Mitogen Regulation of c-Raf-1 Protein Kinase Activity toward Mitogen-activated Protein Kinase-Kinase*  

(Received for publication, February 23, 1993, and in revised form, April 14, 1993)  

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The c-raf-1 protooncogene encodes a Ser/Thr protein kinase. A mitogen-activated protein kinase-kinase (MAPKK) purified from bovine brain is phosphorylated and activated 4–9-fold in vitro by c-Raf-1 from mitogen-treated cells. c-Raf-1 protein kinase activity, measured by the phosphorylation of brain MAPKK substrate, is detectably activated within 1 min after addition of platelet-derived growth factor (PDGF) to 3T3 cells, increasing more rapidly than the endogenous NIH 3T3 cell MAPKK activity. c-Raf-1 activation is also induced by insulin, phorbol ester, thrombin, and endothelin. PDGF, epidermal growth factor, and insulin-stimulated 32P-c-Raf-1 yield very similar, complex tryptic 32P-peptide maps, wherein only 2 of 10 32P-peptides appear entirely de novo after growth factor addition. Mitogen-activated protein kinase/extracellular signal-regulated kinase-2 can phosphorylate c-Raf-1 in vitro on 4–6 tryptic 32P-peptides, all of which comigrate with tryptic 32P-peptides derived from c-Raf-1 labeled in situ. Mitogen-activated protein kinase phosphorylation of c-Raf-1 in vitro, however, does not 1) generate 32P-peptides that comigrate with those that appear de novo after PDGF or insulin treatment in situ; 2) do not convert c-Raf-1 polypeptides to a slower mobility on SDS-polyacrylamide gel electrophoresis as is seen after PDGF or insulin; 3) do not alter c-Raf-1 kinase activity toward MAPKK. Thus, based on overlapping site specificity, Erk-2 is a viable candidate to be among the PDGF-stimulated c-Raf-1 kinases. Although PDGF/insulin-stimulated c-Raf-1 Ser/Thr phosphorylation may be necessary to sustain the active state, a role for mitogen-activated protein kinase/extracellular signal-regulated kinase-2 phosphorylation in the initiation of c-Raf-1 activation is unlikely.

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c-Raf-1 is a ubiquitously expressed Ser/Thr protein kinase which is the normal cellular homolog of the acutely transform-
activation. This MAPKK provides the basis for a sensitive assay for c-Raf-1 kinase activity, enabling a more precise characterization of the changes in c-Raf-1 catalytic function upon stimulation in situ, as well as direct inquiry to the role of the previously described MAP kinase-catalyzed phosphorylation of the c-Raf-1 polypeptide (21, 22) in the regulation of c-Raf-1 protein kinase.

Our results indicate that wild-type c-Raf-1 endogenous to NIH 3T3 cells or H4 cells phosphorylates and activates MAPKK in vitro; this activity of c-Raf-1 is substantially increased upon stimulation by a number of agonists in situ, rising more rapidly than the activity of endogenous MAPKK, consistent with the idea that c-Raf-1 lies upstream of MAPKK in situ. Activation of c-Raf-1 in situ is accompanied by multisite (Ser/Thr) Raf-1 phosphorylation. Although MAPKK probably contributes to the mitogen-stimulated phosphorylation of c-Raf-1, present evidence indicates that MAPK neither initiates nor down-regulates c-Raf-1 activation.

**EXPERIMENTAL PROCEDURES**

Preparation of Bovine Brain MAPKK—All procedures were carried out at 4 °C. Three bovine brains (Pel-Freez, Rogers, AR), frozen as 1-cm slices, were powdered, frozen, and then powder homogenized in 1 liter of buffer A (10 mM HEPES, pH 7.6, 1 mM EDTA, 1.5 mM DTT, 5% (v/v) glycerol, 1 mM PMSF, 2 μM pepstatin, 2 μM leupeptin, 10 kallikrein-inhibiting units/ml aprotinin). The lysate was centrifuged at 3,000 × g for 10 min. Often a fat cake was observed at this point, floating on top of the extract. This was removed and the remaining material was centrifuged for 1.5 h at 100,000 × g. The supernatant was filtered through glass wool and mixed with 300 ml of (settled) DEAE-cellulose equilibrated in buffer A. The slurry was stirred gently for 1 h, and the breakthrough material was collected by suction filtration. Mes was added to the breakthrough from a 1 M, pH 6.5, stock, to a final concentration of 15 mM, and the pH of the extract was adjusted to 6.5. The extract was then mixed with 50 ml of (settled) Fast-S Sepharose which was equilibrated with buffer M (20 mM Mes, pH 6.5, 2 mM EDTA, 1.5 mM DTT, 5% (v/v) glycerol, 0.03% (w/v) Brij 35, 1 mM PMSF). This slurry was gently stirred for 1 h, at which time the resin was poured into a column. The column was washed with 200 ml of buffer M and developed with a 500-ml linear gradient of NaCl (0–250 mM) in buffer M. The flow rate was kept at 1.5 ml/min and 7-ml fractions were collected. Fractions containing maximal MAPKK specific activity were pooled and dialyzed (2 h) into buffer M containing 5 mM MgCl₂, 0.1% Triton X-100, 10 mM MgCl₂. Any remaining MAPK was precipitated from control or PDGF-treated EC4A1 cells and treated with MAPKK (~0.33 unit) purified from insulin-stimulated H4 hepatoma cells (25) or vehicle plus [γ-32P]ATP (100 μM, 11,000 cpm/pmol) and MgCl₂ (10 mM) for 30 min at 30 °C. The immunoprecipitates were washed twice with Raf-1 lysis buffer, twice with LiCl wash buffer, and once with buffer A' (20 mM Mes, pH 6.5, 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.1% Triton X-100, 10 mM MgCl₂). Any remaining MAPK was inactivated by incubating the immunoprecipitates with recombinant rat brain protein-tyrosine phosphatase-1 (300 units/ml; see Ref. 26) for 20 min at 30 °C. The tyrosine phosphatase was washed away as above, except that the buffer A' contained 2 mM NaVO₄. Samples were then assayed for c-Raf-1 activity as described above.

**Tryptic Phosphopeptide Mapping and Phosphoamino Acid Analysis**—MAPKK or c-Raf-1 were excised from dried stained gels and extracted by exhaustive incubation of the macerated, rehydrated gel piece in 1.5% SDS, 20 mM DTT at 55 °C. Proteins were precipitated with chloroform/methanol using phosphotyrosine as a carrier. Digestion with 0.2 mg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was for 16 h at 37 °C. Two-dimensional tryptic phosphopeptide mapping (thin layer electrophoresis, pH 1.9/TLC) was as previously described (27). Phosphoamino acid analysis was performed as described elsewhere (27, 28).

**RESULTS**

**Purification of Bovine Brain MAPKK and Activation by c-Raf-1**—To study mitogen activation of c-Raf-1 and the role of c-Raf-1 in the activation of MAPKK by extracellular agonists, an abundant source of MAPKK was necessary for in vitro assay of c-Raf-1 protein kinase activity. Inasmuch as MAPKKs are highly abundant in brain (29), we examined soluble extracts of bovine brain for erk-specific activator/MAPKK activity. A spontaneously active MAPKK was readily detected in the flow-through fractions of a DEAE-cellulose column and was purified ~200-fold (Table I) to ~30% purity (Fig. 1) as described under "Experimental Procedures." Fig. 1A (right) shows the activity peak from the final Mono-S step; MBP kinase activity in this preparation is negligible in the absence of added prokaryotic recombinant p44 MAPK (bMAPK). Fig. 1A (left) shows the polypeptide species present in the Mono-Q fractions. A dominant 48-kDa polypeptide coelutes with MAPKK activity (compare Fig. 1A, left and right).
Treatment of the purified brain MAPKK with phosphatase-2A failed to decrease activity, in contrast to the extensive phosphatase-2A inactivation observed previously using MAPKK purified from mitogen-treated cells (18). This suggests that the bovine brain MAPKK as isolated has kinase activity independent of its prior phosphorylation (or contains phosphatase-2A-resistant sites). Nevertheless, as observed previously with phosphatase-2A-inactivated MAPKK, the bovine brain MAPKK Mono-S fractions are activated by incubation with c-Raf-1 (Fig. 1B). Thus, c-Raf-1 immunoprecipitated from serum-starved NIH 3T3 cells increases MAPKK activity approximately 2-4-fold in an ATP-dependent reaction, whereas c-Raf-1 immunoprecipitated from PDGF-stimulated cells produces a much larger activation of brain bovine MAPKK, ranging from 5- to 20-fold. Concomitant with the ability of c-ruf-1 to increase MAPKK activity in vitro, c-Raf-1 catalyzes the selective phosphorylation of the coeluting 48-kDa band in the MAPKK preparation, further supporting the identification of the 48-kDa polypeptide as the MAPKK (Fig. 1B, inset).

Activation of MAPKK by c-Raf-1 is reflected both by an enhanced ability of MAPKK to catalyze $^{32}$P incorporation into MAPKK and by the selective phosphorylation of the coeluting 48-kDa polypeptide by c-Raf-1 (Fig. 1B, inset). Assays were performed as described under "Experimental Procedures.

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### Table I

**Purification of MAPKK from bovine brain**

MAPKK was purified from cytosolic extracts of bovine brain as described in "Experimental Procedures." Activity is listed in units which are defined in Refs. 16 and 18. Activity in the crude extract was not detectable, and purification is calibrated from the DEAE step.

| Step                  | Protein activity | Total activity | Recovery |
|-----------------------|------------------|----------------|----------|
|                       | mg               | units/mg       | units    | -fold | %   |
| Crude extract         | 4224             | ND*            | ND       | ND    | ND  |
| DEAE-cellulose        | 1397             | 16             | 23,051   | 1     | 100 |
| Fast-S Sepharose      | 39               | 371            | 14,543   | 23    | 63  |
| DEAE blue 3GA-agarose | 0.39             | 2500           | 975      | 151   | 4   |
| Mono-S                | 0.20             | 3500           | 700      | 212   | 3   |

*Not determined.

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**Fig. 1. Preparation of bovine brain MAPKK, coelution with a 48-kDa c-Raf-1 substrate.** MAPKK was purified as described under "Experimental Procedures." (A) a silver stain of SDS-PAGE of fractions from the Mono-S chromatography of bovine brain MAPKK (Table I). The 48-kDa polypeptide that coelutes with MAPKK in vitro is marked with an arrow. (B) MAPKK assay of the fractions shown at left: (●) plus btMAPK, (○) minus btMAPK. B, coelution of the 48-kDa c-Raf-1 substrate with MAPKK activity. (Left) the same Mono-S fractions from "A" were assayed for activation of MAPKK activity by c-Raf-1 immunoprecipitated from control cells (●) or PDGF-treated cells (○). Basal MAPKK in the fractions is indicated with (▲). Inset, autoradiograph of the SDS-PAGE containing the c-Raf-1-catalyzed phosphorylation of the Mono-S fractions containing MAPKKs. Top panel, autophosphorylation; middle panel, phosphorylation by c-Raf-1 from control cells; bottom panel, phosphorylation by c-Raf-1 from PDGF-treated cells. The 48-kDa MAPKK $^{32}$P-polypeptide is indicated with an arrow. Assays were performed as described under "Experimental Procedures."
Mitogen-regulated c-Raf-1 Activation of MAPKK

Fig. 2. Assay of bovine brain MAPKK for regulation by c-Raf-1. A, activation of MAPKK with c-Raf-1 immunoprecipitated from PMA-stimulated EC4A1 cells, followed by inactivation with phosphatase 2A (PP2A), followed by reactivation with a second c-Raf-1 treatment; MBP kinase assay. MAPKK activity was measured here as activation of the ability to activate MAPK activity. Shown are means ± S.D. for triplicate determinations. For these experiments, ~1 unit of starting MAPKK activity was used.

B, same as part A except that phosphorylation of the btMAPK polypeptide by MAPKK was quantitated. Shown are means ± S.D. for triplicate determinations. C, phosphorylation/dephosphorylation of the 48-kDa c-Raf-1 substrate in parallel with activation/deactivation of MAPKK activity. The position of MAPKK is indicated with an arrow. The data in A are corrected for contaminating MBP kinases in the MAPKK by carrying out the MBP protocol described above without added btMAPK. In addition, the data are corrected for contaminating MBP kinases and MAPKK leaching from the Raf-1 preparation as well as for the basal activity of the btMAPK. These nominal activities are shown in A bars E–K for comparison. The data in B are corrected for btMAPK autophosphorylation (0.1 ± 0.005 pmol/min) and for Raf-1 phosphorylation of btMAPK (0.02 ± 0.01 pmol/min).

from [γ-32P]ATP into the btMAPK polypeptide (Fig. 2B), as well as by the greater ability of MAPKK to activate btMAP kinase catalytic activity toward MBP (Fig. 2A); the greater -fold increase in btMAPK phosphorylation (Fig. 2B) than in MAPK-catalyzed MBP phosphorylation probably reflects a MAPKK-induced increase in MAPK autophosphorylation. Phosphatase-2A treatment of the c-Raf-1-activated MAPKK reverses completely both measures of MAPKK activity, back to their basal level. A second incubation of the phosphatase-2A-treated MAPKK with c-Raf-1 and ATP can restore completely both aspects of MAPKK catalytic function (Fig. 2, A and B).

In parallel to the activation of MAPKK catalytic function, c-Raf-1 catalyzes the phosphorylation of the bovine brain MAPKK (Fig. 2C). The stoichiometry of this phosphorylation is uncertain, primarily because the amount of MAPKK polypeptide is too low to be quantitated accurately; nevertheless, several MAPKK tryptic 32P-peptides are visualized on peptide mapping, and phosphoamino acid analysis of c-Raf-1-phosphorylated 32P-MAPKK shows both 32P-Ser and 32P-Thr phosphate, as before (18). Phosphorylation of MAPKK by c-Raf-1 is also accompanied by a slight slowing of the MAPKK polypeptide on SDS-PAGE, a phenomenon seen with many polypeptides whose activity is regulated by phosphorylation, presumably reflecting a phosphorylation-induced alteration in the SDS binding and/or conformation of the substrate. Treatment of c-Raf-1-activated MAPKK with phosphatase-2A reverses the slowed mobility concomitant with deactivating fully the c-Raf-1-induced increase in MAPKK activity, but results in the hydrolysis of only ~50% of the 32P incorporated into MAPKK in the presence of c-Raf-1. A second incubation with c-Raf-1 and [γ-32P]ATP restores the 32P content of MAPKK to the level achieved by the first cycle of c-Raf-1-catalyzed phosphorylation, concomitant with a slowing of mobility in SDS-PAGE (Fig. 2C) and complete reactivation of the MAPKK activity (Fig. 2A).
The phosphorylated MAPKKs shown in Fig. 2C were subjected to complete tryptic digestion and two-dimensional phosphopeptide mapping; a composite diagram of MAPKK tryptic phosphopeptides is seen in Fig. 3E. Basal autophosphorylation of MAPKK is insignificant (Fig. 2C) and was not analyzed. Phosphorylation of bovine brain MAPKK with c-Raf-1 results in the phosphorylation of seven $^{32}$P-peptides (Fig. 3, A and E); spots 1–5, and spots X and Y; spots 1, X, and Y are quantitatively dominant. Treatment of MAPKK with phosphatase-2A is accompanied by the complete disappearance of $^{32}$P-peptides X and Y with little or no change in the relative $^{32}$P content of spots 1–5. Thus, phosphorylation of one or both of the MAPKK $^{32}$P-peptides X and Y is necessary for c-Raf-1-induced MAPKK activation. Spot 1, which contains about 50% of the $^{32}$P incorporated into MAPKK during the initial phosphorylation by c-Raf-1, ac-

![Fig. 3. Tryptic phosphopeptide mapping of MAPKK after c-Raf-1 activation, protein phosphatase 2A (PP2A) deactivation, and c-Raf-1 reactivation. MAPKK treated with c-Raf-1 as in Fig. 2 was subjected to two-dimensional tryptic phosphopeptide mapping (thin layer electrophoresis (TLE), pH 1.9, horizontal dimension; TLC vertical dimension). A, MAPKK after c-Raf-1 phosphorylation. B, MAPKK deactivated by PP2A. C, PP2A-inactivated MAPKK, reactivated by c-Raf-1. D, mixture of B and C. E, a composite diagram of MAPKK tryptic phosphopeptides. The PP2A-sensitive c-Raf-1 phosphorylation sites are stippled. 600 cpm were loaded on all plates, the origin is marked with an o and a black dot. On the maps, spots X and Y are indicated with arrowheads.](image)
counts for the bulk of the residual \(^{32}\)P-MAPKK after phosphatase-2A deactivation of MAPKK seen in Fig. 2C, lane 3. Reactivation and rephosphorylation of MAPKK catalyzed by a second Raf-1 treatment (see Fig. 2, A–C) results in the selective reincorporation of \(^{32}\)P into spots X and Y (Fig. 3C) with a return in overall MAPKK \(^{32}\)P content to the level achieved in the initial c-Raf-1-catalyzed reaction (Fig. 2C). A mixture of samples from Fig. 3, B and C (Fig. 3D) is identical to Fig. 3A, confirming spots X and Y as the dominant MAPKK \(^{32}\)P-peptides selectively dephosphorylated by phosphatase-2A concomitant with inactivation of MAPKK. Thus, mitogen-stimulated c-Raf-1 catalyzes the phosphorylation of MAPKK at multiple sites that are both resistant and sensitive to phosphatase-2A; occupancy of one or both of the phosphatase-sensitive \(^{32}\)P-peptides (X and/or Y) is essential for the persistence of MAPKK activation, whereas phosphorylation of the phosphatase-2A-resistant sites (especially spot 1) is not per se sufficient to maintain MAPKK activation.

c-Raf-1 Kinase, Assayed by Phosphorylation of MAPKK in Vitro, Is Agonist-stimulated—Based on the results shown in Figs. 1–3, the c-Raf-1-catalyzed phosphorylation of the 48-kDa brain MAPKK serves as a direct assay of c-Raf-1 kinase activity and was employed to characterize further the mitogen activation of c-Raf-1 kinase in situ. PDGF activation of c-Raf-1 was examined in NIH 3T3 cells, as well as a line of NIH 3T3 cells (EC4A1) that stably overexpress recombinant human c-Raf-1. Serum-starved cells were harvested 15 min after addition of PDGF or carrier; c-Raf-1 kinase activity in immunoprecipitates was increased 9–16-fold after PDGF with half-maximal activation observed at approximately 0.3–0.5 ng/ml PDGF (Figs. 4 and 6). The rate of activation of c-Raf-1 after addition of a supramaximal dose of PDGF to serum-starved NIH 3T3 cells was compared to the rate of activation of endogenous MAPKK (Fig. 5A) and PDGF-stimulated tyrosine phosphorylation (Fig. 5B). A doubling in c-Raf-1 kinase is evident as early as 1 min after PDGF addition, rising to a peak at 3 min, approximately 15-fold over basal levels. Endogenous 3T3 cell MAPKK activity also increases 10–15-fold in response to PDGF, but more slowly; MAPKK is not yet half-maximal at 3 min and peaks at 10 min (Fig. 5A). Both c-Raf-1 and MAPKK activities are sustained for at least 30 min, whereas PDGF-stimulated Tyr phosphorylation (Fig. 5B), already maximal at 20 s after mitogen addition, declines rapidly after 3 min.

In addition to PDGF, a variety of agonists whose receptors act through different signal transduction pathways, also activate c-Raf-1 in the NIH 3T3, EC4A1 and H4 hepatoma cells (Fig. 6). Thrombin and endothelin, agents that act through sepsentine receptors that contain seven transmembrane segments and are coupled through heterotrimenic G proteins, each activate c-Raf-1, as does the active phorbol ester, PMA. In H4 hepatoma cells, insulin activates c-Raf-1. Each of these agonist has been shown previously to activate MAP kinase activity.

Effect of Mitogens on Site-specific Phosphorylation of c-Raf-1 in Situ—Numerous studies have shown that the c-Raf-1...
polypeptide is multiply phosphorylated in situ concomitant with mitogen activation (2, 3). We sought to compare the patterns of site-specific c-Raf-1 phosphorylation in situ in response to different agonists to the c-Raf-1 phosphorylation catalyzed by the MAP kinases in vitro.

Addition of PDGF to 3T3 cells stimulates overall 32P incorporation into c-Raf-1 and shifts a portion of the c-Raf-1 polypeptides to a slower mobility on SDS-PAGE (30) (see Fig. 8, inset). The ability of immunoprecipitated c-Raf-1 to catalyze autophosphorylation in vitro is also increased after PDGFTreatment, and the c-Raf-1 polypeptides that undergo the most extensive autophosphorylation are primarily those that exhibit the most retarded mobility on SDS-PAGE (Fig. 8, inset). These findings suggest that these slowly migrating c-Raf-1 polypeptides are the most active c-Raf-1 kinase molecules.

An analogous situation is seen with the S6 kinases (31, 32).

The effect of PDGF on the site-specific phosphorylation of c-Raf-1 32P-labeled in situ was evaluated by two-dimensional peptide mapping of tryptic digests prepared from 32P-c-Raf-1 (diagram of c-Raf-1 32P tryptic peptides is shown in Fig. 7A). The 32P-c-Raf-1 isolated from serum-starved NIH 3T3 cells exhibits four major (spots 1–4) and four minor (spots 5–8) 32P-peptides (Fig. 6B, top left). PDGF induced a 3-fold increase in overall 32P incorporation into immunoprecipitated c-Raf-1 (basal, 548 cpm 32P; PDGF, 1518 cpm 32P); however, when equal counts/min 32P of 32P-c-Raf-1 tryptic digests from control and PDGF-treated cells were compared by peptide mapping, the relative 32P content of spots 1–8 is seen to be unaltered by PDGF, and two additional minor 32P-peptides appear de novo, designated A and B (Fig. 7B, top right). A generally similar result is seen with c-Raf-1 obtained from 32P-labeled EC4A1 cells, before and after EGFTreatment (Fig. 7C). Tryptic digests of overexpressed recombinant human 32P-c-Raf-1 appear almost identical to those seen with endogenous murine c-Raf-1 (compare upper panels in Fig. 6, B and C), except that spot 1 is not detectable in the recombinant human c-Raf-1, whereas spot 7 is visualized as a major 32P-peptide in murine c-Raf-1; thus, spots 1 and 7 may be structurally related. Although absolute recovery of 32P-c-Raf-1 from EC4A1 cells is greater due to c-Raf-1 overexpression, overall stimulation of 32P incorporation into c-Raf-1 by EGFTreatment is (basal, 4000 cpm 32P; EGFTreatment, 6900 cpm 32P); peptide maps of tryptic digests show no significant change in relative 32P content in spots 2–8 after EGFTreatment, with the de novo appearance of spots A and B (B >> A). Insulin stimulation of H4 cells also increases 32P incorporation into the endogenous c-Raf-1 polypeptide (32P-Ser only (Fig. 7E); control, 3600 cpm; insulin, 7100 cpm) and retards the mobility of the c-Raf-1 polypeptide on SDS-PAGE (Fig. 7D, left). Tryptic 32P-peptide maps of endogenous rat c-Raf-1 exhibit a pattern strikingly similar to that seen with the murine and recombinant human c-Raf-1, including the presence of spots A and B (Fig. 7D, right).

MAP Kinase Phosphorylation of c-Raf-1 in Vitro: Site Specificity and Effects on c-Raf-1 Kinase Activity—A Ser/Thr-rich segment of the c-Raf-1 amino-terminal regulatory domain between residues 289 and 315 contains numerous potential MAPK phosphorylation sites (Ser/Thr-Pro) (33–35). As previously observed (21, 22), p42/44 MAPKs phosphorylate c-Raf-1 in vitro (Fig. 7, B and C, bottom left). The p42 MAPK appears to phosphorylate c-Raf-1 immunoprecipitated from serum-starved or PDGF-stimulated cells to a nearly equal extent, suggesting that activation of c-Raf-1 by PDGF does not lead to occupancy of a substantial fraction of the MAP kinase sites on c-Raf-1 (Fig. 8, inset). The sites on c-Raf-1 phosphorylated by MAPKs are amino-terminal to residue 302, inasmuch as neither v-Raf nor the amino-terminal truncated BXB-Raf (in frame deletion of residues 26–302) (36) are phosphorylated by p42 MAP kinase (not shown). The extent (i.e. stoichiometry), however, to which basal or PDGF-stimulated c-Raf-1 is phosphorylated by MAP kinase in vitro is unknown, because a reliable estimate of the amount of c-Raf-1 polypeptide in these reactions is not available. The maximal extent of MAP kinase-catalyzed phosphorylation of c-Raf-1 from serum-starved cells achieved in vitro, however, does not lead to slowing of the mobility of a portion of c-Raf polypeptides on SDS-PAGE, whereas obvious slowing of a substantial fraction of c-Raf-1 molecules is observed after PDGF (Fig. 8, inset) and insulin activation (Fig. 7D) in situ. Examination of the sites phosphorylated by p42 MAP kinase on c-Raf-1, immunoprecipitated from serum-starved cells, by two-dimen-
Mitogen-regulated c-Raf-1 Activation of MAPKK

Fig. 7
The basal activity of the bovine brain MAPKK is resistant to Ser/Thr phosphatase; by contrast, the activity associated with the isolates of MAPKK described previously has exhibited over 90% deactivation on treatment in vitro with comparable amounts of Ser/Thr phosphatase (18). The phosphatase resistance of our preparation of bovine brain MAPKK may be attributable to any of several factors. First, the data in Table I, as well as numerous other studies indicate that MAPKKs and, by implication, MAPKK, are highly abundant in brain (29), a nonproliferating tissue, where they are likely activated by neuronal signal transduction cascades possibly including those initiated at receptors coupled, via heterotrimERIC G proteins, to protein kinase C. MAPKK, in a basal state, present at 0.03% of the total soluble protein, might very well be detectable using the assay conditions described herein. Alternatively, this MAPKK may be a novel isoform with different regulatory properties. Bovine brain MAPKK may be phosphorylated at phosphatase-2A-resistant sites resulting in partial activation. Full activation may be achieved only after subsequent phosphorylation by c-Raf-1. Our data (Fig. 3) indicate that c-Raf-1 itself can phosphorylate MAPKK at phosphatase-2A-resistant sites. Thus, bovine brain MAPKK may be subject to complex regulation. There is good evidence that MAPKKs with radically different modes of regulation exist. Jaiswal et al. (37) recently reported the identification of a MAPKK present in PC12 cells which is regulated by Ser/Thr and Tyr phosphorylation. Finally, our preparation of MAPKK may contain several copurifying isoforms, some of which are already active having been activated in situ which are already active having been activated. Still, our preparation of bovine brain MAPKK is readily responsive to insulin (left) and tryptic phosphopeptide mapping of H4 cell c-Raf-1 from insulin-stimulated cells (right). Cells were labeled with [32P]orthophosphate and treated with PDGF (NIH 3T3 cells), EGF (EC4A1 cells), or insulin (H4 hepatoma cells) as described under "Experimental Procedures." E, phosphoamino acid analysis of c-Raf-1 from insulin-stimulated cells.
activated by phosphorylation in vitro with c-Raf-1, as observed previously with MAPKK from mitogen-treated cells (18) and skeletal muscle (20); bovine brain MAPKK then can be deactivated by phosphatase-2A and activated a second time by c-Raf-1. Bovine brain MAPKK is phosphorylated on multiple sites by c-Raf-1 (Fig. 3). Phosphorylation at the sites encompassed on 32P-peptides X and/or Y are necessary for MAPKK activation, whereas the role of the phosphatase-2A-resistant phosphorylation sites on spot 1, the other major site(s) of c-Raf-1-catalyzed 32P incorporation, is not yet known.

Measurement of 32P incorporation into brain MAPKK provides a sensitive assay of c-Raf-1 kinase activity and enables the demonstration of a far greater stimulation of c-Raf-1 kinase activity by PDGF treatment in situ than that indicated by previous work which employed model peptide or protein substrates (2, 3). In response to PDGF, clear-cut activation of c-Raf-1 kinase is evident at 1 min, preceding detectable activation of the endogenous MAPKK. This temporal pattern is consistent with an in vitro role for c-Raf-1 as a physiologic activator of MAPK. Whether A-Raf, B-Raf, or Ser/Thr protein kinases other than c-Raf isoforms can serve as MAPKK activator is not yet known. While other MAPKK isoforms with different regulatory properties exist (37), our data strongly support the contention that c-Raf-1 is a major MAPKK activator in several signal transduction pathways. Whether the Tyr-phosphorylated MAPKK in PC-12 cells is in part regulated by c-Raf-1 remains to be determined. We have observed 2 that expression in NIH 373 cells of dominant negative forms of c-raf can prevent the activation of cotransfected Erk-1 engendered by activated ras, v-src, etc., suggesting that c-Raf-1 is a necessary immediate upstream activator of MAPKK in response to those agents, at least in NIH 373 cells. It has been suggested that agonists acting through heterotrimeric G proteins may recruit a MAPKK activator other than c-Raf-1 (38, 39); the present work shows that thrombin and endothelin do activate c-Raf-1 kinase (Fig. 6).

Interestingly, PDGF activation of c-Raf-1 and MAPKK is sustained for at least 30 min, whereas PDGF-stimulated Tyr phosphorylation declines rapidly after 3 min. This indicates that PDGF-induced Tyr phosphorylation, although indispensable for activation of c-Raf-1, is not necessary to sustain the activation of c-Raf-1. The biochemical mechanisms which account for the initiation and maintenance of c-Raf-1 activation after the addition of PDGF are not yet known. In addition to PDGF, many other growth factors, including EGF, colony-stimulating factor-1, and insulin, as well as cytokines, activation of antigen receptors, active phospholipases, all act to increase c-Raf-1 Ser/Thr phosphorylation (2, 3). Previous studies have demonstrated that phosphatase-1 specifically dephosphorylates c-Raf-1 from insulin-treated cells concomitant with a deactivation of c-Raf-1 kinase (40), indicating that Ser/Thr phosphorylation of c-Raf-1 is necessary to maintain the activated state. Nevertheless, the role of c-Raf-1 phosphorylation in initiating the active state of the c-Raf-1 protein kinase is uncertain, as is the identity of the protein kinase(s) that catalyze c-Raf-1 (Ser/Thr) phosphorylation in situ. c-Raf-1 catalyzes an autophosphorylation in vitro, and a contribution of this reaction to PDGF activation in situ has been observed (18). The data of Izumi et al. (30), indicate, however, that the pattern of site-specific c-Raf-1 phosphorylation seen after PDGF stimulation in situ is the same for recombinant wild-type c-Raf-1 as for a c-raf-1 mutant whose ATP binding site has been mutagenically inactivated. This suggests strongly that c-Raf-1 autophosphorylation in situ contributes negligibly to overall c-Raf-1 phosphorylation, and that insulin/ growth factor-stimulated c-Raf-1 phosphorylation is mediated almost entirely by other mitogen-activated Ser/Thr kinases. It is noteworthy that the tryptic 32P-phosphopeptide maps of c-Raf-1 isolated after PDGF, insulin (30), and EGF stimulation are very similar (Fig. 7), suggesting that these three agonists, acting in two different cell types (H4 hepatoma and 3T3 cells), activate a common set of c-Raf-1 kinases.

For evidence for an important role for kinase C in the c-Raf-1 activation is available in some systems, such as T and B cells, where down-regulation of protein kinase C with TPA prevents activation of c-Raf-1 through the antigen receptor (2, 3). Because of the regulatory requirement for c-Raf-1 activation with TPA treatment (but not either alone). A mutant c-Raf-1 (Ser-499 → Ala) cannot be activated by coexpression with baculoviral-encoded protein kinase Ca plus treatment with TPA, but can still be activated if coinfected with baculoviral e-ras and lck in a triple infection. Kinase Ca can directly phosphorylate and activate purified c-Raf-1 in vitro; Raf-1 (Ser-445 → Ala), although phosphorylated is not activated. Thus under some circumstances, kinase Ca may act directly on c-Raf-1; this is not, however, the mechanism underlying activation by insulin or PDGF in situ. Several studies have shown that down-regulation of 3T3 cells with TPA does not prevent activation of endogenous c-Raf-1 by PDGF (2, 3); moreover, the mutant c-raf-1 (Ser-499 → Ala), although refractory to activation by kinase C-mediated phosphorylation in vitro is activated by serum, when expressed in NIH 3T3 cells.

With regard to the role of MAPK, the present data indicate that MAPK is capable of phosphorylating c-Raf-1 in situ at multiple sites, all of which are located on 32P-peptides that comigrate with c-Raf-1 32P-peptides labeled in situ. This is consistent with earlier reports (21, 22) and provides significant evidence that MAPK, or a protein kinase with similar specificity, participates in the insulin, PDGF and EGF-stimulated (Ser/Thr) phosphorylation of c-Raf-1 that occurs in situ. Nevertheless, MAPK phosphorylation of c-Raf-1 does not phosphorylate c-Raf-1 in the, the new 32P-peptides that appear only after mitogen activation in situ; does not generate a population of c-Raf-1 molecules with slowed mobility on SDS-PAGE, as is seen on activation in situ with PDGF; and neither increases nor decreases c-Raf-1 kinase activity toward MAPKK. Thus phosphorylation of c-Raf-1 in situ catalyzed by MAPK alone does not reproduce the activation of c-Raf-1 generated by PDGF in situ.

Based on the peptide maps shown in Fig. 7, it appears likely that at least one Ser/Thr kinase other than MAPK participates in the phosphorylation of c-Raf-1 (i.e. a spot A/B kinase); conceivably, c-Raf-1 phosphorylation by this second enzyme may mediate the initial activation of c-Raf-1 protein kinase. Alternatively, the initial activation of c-Raf-1 may be due to a noncovalent protein-protein, or ligand-protein interaction between the amino-terminal (CR-1) regulatory domain of c-Raf-1 and a mitogen-generated upstream activator, which alters c-Raf-1 conformation, and enables it to undergo multisite phosphorylation. By this scenario, the growth factor-stimulated phosphorylation of c-Raf-1 does not initiate c-Raf-1 activation, but may contribute to the persistent activation of c-Raf-1 observed after PDGF-stimulated, PDGF receptor Tyr phosphorylation has waned. Although the very similar pattern of c-Raf-1 (Ser/Thr) phosphorylation seen after insulin, EGF, or PDGF treatment argues for a common mode of c-Raf-1 activation by different extracellular

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2 U. R. Rapp, unpublished results.
3 J. Troppmair and U. R. Rapp, manuscript in preparation.
4 J. Troppmair and U. R. Rapp, manuscript in preparation.
5 W. Kelch and U. R. Rapp, manuscript in preparation.
stimuli, the existence of multiple alternative mechanisms of c-Raf-1 activation remains a viable possibility. Such a scenario would reflect the central role of c-Raf-1 in growth control and the diverse upstream elements which must recruit c-Raf-1 to successfully initiate mitogenesis.

Acknowledgments—We thank David Brautigan and Jian Chen for their generous gift of purified phosphatase-2A, Thomas Ingebritsen for recombinant rat brain protein-tyrosine phosphatase-1, Anna-Maria Forte and Xia-Mei Liu for technical assistance, Karen Delillo for photography and technical assistance, and Martha Chambers for assistance with the manuscript.

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