity of Raf-1 immunoprecipitated from cells infected only with baculovirus encoding Raf-1 was very low (Fig. 3, lane 1). The autokinase activity

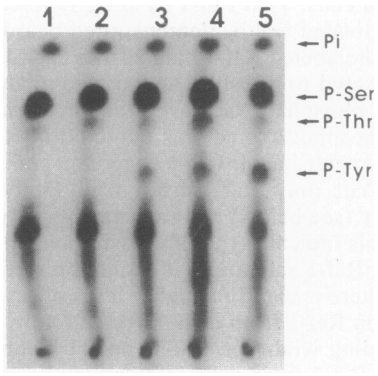

FIG. 2. Phospho amino acid analysis of metabolically labeled Raf-1 protein bands cut out from the immunoblot described in Fig. 1. Lane numbers correspond to those in Fig. 1.

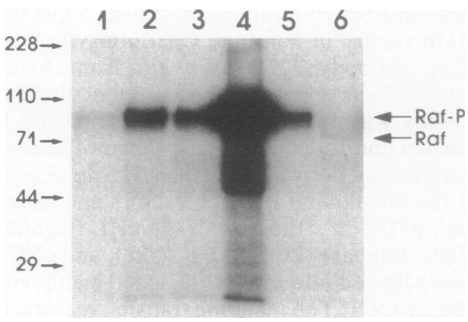

FIG. 3. Autoradiogram of Raf-1 *in vitro* kinase activity. Kinase assays were performed *in vitro* with Raf-1 immunoprecipitated from cells infected with baculoviruses encoding either Raf-1 alone (lane 1), Raf-1 and p21$^{c\text{-}ras}$ (lane 2), Raf-1 and pp60$^{v\text{-}src}$ (lane 3), Raf-1, p21$^{c\text{-}ras}$, and pp60$^{v\text{-}src}$ (lane 4), Raf-1, dominant inhibitory mutant of p21$^{c\text{-}ras}$ (p21$^{ras\text{-}N17}$), and pp60$^{v\text{-}src}$ (lane 5), or kinase-inactive mutant of Raf-1 (Raf-301), p21$^{c\text{-}ras}$, and pp60$^{v\text{-}src}$ (lane 6). The products were resolved by SDS/PAGE, immunoblotted, and exposed to film.

of Raf-1 immunoprecipitated from cells infected with baculoviruses encoding either Raf-1 and p21$^{c\text{-}ras}$ or Raf-1 and pp60$^{v\text{-}src}$ was increased (lanes 2 and 3). Thus either pp60$^{v\text{-}src}$ or p21$^{c\text{-}ras}$ was able to increase the activity of Raf-1 in Sf9 cells to a limited extent. The autokinase activity of Raf-1 immunoprecipitated from Sf9 cells triply infected with baculoviruses encoding Raf-1, p21$^{c\text{-}ras}$, and pp60$^{v\text{-}src}$ (lane 4) was dramatically increased compared with the activity from doubly (lanes 2 and 3) or singly (lane 1) infected cells. The activity observed in Fig. 3 most likely reflects the kinase activity of Raf-1, since the kinase-inactive mutant of Raf-1 (Raf-301) was still inactive when coexpressed with both p21$^{c\text{-}ras}$ and pp60$^{v\text{-}src}$ (lane 6). Raf-1 phosphorylates itself equally on serines and threonines as shown by phospho amino acid analysis of *in vitro* phosphorylated Raf-1 from Sf9 cells (data not shown) and as reported recently for Raf-1 from NIH 3T3 cells (10).

Several points about the activation of Raf-1 deserve mention. The production of pp60$^{v\text{-}src}$, p21$^{c\text{-}ras}$, and Raf-1 in the same cell is important, since Raf-1 activity assayed from lysates of cells infected with each of the viruses separately and mixed just before lysis was not elevated (data not shown). Both kinase-inactive pp60$^{c\text{-}src}$ (pp60$^{src\text{-}295}$) (data not shown) and a dominant inhibitory mutant of p21$^{c\text{-}ras}$ (p21$^{ras\text{-}N17}$) (see Fig. 5, lane 4) were unable to activate Raf-1. An immunoblot of Raf-1 immunoprecipitates after an *in vitro* kinase reaction, from cells infected with viruses in the combinations indicated, is shown in Fig. 4*A*. The relative amounts of p21^{ras} and pp60$^{v\text{-}src}$ present in each of the lysates used are shown in Fig. 4*B*. As can be seen, the amounts of Raf-1, p21^{ras}, and pp60$^{v\text{-}src}$ proteins in the samples were similar. Only in the case of cells infected triply with Raf-1, p21$^{c\text{-}ras}$, and pp60$^{v\text{-}src}$ was a significant proportion of Raf-1 autophosphorylated and shifted in mobility after an *in vitro* kinase assay. The immunoblotted Raf-1 bands that are retarded in mobility (labeled Raf-P in Fig. 4*A*) correspond to the labeled bands in the autoradiogram (Fig. 3). Raf-1 is autophosphorylated (and hence retarded in mobility) to a much larger extent *in vitro* than in intact cells. This may be due to absence of inhibitors or phosphatases *in vitro* that might attenuate autophosphorylation in cells. Alternatively, Raf-1 may not be a substrate for itself in the cell. Nevertheless, in the absence of exogenous or endogenous substrates specific for the Raf-1 kinase, the *in vitro* autokinase assay provides a sensitive method for separating and quantitatively differentiating between the activated Raf-1 protein and the inactive Raf-1.

Activation of Raf-1 by pp60$^{v\text{-}src}$ in Double Infections is Independent of p21^{ras} Function. In mammalian cells, pp60$^{v\text{-}src}$ requires p21^{ras} function to effect cell transformation, since

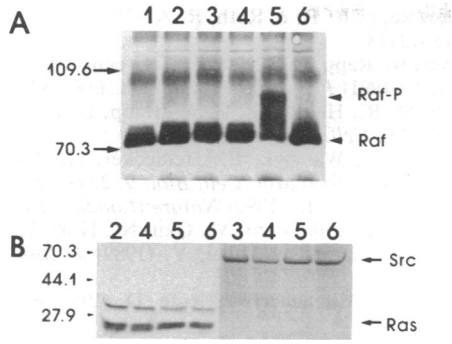

FIG. 4. (*A*) Immunoblot of Raf-1 after a kinase assay performed *in vitro* with Raf-1 immunoprecipitated from cells infected with baculoviruses encoding either Raf-1 alone (lane 1), Raf-1 and $p21^{c\text{-}ras}$ (lane 2), Raf-1 and $pp60^{v\text{-}src}$ (lane 3), Raf-1, dominant inhibitory mutant $p21^{ras\text{-}N17}$, and $pp60^{v\text{-}src}$ (lane 4), Raf-1, $p21^{c\text{-}ras}$, and $pp60^{v\text{-}src}$ (lane 5), or kinase-inactive mutant Raf-301, $p21^{c\text{-}ras}$, and $pp60^{v\text{-}src}$ (lane 6). The products were resolved by SDS/PAGE and immunoblotted with anti-Raf antibody. (*B*) Immunoblot of $p21^{ras}$ and $pp60^{v\text{-}src}$. Whole cell lysates in the same ratios as used for the Raf-1 kinase assay shown in *A* were separated by SDS/PAGE and immunoblotted with either anti-Ras antibody (ras10) or anti-Src antibody (src327). The numbering scheme of the lanes corresponds to that in *A*.

anti-Ras antibodies are able to block $pp60^{v\text{-}src}$-induced transformation (25). It is known that $pp60^{c\text{-}src}$ can associate with and phosphorylate the GTPase-activating protein (GAP), and it has been hypothesized that this might lead to the activation of $p21^{c\text{-}ras}$ (36). In Sf9 cells too it appears that $pp60^{v\text{-}src}$ in the absence of $p21^{c\text{-}ras}$ is not able to fully activate Raf-1 (Fig. 3, compare lanes 3 and 4). We have used a dominant inhibitory mutant of $p21^{c\text{-}ras}$ ($p21^{ras\text{-}N17}$) to understand the pathway used by $pp60^{v\text{-}src}$ to activate Raf-1. $p21^{ras\text{-}N17}$ is an inactive mutant of $p21^{c\text{-}ras}$ that inhibits wild-type $p21^{c\text{-}ras}$ activity in cells by competing for $p21^{c\text{-}ras}$ nucleotide-releasing factors (32). It is possible that the only pathway for activation of Raf-1 by $pp60^{v\text{-}src}$ is via $p21^{c\text{-}ras}$. Thus, in Sf9 cells doubly infected with baculoviruses encoding Raf-1 and $pp60^{v\text{-}src}$, low endogenous levels of $p21^{c\text{-}ras}$ would be the limiting component in the pathway for activation of Raf-1 by $pp60^{v\text{-}src}$. If such were the case, a triple infection of Sf9 cells with baculoviruses encoding Raf-1, $pp60^{v\text{-}src}$, and $p21^{ras\text{-}N17}$ might be expected to negate the $pp60^{v\text{-}src}$-induced activation of Raf-1. As seen in Fig. 3 (lane 5) this is not the case. Thus it appears that $pp60^{v\text{-}src}$ is able to activate Raf-1 via a Ras-independent pathway.

A second approach was taken to determine whether activation of Raf-1 by $pp60^{v\text{-}src}$ is independent of endogenous $p21^{ras}$. If the only role of $pp60^{v\text{-}src}$ were to activate endogenous $p21^{ras}$, then $p21^{v\text{-}ras}$ would not require $pp60^{v\text{-}src}$ to fully activate Raf-1, since $p21^{v\text{-}ras}$ is constitutively activated. Activity of Raf-1 immunoprecipitated from cells doubly infected with baculoviruses encoding Raf-1 and $p21^{v\text{-}ras}$ should be the same as that from cells triply infected with baculoviruses encoding Raf-1, $pp60^{v\text{-}src}$, and $p21^{c\text{-}ras}$. We found that $p21^{v\text{-}ras}$, $p21^{c\text{-}ras}$, and $pp60^{v\text{-}src}$ each have approximately the same effect on the activity of Raf-1 (Fig. 5, lanes 1, 3, and 2). Thus, $p21^{v\text{-}ras}$ does not activate Raf-1 to the extent of $p21^{c\text{-}ras}$ and $pp60^{v\text{-}src}$ combined (lanes 1 and 5). These results clearly indicate that although $p21^{ras}$ is essential for activation of Raf-1, full activation of Raf-1 requires both a tyrosine-kinase activity and a Ras-dependent activity.

DISCUSSION

In this report we show that in Sf9 cells, both $p21^{ras}$ and the tyrosine-kinase $pp60^{v\text{-}src}$ are required for full activation of the Raf-1 kinase. The characteristic shift in electrophoretic mobility of Raf-1 [which is correlated with activation of Raf-1 in mammalian cells (4)] is observed only in the presence of both $p21^{ras}$ and $pp60^{v\text{-}src}$. This shift is accompanied by a dramatic increase in the *in vitro* kinase activity of Raf-1. Thus, both $p21^{ras}$ and $pp60^{v\text{-}src}$ are necessary, but neither alone is sufficient, for full activation of Raf-1 kinase.

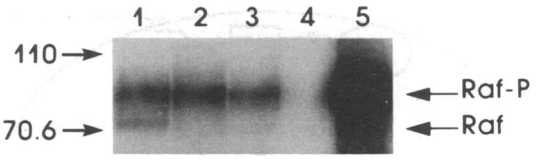

FIG. 5. Autoradiogram of Raf-1 *in vitro* kinase activity. Kinase assays were performed *in vitro* on Raf-1 immunoprecipitated from cells infected with baculoviruses encoding either Raf-1 and $p21^{v\text{-}ras}$ (lane 1), Raf-1 and $pp60^{v\text{-}src}$ (lane 2), Raf-1 and $p21^{c\text{-}ras}$ (lane 3), Raf-1 and dominant inhibitory mutant $p21^{ras\text{-}N17}$ (lane 4), or Raf-1, $p21^{c\text{-}ras}$, and $pp60^{v\text{-}src}$ (lane 5). The products were resolved by SDS/PAGE, immunoblotted, and exposed to film.

We have shown that activation of Raf-1 is dependent on tyrosine-kinase activity. Kinase-inactive $pp60^{c\text{-}src}$ did not activate Raf-1. A small amount of tyrosine phosphorylation of Raf-1 was detected in all cells coexpressing Raf-1 and $pp60^{v\text{-}src}$. It is unclear what role, if any, tyrosine phosphorylation of Raf-1 plays in the activation of Raf-1. Nevertheless, the data indicate that if tyrosine phosphorylation of Raf-1 is important for Raf-1 activation, it is not sufficient for full activation. Clearly, $pp60^{v\text{-}src}$ function involves activities other than the activation of $p21^{c\text{-}ras}$. Conversely, for full activation of Raf-1, $p21^{v\text{-}ras}$ alone, like $p21^{c\text{-}ras}$, is insufficient and requires $pp60^{v\text{-}src}$.

It is not clear whether the shift in the electrophoretic mobility of metabolically labeled Raf-1, observed from cells infected with Raf-1, $p21^{c\text{-}ras}$, and $pp60^{v\text{-}src}$, is due to Raf-1 autokinase activity or is due to activation of a Raf-1-dependent kinase. Since this shift is marked by an increase in threonine phosphorylation and since the autokinase activity of Raf-1, as seen *in vitro*, is marked by high levels of threonine phosphorylation, it is possible that some (but possibly not all) of the shift seen is due to autophosphorylation. In any case, the shift in electrophoretic mobility of Raf-1 from intact cells correlates well with activation of Raf-1 serine/threonine autokinase activity as measured *in vitro*.

Our results provide a possible explanation for a paradox in the Raf-1 field. It has been reported that $p21^{v\text{-}ras}$ expression is not sufficient to activate Raf-1 in some cell lines (33), whereas $p21^{v\text{-}ras}$ expression is sufficient in others (4). It is possible that Raf-1 activation in $p21^{v\text{-}ras}$-transformed cells is conditional on the level of tyrosine kinase activity, present or induced, in the individual cell lines.

In the Sf9 system, $p21^{v\text{-}ras}$ is nearly as effective as $p21^{c\text{-}ras}$ in activating Raf-1. It is likely that GAP, which would normally regulate $p21^{c\text{-}ras}$ activity, is either absent or limiting in Sf9 cells, rendering $p21^{c\text{-}ras}$ almost as active as $p21^{v\text{-}ras}$ (34). This suggests that the Sf9 cell system and the methods described in this report can be used to analyze effects of regulatory proteins such as GAP by using Raf-1 activity as an assay.

From these results a general model can be proposed. In this model two signals, a Ras-dependent signal and a tyrosine kinase-dependent signal, are required for full activation of Raf-1 (Fig. 6). Of the two signals, the one propagated by $p21^{ras}$ is almost certainly indirect, involving at least one intermediate. This intermediate could be a signal generator such as a lipase or an intermediate kinase. The presence of a cysteine motif in the regulatory portion of Raf-1, similar to that utilized for lipid binding by protein kinase C (35), suggests a similar lipid-mediated mode of activation for Raf-1. The second signal, originating from $pp60^{v\text{-}src}$ and

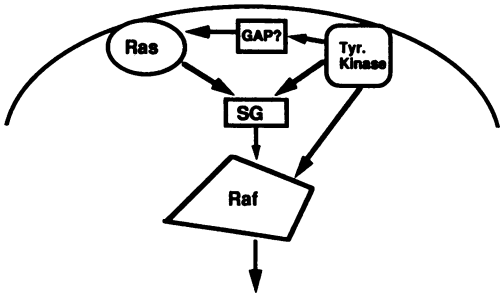

FIG. 6. Pathways for activation of Raf-1 by $pp60^{v\text{-}src}$ and $p21^{c\text{-}ras}$. SG, signal generator.

propagated independently of $p21^{ras}$, could be direct tyrosine phosphorylation of Raf-1. However, in mammalian cells Raf-1 activation is seen even in the absence of tyrosine phosphorylation of Raf-1 (6, 7). Hence both $p21^{ras}$ and $pp60^{v\text{-}src}$ may function indirectly, perhaps via a common intermediate such as a signal generator, to activate Raf-1. A closer examination of the activating modification(s) of Raf-1 should provide further clues on the pathways of activation.

We thank G. Heidecker and U. Rapp for the Raf-301 mutant, L. Feig for *ras* mutants, and Ray Erickson for the baculovirus encoding $pp60^{v\text{-}src}$. We thank Sadhana Agarwal, Loren Williams, and Ken Wood for valuable suggestions and critical review of the manuscript. This work was supported by National Institutes of Health grants to T.M.R. (CA43803 and CA5066).

1. Li, P., Wood, K., Mamon, H., Haser, W. & Roberts, W. (1991) *Cell* **64**, 479–482.
2. Ray, L. B. & Sturgill, T. W. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3753–3757.
3. Pelech, S. L., Sanghera, J. S. & Daya-Makin, M. (1990) *Biochem. Cell Biol.* **68**, 1297–1330.
4. Morrison, D. K., Kaplan, D. R., Rapp, U. R. & Roberts, T. M. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8855–8859.
5. Morrison, D. K., Kaplan, D. R., Escobedo, J. A., Rapp, U. R., Roberts, T. M. & Williams, L. T. (1989) *Cell* **58**, 649–657.
6. App, H., Hazan, R., Zilberstein, A., Ullrich, A., Schlessinger, J. & Rapp, U. R. (1991) *Mol. Cell. Biol.* **11**, 913–919.
7. Bacharini, M., Sabatini, D. M., App, H., Rapp, U. R. & Stanley, R. E. (1990) *EMBO J.* **9**, 3649–3657.
8. Blackshear, P. J., Haupt, D. M., App, H. & Rapp, U. R. (1990) *J. Biol. Chem.* **265**, 1231–1234.
9. Carroll, M. P., Clark-Lewis, I., Rapp, U. R. & Stratford May, W. (1990) *J. Biol. Chem.* **265**, 19812–19817.
10. Isumi, T., Tamemoto, H., Nagao, M., Kadowaki, T., Takaku, F. & Kasuga, M. (1991) *J. Biol. Chem.* **266**, 7933–7939.
11. Kovacina, K. S., Yonezawa, K., Brautigan, D. L., Tonks, N. K., Rapp, U. R. & Roth, R. A. (1990) *J. Biol. Chem.* **265**, 12115–12118.
12. Turner, B., Rapp, U. R., App, H., Greene, M., Dobashi, K. & Reed, J. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1227–1231.
13. Smith, M. R., Heidecker, G. A., Rapp, U. R. & Kung, H.-F. (1990) *Mol. Cell. Biol.* **10**, 3828–3833.
14. Wasylyk, C., Wasylyk, B., Heidecker, G., Huleihel, M. & Rapp, U. R. (1989) *Mol. Cell. Biol.* **9**, 2247–2250.
15. Jamal, S. & Ziff, E. (1990) *Nature (London)* **344**, 463–466.
16. Kaibuchi, K., Fukumoto, Y., Oku, N., Hori, Y., Yamamoto, T., Toyoshima, K. & Takai, Y. (1989) *J. Biol. Chem.* **264**, 20855–20858.
17. Sariban, E., Mitchell, T. & Kufe, D. (1987) *Blood* **69**, 1437–1440.
18. Huleihel, M., Goldsborough, M., Cleveland, J. L., Gunnell, M., Bonner, T. & Rapp, U. R. (1986) *Mol. Cell. Biol.* **6**, 2655–2662.
19. Rapp, U. R., Goldsborough, M. D., Mark, G. E., Bonner, T. I., Groffen, J., Reynolds, F. H. & Stephenson, J. R. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 4218–4222.
20. Bonner, T. I., Kerby, S. B., Sutrave, P., Gunnell, M. A., Mark, G. & Rapp, U. R. (1985) *Mol. Cell. Biol.* **5**, 1400–1407.
21. Heidecker, G., Huleihel, M., Cleveland, J. L., Beck, P., Lloyd, P., Pawson, T. & Rapp, U. R. (1990) *Mol. Cell. Biol.* **10**, 2503–2512.
22. Stanton, V. P. & Cooper, G. M. (1987) *Mol. Cell. Biol.* **7**, 1171–1179.
23. Stanton, V. P., Nichols, D. W., Laudano, A. P. & Cooper, G. M. (1989) *Mol. Cell. Biol.* **9**, 639–647.
24. Stacy, D. W., Tsai, M. H., Yu, C. L. & Smith, J. K. (1988) *Cold Spring Harbor Symp. Quant. Biol.* **53**, 871–881.
25. Smith, M. R., DeGudicibus, S. J. & Stacy, D. W. (1986) *Nature (London)* **320**, 540–543.
26. Kolch, W., Heidecker, G., Lloyd, P. & Rapp, U. R. (1991) *Nature (London)* **349**, 426–428.
27. Summers, M. D. & Smith, G. E. (1987) *A Manual of Methods for Baculovirus Vector and Insect Cell Culture Procedures* (Texas Agric. Exp. Sta., College Station).
28. Schultz, A. M., Copeland, T., Mark, G. E., Rapp, U. R. & Oroszlan, S. (1985) *Virology* **146**, 78–89.
29. Feig, L. A., Pan, B. T., Roberts, T. M. & Cooper, G. M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4607–4611.
30. Piwnica-Worms, H., Williams, N. G., Cheng, S. H. & Roberts, T. M. (1990) *J. Virol.* **64**, 61–68.
31. Kamps, M. P. & Sefton, B. M. (1989) *Anal. Biochem.* **176**, 22–27.
32. Feig, L. A. & Farnsworth, C. L. (1991) *Mol. Cell. Biol.* **11**, 4822–4829.
33. Reed, J. C., Yum, S., Cuddy, M. P., Turner, B. C. & Rapp, U. R. (1991) *Cell Growth Differ.* **2**, 235–243.
34. Zhang, K., DeClue, J. E., Vass, W. C., Papageorge, A. G., McCormick, F. & Lowy, D. R. (1990) *Nature (London)* **346**, 754–756.
35. Lee, M. H. & Bell, R. M. (1986) *J. Biol. Chem.* **261**, 14867–14870.
36. Brott, B. K., Decker, S., Shafer, J., Gibbs, J. B. & Jove, R. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 755–759.