Norris, K., & Hornsby, P. (1990) *Mutat. Res. 237*, 95-106.
Paoletti, F., Aldinucci, D., Mocali, A., & Caparrini, A. (1986) *Anal. Biochem. 154*, 536-541.
Peterson, G. (1977) *Anal. Biochem. 83*, 346-356.
Phillips, J., Campbell, S., Michaud, D., Charbonneau, M., & Hilliker, A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2761-2765.
Scott, M., Meshnik, S., & Eaton, J. (1987) *J. Biol. Chem. 262*, 3640-3645.
Sherman, L., Dafni, N., Lieman-Hurwitz, J., & Groner, Y. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5465-5469.
Shibanuma, M., Kuroki, T., & Nose, K. (1988) *Oncogene 3*, 17-21.
Simon, R., Scoggin, C., & Patterson, D. (1981) *J. Biol. Chem. 256*, 7181-7186.
Sinet, P. (1982) *Ann. N.Y. Acad. Sci. 396*, 83-94.
Thomas, M., Shirley, P., Hedrick, C., & DeChatelet, L. (1986) *Biochemistry 25*, 8042-8048.
Van Beveren, C., van Straaten, F., Curran, T., Müller, T., & Verma, I. (1983) *Cell 32*, 1241-1255.
Zimmerman, R., & Cerutti, P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2085-2087.

Tyrosine and Threonine Phosphorylation of an Immunoaffinity-Purified 44-kDa MAP Kinase†

Jean-Claude Scimeca, Robert Ballotti, Tien T. Nguyen, Chantal Filloux, and Emmanuel Van Obberghen*
INSERM U145, Faculté de Médecine, 06034 Nice Cédex, France
Received May 28, 1991; Revised Manuscript Received July 22, 1991

ABSTRACT: We have approached the functioning of a MAP kinase, which is thought to be a "switch kinase" in the phosphorylation cascade initiated from various receptor tyrosine kinases including the insulin receptor. To do so, antipeptide antibodies were raised against the C-terminal portion of ERK1 (extracellular signal-regulated kinase 1), a protein kinase belonging to the family of MAP kinases. With these antipeptide antibodies, we observed the following: (i) a 44-kDa protein can be specifically recognized both under native and denaturing conditions; (ii) a 44-kDa phosphoprotein can be revealed in ^{32}P-labeled cells; its phosphorylation is stimulated by insulin, sodium orthovanadate, and okadaic acid; (iii) a MBP kinase activity can be precipitated, which phosphorylates MBP on threonine residues, and which is stimulated by insulin, sodium orthovanadate, okadaic acid, and fetal calf serum; (iv) this MBP kinase activity appears to be correlated with the in vivo induced phosphorylation of the 44-kDa protein. We next studied the in vitro phosphorylation of this 44-kDa/ERK1-immunoreactive protein. A time- and manganese-dependent phosphorylation was stimulated by the in vitro addition of sodium orthovanadate. Phosphoamino acid analysis of the in vitro phosphorylated 44-kDa protein revealed both threonine and tyrosine phosphorylation. Importantly, this in vitro phosphorylation of MAP kinase results in activation of phosphorylation of added MBP substrate. As a whole, our data indicate that the 44-kDa phosphoprotein identified by our antipeptide antibodies very likely corresponds to a MAP kinase. The observation that the immunoaffinity-purified 44-kDa MAP kinase exhibits in vitro phosphorylation on threonine and tyrosine residues suggests that this MAP kinase possesses both a threonine and tyrosine kinase autophosphorylation activity or that one or more MAP kinase kinase(s) copurify(ies) with the 44-kDa MAP kinase.

A growing list of hormones and growth factors initiate their biological effects by interacting with their specific transmembrane receptors, which carry protein tyrosine kinase activity (Ullrich & Schlessinger, 1990; Yarden & Ullrich, 1988). One of the major goals of the current research focused on cell metabolism and growth concerns the eludication of the mechanism by which these hormones and growth factor receptors stimulate pleiotropic cell responses. For insulin, it is now well established that the earliest post-binding event identified so far is activation of the receptor tyrosine kinase, with ensuing receptor tyrosine autophosphorylation (Gammeltoft & Van Obberghen, 1986; Rosen, 1987). Concurrent with this insulin receptor phosphorylation, the state of phosphorylation of a series of cellular proteins changes, and this concerns for the most part proteins phosphorylated on serine and/or threonine residues (Czech et al., 1988; Pelech et al., 1987; Rosen, 1987). One of the most pressing problems to be solved in this context is the identification of the serine/threonine protein kinases and phosphatases, which are the missing components in the phosphorylation cascade initiated from the insulin receptor tyrosine kinase.

MAP kinase is a serine/threonine kinase transiently activated in many cell types by a variety of extracellular signals such as insulin, EGF,[1] PDGF, FGF, NGF, or TPA (Erickson

† This research was supported by funds from INSERM, Université de Nice, and Association pour la Recherche sur le Cancer (Grant 6760). J.-C. Scimeca is recipient of a fellowship from Ligue Nationale Française Contre le Cancer, Fédération des Comités Départmentaux, Comité Départemental du Var. Dr. T. T. Nguyen is recipient of a postdoctoral fellowship from the Medical Research Council of Canada.
* Corresponding author.

[1] Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; NGF, nerve growth factor; MAP kinase, mitogen-activated protein kinase; TPA, *O*-tetradecanoylphorbol 13-acetate; MBP, myelin basic protein; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; PMSF, phenylmethanesulfonyl fluoride; C peptide, ERK1 peptide/sequence 356-367; TLC, thin-layer chromatography; ERK1, extracellular signal-regulated kinase 1; PNPP, *p*-nitrophenyl phosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

0006-2960/91/0430-9313$02.50/0

et al., 1990; Hoshi et al., 1988; L'Allemain et al., 1991; Miyasaka et al., 1990; Ray & Sturgill, 1987; Rossomando et al., 1989). It appears to be a promising candidate for an important "switch kinase". Recently, the nearly complete ERK1 (extracellular signal-regulated kinase 1) sequence was deduced from a rat brain cDNA, and sequence comparison indicated homologies with two yeast kinases involved in cell-cycle control and with human cdc2 protein kinase (Boulton et al., 1990). The cloning was performed after obtaining the partial peptide sequence of a 43-kDa MAP kinase protein purified from insulin-treated rat fibroblasts transfected with human insulin receptor cDNA (Boulton et al., 1991). In addition to the clone coding for ERK1, clones coding for highly related kinases were isolated. Taken together, these data strongly suggest the existence of a family of MAP kinases, ERK1 being one of them.

Data in the literature indicate that in intact cells MAP kinase is phosphorylated on threonine and on tyrosine residues and that both phosphorylations are important for its catalytic activity (Anderson et al., 1990; Ray & Sturgill, 1988). At present it is not clear whether receptor tyrosine kinases are directly responsible for MAP kinase phosphorylation on tyrosine or whether intermediate nonreceptor tyrosine kinases are involved. To approach the precise position of MAP kinase in the phosphorylation cascade initiated from tyrosine kinase receptors, we have studied an immunoaffinity-purified ERK1 using antipeptide antibodies to the carboxyl-terminus of the ERK1 sequence. These sequence-specific antibodies were able to detect a 44-kDa protein in immunoblot experiments and also to precipitate a 44-kDa protein from extracts derived from cells biosynthetically labeled with [^{35}S]methionine. Further, our antiserum precipitated a protein kinase activity, which phosphorylated MBP on threonine residues. ^{32}P-labeling in intact cells revealed an insulin, sodium orthovanadate, and okadaic acid stimulated phosphorylation of a ERK1-immunoreactive 44-kDa protein, which persisted after alkali treatment. We found that the MBP kinase activity of immunopurified 44-kDa protein appeared to be correlated with its in vivo phosphorylation level. Next we tested the in vitro phosphorylation of immunopurified MAP kinase. We found a manganese-dependent phosphorylation of the purified 44-kDa MAP kinase, which was time-dependent and stimulated by the addition, in vitro, of sodium orthovanadate. Phosphoamino acid analysis indicated that ^{32}P was incorporated on threonine but also on tyrosine residues. The phosphothreonine and phosphotyrosine amounts appear to reach quantitatively comparable levels. Finally, this in vitro phosphorylation of MAP kinase activates the enzyme for exogenous substrate phosphorylation.

Materials and Methods

Materials

Sodium orthovanadate, myelin basic protein (MBP) from bovine brain, protein A–Sepharose and Kemptide (sequence LRRASLG) were from Sigma. Bovine serum albumin for immunoblot experiments was from Intergen Co. (Providence, RI). NIH 3T3 cells (clone HIR 3.5) transfected with an expression plasmid encoding the human insulin receptor and expressing approximately 6×10^6 receptors/cell were provided by Dr. J. Whittaker, Stony Brook, NY (Whittaker et al., 1987). HIR cells, which exhibit several typical insulin-induced biological responses (Hofmann et al., 1989), were cultured to confluence in DMEM/10% FCS. Synthetic peptide and coupled peptide for rabbit immunization were obtained from Neosystem (Strasbourg, France). Rabbit immunization is described under Methods. Okadaic acid, produced by Dr. Y. Tsukitani (Fujisawa Pharmaceuticals Co., Tokyo, Japan), was a gift from Dr. P. Cohen (Dundee, U.K.).

Methods

Preparation of an Antipeptide Antibody to ERK1. According to the sequence of the MAP kinase published by Boulton et al. (1990), a 12 amino acid peptide corresponding to the C-terminal portion of the kinase was chosen (amino acids 356–367; TARFQPGAPEAP = C peptide). For production of antibodies, the peptide was coupled to Keyhole Limpet hemocyanin via the Thr 356 residue with glutaraldehyde. For each rabbit injection, 150 μg of antigen was used. After an initial intradermal injection in complete Freund's adjuvant, the animal was given boosts of peptide antigen in incomplete Freund's adjuvant. Blood was collected 10 days after each injection; preimmune serum served as a control.

Immunoblotting of Cytosols. Confluent HIR cells were washed twice in ice-cold PBS (140 mM NaCl, 3 mM KCl, 6 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4) and then once in buffer A (50 mM Hepes, pH 7.4, 150 mM NaCl, 2 mg/mL leupeptin, 2 mg/mL aprotinin, and 0.18 mg/mL PMSF). Cells were scraped in buffer A and, after sonification (15 s), cell extracts were centrifuged for 30 min at 20000*g*. Pellets were discarded and Laemmli buffer (3% SDS final) was added to supernatants corresponding to cytosolic fractions. Samples were then subjected to SDS–PAGE under reducing conditions.

Following electrophoresis, proteins were transferred to an Immobilon membrane (Immobilon PVDF Millipore), which was saturated overnight in 10 mM Tris, pH 7.4, 140 mM NaCl, 5% BSA, and 0.5% gelatin (saturating buffer). Rabbit serum was then diluted 1000 times in saturating buffer and incubated with transfer membrane for 90 min at room temperature. After three 20-min washes with 10 mM Tris, pH 7.4, 140 mM NaCl, 0.5% BSA, 0.05% gelatin, and 0.5% NP40 (washing buffer), the membrane was incubated for 1 h with biotinylated swine anti-rabbit immunoglobulins (Dakopatts) diluted 1000 times in saturating buffer. After additional washes as described above, the membrane was incubated for 45 min with avidin and biotinylated horseradish peroxydase (ABComplex from Dakopatts) each diluted 5000 times in saturating buffer. The membrane was then washed twice with washing buffer and once with 0.1 M Tris, pH 7.4. The binding of antibodies was revealed by incubation with 0.1M Tris, pH 7.4, 0.5 mg/mL diaminobenzidine, and 0.03% H_2O_2. The reaction was terminated by extensive washes with water.

^{32}P-Labeling in Intact Cells. Confluent HIR cells, growing in 12-well dishes, were starved overnight in DMEM/0.2% BSA medium (BSA 7030 from Sigma). Cells were then washed twice with DMEM without phosphate and incubated for 3.5 h in this medium containing 500 μCi of [^{32}P]orthophosphate (1.7 mCi/mL). At the end of labeling, effectors were added for 5 min, except for okadaic acid, which was added for 1 h. After three washes with ice-cold PBS, proteins were solubilized for 15 min on ice in 50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM sodium orthovanadate, 100 mM NaF, 1% Triton X-100, 2 mg/mL aprotinin, 20 μM leupeptin, and 0.18 mg/mL PMSF. Cell extracts were incubated for 90 min with antibodies adsorbed on protein A–Sepharose. After five washes with solubilization buffer, Laemmli buffer (3% SDS) was added to dried pellets, and proteins were submitted to SDS–PAGE under reducing conditions. For KOH treatment, the gels were destained and incubated for 1 h at 55 °C in 1 M KOH.

MBP Kinase Assay. Confluent cells growing in 12-well dishes were starved overnight in DMEM/0.2% BSA, and, after incubation with effectors, total cell extracts were prepared and submitted to immunoprecipitation with the antipeptide to ERK1 carboxyl-terminus as described for the ^{32}P-labeling experiments. After washes, the immunoprecipitates were eluted with C peptide at 4 °C for 30 min in a medium containing the peptide (10^{-5} M final concentration) and 25 mM Hepes, pH 7.5, 25 mM NaCl, 40 mM PNPP, 2 mM EGTA, 1 mM DTT, 2 mg/mL aprotinin, 20 μM leupeptin, and 0.18 mg/mL PMSF. MBP phosphorylation was then performed for 15 min at room temperature in presence of 150 μg/mL MBP, 10 mM magnesium acetate, and [γ-^{32}P]ATP (5 μM, 33 Ci/mmol). The phosphorylation reaction was terminated by adding Laemmli buffer (3% SDS final), and samples were submitted to SDS–PAGE.

Phosphoamino Acid Analysis. MBP phosphorylation was performed as described above, and samples were submitted to SDS–PAGE. After electrophoresis, ^{32}P-labeled MBP was localized by autoradiography, and gel pieces corresponding to the labeled MBP were excised. Phosphoproteins were eluted from the gel by an overnight incubation at 37 °C in 50 mM NH_4HCO_3, pH 8. Eluted MBP was precipitated for 30 min on ice in presence of 10% TCA and 25 μg of bovine γ-globulin. Pellets were washed once with 100% ethanol and once with ethanol/ethylic ether (1:1). MBP was hydrolyzed for 90 min at 110 °C in 6 N HCl. Phosphoamino acids were separated on cellulose thin-layer plates by electrophoresis at pH 3.5 for 2 h at 1000 V and were analyzed by autoradiography (Cooper et al., 1983).

In Vitro Phosphorylation Assay of Immunoprecipitated ERK1. Total cell extracts from HIR cells were submitted to immunoprecipitation by anti-ERK1 antipeptides. The immune pellets were washed five times in solubilization buffer. After an additional wash in HNTG buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 2 mg/mL leupeptin, 2 mg/mL aprotinin, and 0.18 mg/mL PMSF), ERK1 was eluted by a 30-min incubation at room temperature in HNTG buffer containing 10 μM C peptide and 0.2 mM sodium orthovanadate. In vitro phosphorylation was performed at room temperature for the indicated time in presence of 5 mM $MnCl_2$ and [γ-^{32}P]ATP (5 μM, 33 Ci/mmol). The reaction was terminated by addition of 4× concentrated Laemmli buffer, and samples were submitted to SDS–PAGE.

Results and Discussion

Antipeptides to ERK1 Recognize a Native and Denatured 44-kDa Protein from Mouse Fibroblasts. First, we tested the presence of specific antibodies by immunoblot experiments. As described under Methods, cytosols from HIR cells, rat brain, or rat spinal cord were submitted to SDS–PAGE, and proteins were transferred to blotting membrane. Incubation with immune serum revealed one specific band in the three tissues with a molecular weight estimated to be 44K (Figure 1). Compared to mouse fibroblasts (lane A), this protein is more abundant in the rat central nervous system with a even stronger expression in spinal cord (lanes B and C). These results are in agreement with the findings of Boulton et al. (1990), who reported a high level of ERK1 transcript expression in rat central nervous system, i.e., three to six times higher, respectively, in brain and spinal cord, compared to other tissues. Next we evaluated the specificity of our antibodies by their ability to precipitate the 44-kDa protein in presence of various peptides. To do so, cytosols derived from HIR cells were submitted to immunoprecipitation by antibodies adsorbed on protein A–Sepharose beads. After washes,

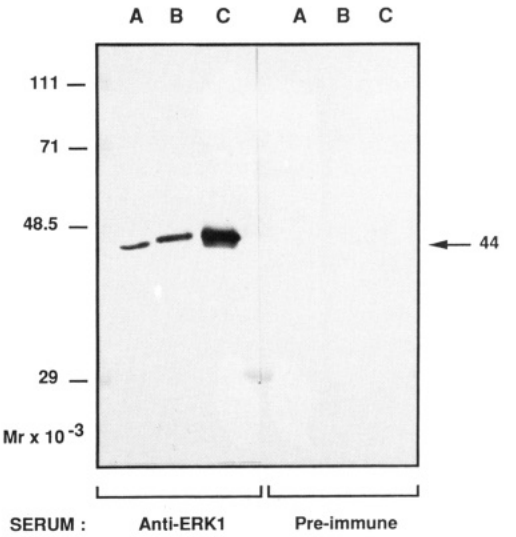

FIGURE 1: Immunoblot with antipeptide to ERK1/sequence 356–367. 25 μg of cytosol proteins from HIR cells (A), rat brain (B), or rat spinal cord (C) were submitted to SDS–PAGE under reducing conditions, transferred to a PVDF membrane, and immunoblotted with preimmune or immune antibodies.

Laemmli buffer was added to the pellets, the proteins were submitted to SDS–PAGE, and immunoblotting was performed with our antipeptides as described above. We found that immune antibodies were able to precipitate a 44-kDa protein in absence of peptide, and that this immunoprecipitation was virtually abolished in presence of the C peptide or of the coupled C peptide used for rabbit immunization (data not shown). In contrast, this 44-kDa protein immunoprecipitation was not affected by two nonrelated peptides: (i) a 12 amino acid peptide corresponding to the sequence from another MAP kinase region, and (ii) Kemptide.

As a whole, our data indicate that our antipeptide to the carboxyl-terminus of ERK1 immunoprecipitates native protein and recognizes on immunoblot a denatured protein of 44 kDa in various tissues. In addition, using [^{35}S]methionine biosynthetic labeling of HIR cells, we found specific immunoprecipitation of a 44-kDa protein (data not shown). In fact, Boulton et al. (1990) published an NH_2-terminal incomplete sequence with a predicted molecular mass of 42 kDa for the cloned protein. Thus, it is entirely possible that the complete sequence could encode for a protein with a molecular mass consistent with the 44-kDa species we observed here. The antipeptides we raised appeared to recognize only one molecular species with a molecular mass estimated to be 44 kDa. This could be explained by a differential expression of the ERK proteins, depending on the tissues, or by variations in the C-terminus among the different members of the family.

Immunoprecipitated 44-kDa Protein Contains MAP-Kinase-Like Activity. To confirm the specific immunoprecipitation of a MAP kinase activity, we have tested whether eluates obtained from anti-ERK1 immunoprecipitates were able to phosphorylate MBP, considered as the best substrate for in vitro MAP kinase activity. Confluent HIR cells were starved overnight in DMEM/0.2% BSA. After incubation with insulin or sodium orthovanadate for 5 min, cell extracts were subjected to immunoprecipitation with antipeptides to ERK1, and eluates were prepared as described under Methods. Figure 2, top panel, shows MBP phosphorylation induced by preparations obtained from these cells. A strong stimulation of MBP phosphorylation is found in response to insulin (lane 2) and sodium orthovanadate (lane 3). Phosphoamino acid analysis performed in these conditions indicates that all the

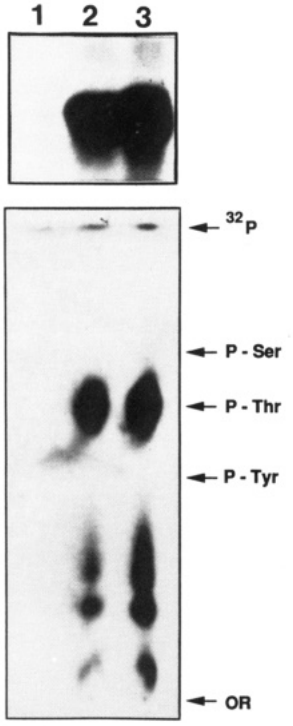

FIGURE 2: MBP phosphorylation by eluates from anti-ERK1 immunoprecipitates and phosphoamino acid analysis of MBP. Cells growing in 12-well dishes were incubated for 5 min with buffer (lane 1), 0.1 μM insulin (lane 2), or 10 mM sodium orthovanadate (lane 3); cell extracts were submitted to immunoprecipitation, and eluates were incubated with MBP for 15 min at room temperature. After SDS–PAGE and autoradiography (top), phosphorylated MBP was eluted and hydrolyzed for 90 min at 110 °C in 6 N HCl; phosphoamino acids were separated by TLC for 2 h at 1000 V, standards were detected with ninhydrin, and the cellulose thin-layer plate was autoradiographed (bottom).

^{32}P incorporation into MBP occurred on threonine residues for both effectors (bottom panel). As it is known that MAP kinase efficiently phosphorylates MBP and that MAP-kinase-induced ^{32}P incorporation into MBP takes place on threonine residues (Boulton et al., 1991; Erickson et al., 1990), our results further support the notion that the 44-kDa protein recognized by our antipeptides indeed corresponds to a MAP kinase. In additional experiments, we compared MBP kinase activity from an immunoprecipitate eluted by C peptide, as described above, with the activity measured in the noneluted immunoprecipitate. We found a comparable level of ^{32}P incorporation into MBP under the two conditions, indicating that our antibodies do not modulate (stimulate or inhibit) the MBP kinase activity of the 44-kDa enzyme (data not shown).

Phosphorylation of a 44-kDa Protein in Intact Mouse Fibroblasts. We then extended our studies to the phosphorylation of the ERK1-immunoreactive 44-kDa protein in intact HIR cells and its possible regulation by insulin and the following two phosphatase inhibitors: (i) okadaic acid, an inhibitor of serine/threonine phosphatases 1 and 2A (Cohen et al., 1990), and (ii) sodium orthovanadate, a tyrosine phosphatase inhibitor (Swarup et al., 1982).

^{32}P labeling of HIR cells was performed as described under Methods, and cells were incubated with insulin or sodium orthovanadate for 5 min, or with okadaic acid for 1 h. After solubilization, the cell extracts were subjected to immunoprecipitation by anti-ERK1 antipeptides. Samples were then equally divided on two gels in order to compare alkali-treated and nontreated gels. Figure 3 illustrates that 44-kDa protein phosphorylation was stimulated by the three agents. Note that the three agents increased the labeling of a 97-kDa protein,

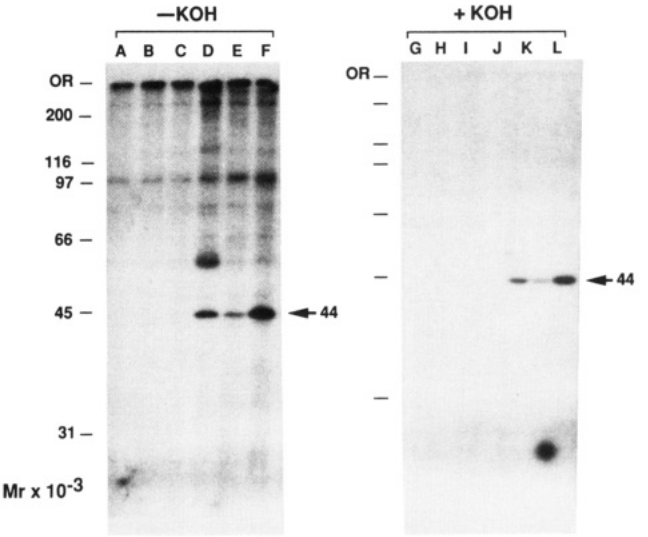

FIGURE 3: Phosphorylation of a 44-kDa protein in intact mouse fibroblasts. HIR cells growing in 12-well dishes were incubated with ^{32}P for 3 h 45 min with buffer (A, G), 0.04% DMSO (B, H), 0.1 μM okadaic acid (C, I), 1 μM okadaic acid (D, J), 0.1 μM insulin (E, K), or 10 mM sodium orthovanadate (F, L). Incubations were for 5 min for insulin and sodium orthovanadate and for 1 h for okadaic acid. Cell extracts were submitted to immunoprecipitation by anti-ERK1 antipeptides. Laemmli buffer was added to washed pellets, and samples were divided on two gels for alkali treatment (right panel) after SDS–PAGE under reducing conditions. An autoradiograph of the gel is shown.

and especially okadaic acid also enhanced the labeling of a 55-kDa protein. After KOH treatment, only the 44-kDa phosphorylation remained visible, suggesting that phosphorylation occurred essentially on tyrosine and/or threonine residues. Interestingly, okadaic acid, which is usually described as a serine/threonine phosphatase inhibitor, also increased the tyrosine phosphorylation of the 44-kDa MAP kinase under study. These findings are consistent with the results obtained by Haystead et al. (1990) in isolated adipocytes, demonstrating a stimulatory effect of okadaic acid on both MBP kinase activity and tyrosine phosphorylation of a 38- and a 42-kDa protein. The significance of the observations made with okadaic acid remains unclear, since phosphatase 2A, a target for okadaic acid, also displays phosphotyrosine phosphatase activity in vitro, depending on the Mn^{2+} concentration (Ahn et al., 1991). Okadaic acid action could be explained by a direct inhibition of a tyrosine phosphatase or, alternatively, by an interaction with a serine/threonine phosphatase, which ultimately regulates the tyrosine phosphorylation state of MAP kinase. Whatever the precise model, the action of okadaic acid and sodium orthovanadate on MAP kinase in vivo tyrosine phosphorylation and MAP kinase activity points to the involvement of protein(s) phosphatase(s) in the enzyme regulation.

Data illustrated in Figures 2 and 3 suggest that increased phosphorylation of the 44-kDa protein is concomitant with a stimulation of its MBP kinase activity. To further document this, we performed ^{32}P labeling in intact cells, followed by immunoprecipitation of the in vivo labeled 44-kDa protein and measurement of its MBP kinase activity. As illustrated in Figure 4, an increased phosphorylation of 44-kDa protein was found in response to insulin, sodium orthovanadate, okadaic acid, and fetal calf serum (top panel). When MBP kinase activity was studied with the same eluates, we observed a direct correlation between 44-kDa protein phosphorylation and ^{32}P incorporation in MBP (bottom panel). These results mean that the level of in vivo phosphorylation of the 44-kDa protein

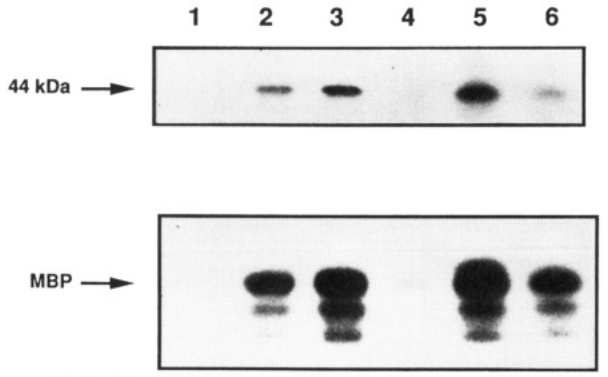

FIGURE 4: In vivo 44-kDa protein phosphorylation and MBP kinase activity. HIR cells growing in 12-well dishes were incubated with ^{32}P for 3 h 45 min, with buffer (5 min), 0.1 μM insulin (5 min), 10 mM sodium orthovanadate (5 min), 0.04% DMSO (1 h), 2 μM okadaic acid (1 h), and 10% FCS (5 min), respectively, from lane 1 to lane 6. The 44-kDa protein was precipitated with anti-ERK1 antipeptides, and MBP phosphorylation with the eluates was performed for 15 min at 24 °C. The reaction was stopped with Laemmli buffer, and samples were analyzed by SDS–PAGE under reducing conditions followed by autoradiography.

parallels its in vitro MBP kinase activity. As mentioned earlier, the okadaic acid induced stimulation of MBP kinase activity we illustrate here is reminescent of the okadaic acid action in isolated adipocytes reported by Haystead et al. (1990) on two MBP kinases. However, molecular weights of 38K and 42K were determined for these kinases. Provided that the activities characterized by Haystead et al. (1990), at least the 42K kinase, are related to the MAP kinase family, our observations are compatible with a common mechanism of okadaic acid action on these kinases.

In Vitro Phosphorylation of Immunoprecipitated ERK1. We next addressed the issue of in vitro phosphorylation of immunopurified MAP kinase. To do so, ERK1 was isolated from quiescent fibroblast extracts by incubation with anti-peptides followed by specific elution from the protein A–Sepharose/antibody pellet. As illustrated in Figure 5A, a time-dependent phosphorylation of the eluted 44-kDa MAP kinase occurred in presence of 5 mM $MnCl_2$ and 0.2 mM sodium orthovanadate (panel A), conditions leading to the maximum signal we observed. When sodium orthovanadate was omitted, the phosphorylation was still found, but with a weaker intensity (panel C). By contrast, when $MnCl_2$ was replaced by magnesium acetate, a dramatic decrease in ^{32}P incorporation was seen at all time points (panel B). Next, phosphoamino acid analysis of in vitro phosphorylated MAP kinase was performed. Immunopurified ERK1 was phosphorylated for 45 min and submitted to phosphoamino acid analysis. Figure 5B indicates that ^{32}P incorporation occurred both on threonine residues and on tyrosine residues. Note that a faint serine phosphorylation could be detected on long-term film exposures. The phosphotyrosine content appears to be nearly equal to the phosphothreonine amount, which is in agreement with the in vivo phosphorylation of the regulatory phosphorylation sites of 42-kDa MAP kinase (Payne et al., 1991). We have evaluated whether the in vitro phosphorylation of ERK1 leads to activation of phosphorylation of added substrate such as MBP. To do so, immunopurified ERK1 was phosphorylated for increasing periods of time, and kinase activity toward MBP was then measured after an additional 5-min incubation with the substrate. As illustrated in Figure 6, MBP phosphorylation was enhanced by increasing ERK1 phosphorylation time, indicating that the in vitro phosphorylation of ERK1 stimulates its exogenous substrate phosphorylation activity.

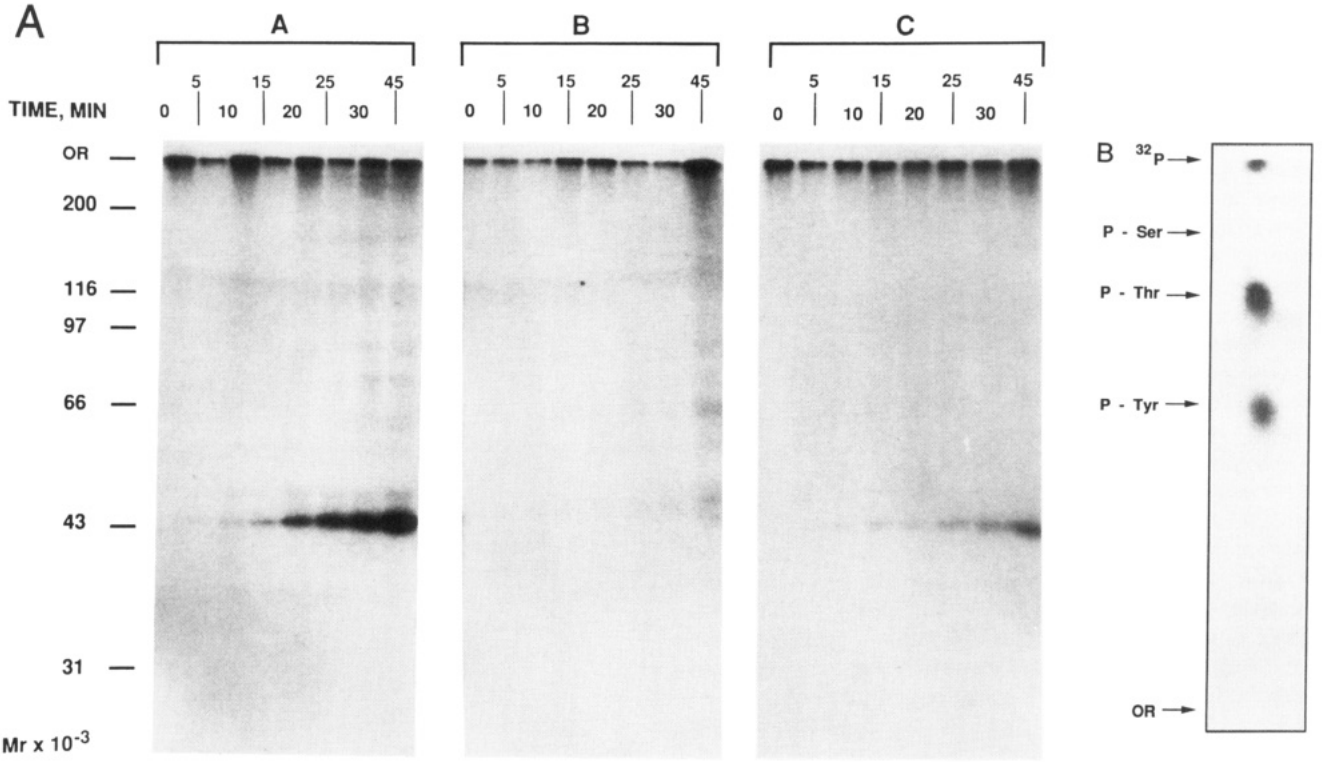

FIGURE 5: In vitro 44-kDa protein phosphorylation and phosphoamino acid analysis. Immunoprecipitated and eluted 44-kDa protein from HIR cells was incubated for 0–45 min at room temperature in presence of [γ-^{32}P]ATP (5 μM, 33 Ci/mmol) and 5 mM $MnCl_2$/0.2 mM sodium orthovanadate (A), 5 mM $MgCl_2$/0.2 mM sodium orthovanadate (B), or 5 mM $MnCl_2$ (C). Phosphorylation was stopped with 4× concentrated Laemmli buffer, and the 44-kDa protein was analyzed by SDS–PAGE under reducing conditions (panel A). The 44-kDa protein was phosphorylated for 45 min at room temperature in presence of [γ-^{32}P]ATP (5 μM, 33 Ci/mmol) and 5 mM $MnCl_2$/0.2 mM sodium orthovanadate; after SDS–PAGE and elution, the 44-kDa protein was hydrolyzed in 6 N HCl for 90 min at 110 °C, and phosphoamino acids were analyzed by TLC (panel B).

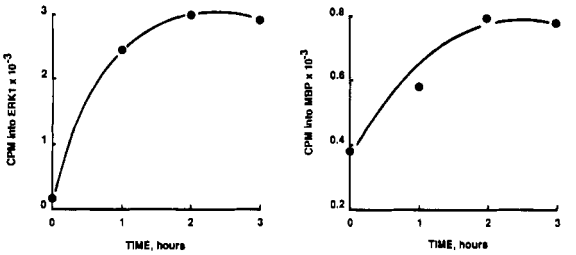

FIGURE 6: MBP kinase activity of ERK1 after increasing in vitro phosphorylation times. Immunopurified ERK1 was incubated in presence of [γ-^{32}P]ATP (5 μM, 33 Ci/mmol) and 5 mM $MnCl_2$/0.2 mM sodium orthovanadate at 30 °C for increasing periods of time. MBP and magnesium acetate were then added, and substrate phosphorylation was performed at 25 °C for 5 min. After SDS–PAGE, phosphoproteins corresponding to ERK1 (left panel) and MBP (right panel) were excised, and incorporated ^{32}P was determined by Cerenkov counting.

Taken together, our data show that a time- and manganese-dependent in vitro phosphorylation of a 44-kDa ERK1-immunoreactive protein occurs, the phosphorylation of which is stimulated by sodium orthovanadate. Interestingly, the ERK1 phosphorylation was equal on threonine and on tyrosine residues and results in activation of phosphorylation of added substrate. This dual in vitro phosphorylation could indicate that the 44-kDa ERK1 is endowed with the capacity to autophosphorylate on both tyrosine and threonine residues. This unusual enzymatic property has recently been found in other kinases related to cell cycle regulation, including the fission yeast p107$^{wee\text{-}1}$ (Featherstone & Russell, 1991) and the mammalian *clk* gene product (Ben-David et al., 1991). It should be noted that, at present, we have been unable to show that immunopurified 44-kDa ERK1 phosphorylates the copolymer (Glu, Tyr) (4:1) (data not shown). This could suggest that, if the dual enzyme specificity proves to be correct, the activity toward tyrosine might be limited to autophosphorylation. Alternatively, our data could indicate that a threonine kinase and a tyrosine kinase copurify with ERK1. Since in Western blot experiments we detect only one specific molecular species at 44-kDa, these additional kinases do not appear to be recognized by our ERK1 antipeptides but rather could be associated to MAP kinase and consequently coprecipitated. However, it remains possible that they interact directly with the antibodies but that a conformational epitope disappears after denaturation on SDS–PAGE. At the present time, we have no evidence to prove that the threonine phosphorylation is due to an autophosphorylation activity of MAP kinase. Hence a MAP kinase kinase with threonine specificity could be coprecipitated. However, it is possible that only one kinase kinase with a dual specificity could be associated with MAP kinase. As mentioned above, Ben-David et al. (1991) have recently reported the existence of a mammalian kinase related to cell-cycle regulators and with potential for serine-/threonine and tyrosine phosphorylation. Such a kinase could exist in a complex with MAP kinase in basal conditions, consistent with its coprecipitation from quiescent fibroblasts. Upon activation by an extracellular stimulus, this threonine-/tyrosine kinase would phosphorylate MAP kinase on threonine and tyrosine residues, leading to activation of the MAP kinase.

In summary, using antipeptides against the C-terminal portion of ERK1, we have observed in vitro phosphorylation of a 44-kDa MAP kinase, after its immunopurification from quiescent mouse fibroblast extracts. This in vitro phosphorylation occurred both on threonine and tyrosine residues. Previous studies have shown that in vivo activated pp42/MAP kinase contained both phosphothreonine and phosphotyrosine (Ray & Sturgill, 1988). Further, the threonine and tyrosine regulatory phosphorylation sites of pp42/MAP kinase have been identified, and the sequence that contains them is perfectly conserved in the ERK1 protein we are studying (Payne et al., 1991). More important, we show here that the in vitro phosphorylation of ERK1 leads to activation of phosphorylation of added MBP substrate. Hence, the in vitro phosphorylation is likely to be physiologically relevant. Ahn et al. (1991) have recently proposed the existence of activators that regulate MBP kinases 1 and 2 by phosphorylation. In preliminary experiments, we have gathered evidence for the presence of at least one phosphoprotein in the 90-kDa range, which can be coprecipitated with the 44-kDa MAP kinase under certain conditions. Studies are in progress to investigate whether the copurifying 90-kDa protein is a MAP kinase activator or a kinase kinase and whether it regulates the MAP kinase in vitro threonine/tyrosine phosphorylation.

ACKNOWLEDGMENTS

We thank Dr. P. Cohen for okadaic acid, Dr. J. Whittaker for HIR cells, Dr. Y. Le Marchand-Brustel for reviewing the manuscript, J. Duch and L. Capolongo for secretarial assistance, and G. Visciano for illustration work.

REFERENCES

Ahn, N. G., Seger, R., Bratlien, R. L., Diltz, C. D., Tonks, N. K., & Krebs, E. G. (1991) *J. Biol. Chem. 266*, 4220–4227.

Anderson, N. G., Maller, J. L., Tonks, N. K., & Sturgill, T. W. (1990) *Nature 343*, 651–653.

Ben-David, Y., Letwin, K., Tannock, L., Bernstein, A., & Pawson, T. (1991) *EMBO J. 10*, 317–325.

Boulton, T. G., Yancopoulos, G. D., Gregory, J. S., Slaughter, C., Moomaw, C., Hsu, J., & Cobb, M. H. (1990) *Science 249*, 64–67.

Boulton, T. G., Gregory, J. S., & Cobb, M. H. (1991) *Biochemistry 30*, 278–286.

Cohen, P., Holmes, C. F. B., & Tsukitani, Y. (1990) *Trends Biochem. Sci. 15*, 98–102.

Cooper, J. A., Sefton, B. M., & Hunter, T. (1983) *Methods Enzymol. 99*, 387–402.

Czech, M. P., Klarlund, J. K., Yagaloff, K. A., Bradford, A. P., & Lewis, R. E. (1988) *J. Biol. Chem. 263*, 11017–11020.

Erickson, A. K., Payne, D. M., Martino, P. A., Rossomando, A. J., Shabanowitz, J., Weber, M. J., Hunt, D. F., & Sturgill, T. W. (1990) *J. Biol. Chem. 265*, 19728–19735.

Featherstone, C., & Russell, P. (1991) *Nature 349*, 808–811.

Gammeltoft, S., & Van Obberghen, E. (1986) *Biochem. J. 235*, 1–11.

Haystead, T. A. J., Weiel, J. E., Litchfield, D. W., Tsukitani, Y., Fischer, E. H., & Krebs, E. G. (1990) *J. Biol. Chem. 265*, 16571–16580.

Hofmann, C., White, M. F., & Whittaker, J. (1989) *Endocrinology 124*, 257–264.

Hoshi, M., Nishida, E., & Sakai, H. (1988) *J. Biol. Chem. 263*, 5396–5401.

L'Allemain, G., Sturgill, T. W., & Weber, M. J. (1991) *Mol. Cell. Biol. 11*, 1002–1008.

Miyasaka, T., Chao, M., Sherline, P., & Saltiel, A. (1990) *J. Biol. Chem. 265*, 4730–4735.

Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J.-H., Shabanowitz, J., Hunt, D. F., Weber, M. J., & Sturgill, T. W. (1991) *EMBO J. 10*, 885–892.

Pelech, S. L., Tinker, D. A., Chan, C. P., & Krebs, E. G. (1987) *Insulin, insulin-like growth factors, and their re-*

ceptors in the central nervous system (Raizada, M. K., Phillips, M. I., & LeRoith, D., Eds.) pp 27–46, Plenum Publishing Corp., New York.
Ray, L. B., & Sturgill, T. W. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1502–1506.
Ray, L. B., & Sturgill, T. W. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3753–3757.
Rosen, O. M. (1987) *Science 237*, 1452–1458.
Rossomando, A. J., Payne, D. M., Weber, M. J., & Sturgill, T. W. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6940–6943.
Swarup, G., Cohen, S., & Garbers, D. L. (1982) *Biochem. Biophys. Res. Commun. 107*, 1104–1109.
Ullrich, A., & Schlessinger, J. (1990) *Cell 61*, 203–212.
Whittaker, J., Okamoto, A. K., Thys, R., Bell, G. I., Steiner, D. F., & Hofmann, C. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5237–5241.
Yarden, Y., & Ullrich, A. (1988) *Annu. Rev. Biochem. 57*, 443–478.

Inhibition of Substrate Binding to the Adrenal Cytochrome $P450_{C-21}$ by Acrylamide and Its Implications for Solvent Accessibility of the Binding Site in the Microsomes†

Shakunthala Narasimhulu
Harrison Department for Surgical Research, University of Pennsylvania, Philadelphia, Pennsylvania 19104
Received August 2, 1990; Revised Manuscript Received April 8, 1991

ABSTRACT: The present study offers evidence indicating that acrylamide, a highly polar molecule and an efficient quencher of tryptophanyl fluorescence, inhibits substrate binding to $P450_{C-21}$ in bovine adrenocortical microsomes, in a competitive manner similar to that in the purified enzyme. Resolution of the fluoresence-quenching data revealed an acrylamide quenching constant (K_2 = 9.9 M, that is, the association constant for the quencher–fluorophore complex) that was similar to the reciprocal of its inhibition constant ($1/K_i = K_a = 8.3 \pm 0.9$ M) for substrate binding. The substrate inhibited the fluoresence quenching by acrylamide as indicated by its concentration-dependent decrease in K_2. The inhibition was in accordance with partial competition. These results are essentially similar to those previously observed in the purified lipid-free enzyme. In addition, the substrate dissociation, acrylamide inhibition, and fluorescence-quenching constants and the tryptophanyl fluorescence maximum (340–342 nm) were essentially the same in the microsomes and the lipid-free purified enzyme. These results indicate that the substrate-binding site of $P450_{C-21}$ and the concerned tryptophan are accessible to the highly polar molecule in the microsomal membranes, similar to that in the lipid-free purified enzyme. This implies that the substrate-binding site is not shielded by lipids in such a way that only the substrate in the lipid phase can gain access to the binding site. This conclusion is consistent with the currently favored model, for membrane topology of mammalian P450 enzymes, in which P450 is anchored to the membrane through a short N-terminal sequence while the remaining portion of the molecule is exposed to polar environment.

Cytochrome P450, the oxygen-activating component of a variety of mixed function oxidases, is ubiquitous in nature. Mammalian cytochrome P450 enzymes are mainly intrinsic membrane proteins, whereas bacterial P450's are water soluble. Binding of substrates to the soluble (Katagiri et al., 1968) as well as membrane-bound P450's (Narasimhulu, 1963, 1965; Remmer et al., 1966; Schenkman et al., 1967; Peterson et al., 1971) results in the characteristic blue-shift of the Soret absorption band. With this spectral shift as the criterion for binding, various aspects of substrate–P450 binding equilibria have been studied. Most substrates of P450 enzymes, including those of water-soluble ones, are largely hydrophobic. Studies using hepatic microsomal P450's have shown correlation of substrate hydrophobicity and substrate affinity (Al-Gailany et al., 1978; White et al., 1980), indicating that substrate-binding sites of P450 enzymes are probably hydrophobic. At present, most direct evidence regarding the nature of the binding site and its accessibility to substrate comes from a high-resolution X-ray crystallographic study of $P450_{cam}$ (Poulos, 1986). Studies on the crystal structure of substrate-bound $P450_{cam}$ have shown that the site, in which the substrate resides upon binding, is lined with hydrophobic residues. In addition, these studies have shown that camphor also makes polar contacts (with the hydroxyl group of Tyr-96). This suggests that polar interactions may also be involved in the substrate binding dynamics. Similar direct evidence is not yet available for the membrane P450's. However, there are significant similarities between membrane P450's and $P450_{cam}$. For example, sequence alignment studies have shown that all eukaryotic P450 species show clear homologies with the bacterial enzyme in the vicinity of the cysteine heme ligand (Cys-357 in $P450_{cam}$) and the oxygen-binding site (helix I), whereas other regions are less well conserved (Poulos et al.,

† This work was supported in part by ONR Contract N00014-75C-0322, by NIH Grant AM18545, in part by a grant from the Research Foundation of the University of Pennsylvania, Harrison Department for Surgical Research, and by NIH Grant RR01348.

0006-2960/91/0430-9319$02.50/0