CELL REGULATION, Vol. 2, 357–371, May 1991

Identification of multiple extracellular signal-regulated kinases (ERKs) with antipeptide antibodies*

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A protein kinase characterized by its ability to phosphorylate microtubule-associated protein-2 (MAP2) and myelin basic protein (MBP) is thought to play a pivotal role in the transduction of signals from many receptors in response to their ligands. A kinase with such activity, named extracellular signal-regulated kinase 1 (ERK1), is activated rapidly by numerous extracellular signals, requires phosphorylation on tyrosine to be fully active, and in vitro can activate a kinase (a ribosomal S6 protein kinase) that is downstream in phosphorylation cascades. From the protein sequence predicted by the rat ERK1 cDNA, peptides were synthesized and used to elicit antibodies. The antibodies recognize both ERK1; a closely related kinase, ERK2; and a third novel ERK-related protein. Using these antibodies we have determined that ERK1 and ERK2 are ubiquitously distributed in rat tissues. Both enzymes are expressed most highly in brain and spinal cord as are their mRNAs. The third ERK protein was found in spinal cord and in testes. The antibodies detect ERKs in cell lines from multiple species, including human, mouse, dog, chicken, and frog, in addition to rat, indicating that the kinases are conserved across species. ERK1 and ERK2 have been separated by chromatography on Mono Q. Stimulation by insulin increases the phosphorylation of both kinases on tyrosine residues, as assessed by immunoblotting with phosphotyrosine antibodies, and retards their elution from Mono Q. Each of these ERKs appears to account for a distinct peak of MBP kinase activity. The activity in each peak is diminished by incubation with either phosphatase 2a or CD45. Therefore, both enzymes have similar modes of regulation and appear to contribute to the growth factor-stimulated MAP2/MBP kinase activity measured in cell extracts.

* In partial fulfillment of the requirements for the Ph.D. degree in Molecular and Cell Biology at UT at Dallas, Richardson, TX (T.G. Boulton).

Introduction

The molecular events triggered by extracellular signals that activate tyrosine phosphorylation have been the subject of intense study since Ushiro and Cohen (1980) discovered that the epidermal growth factor receptor had, in common with the transforming protein of Rous sarcoma virus, pp60$^{v\text{-}src}$, protein-tyrosine kinase activity (Collett *et al.*, 1980; Hunter and Sefton, 1980; Levinson *et al.*, 1980). Receptors for insulin and many other growth factors also either contain intrinsic tyrosine kinase activity or activate tyrosine phosphorylation (Kasuga *et al.*, 1982; Ek and Heldin, 1984; Maher, 1988; Veillette *et al.*, 1988; Ullrich and Schlessinger, 1990). Thus, it has been postulated that the effects of these factors are mediated by phosphorylation of specific downstream targets on tyrosine residues. Many of the phosphorylations that occur in response to insulin and other extracellular cues are not on tyrosine but on serine/threonine residues (e.g., those on ribosomal protein S6 kinases [Ballou *et al.*, 1988], phosphatase 1 G binding protein [Dent *et al.*, 1990], and acetyl coA-carboxylase [Borthwick *et al.*, 1990]). As a result, the cadre of protein-serine/threonine kinases that are phosphorylated on tyrosine and activated in response to growth-promoting agents are key intermediates in these pathways.

Ray and Sturgill (1987), Cicirelli *et al.* (1988), and others (Hoshi *et al.*, 1988) have identified a microtubule-associated protein 2 (MAP2)[1]/myelin basic protein (MBP) kinase that appears to have the requisite properties; after insulin or nerve growth factor stimulation, it is rapidly activated and contains phosphate on tyrosine as well as on serine/threonine residues (Ray and Sturgill, 1988; Boulton *et al.*, 1991b). Further, at least one potential target for this kinase, a

[1] Abbreviations: CHO, Chinese hamster ovary; DTT, dithiothreitol; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; ERK, extracellular signal-regulated kinase; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MAP2, microtubule-associated protein-2; MBP, myelin basic protein; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane.

ribosomal protein S6 kinase (Sturgill *et al.,* 1988; Gregory *et al.,* 1989; Ahn and Krebs, 1990), has been identified, suggesting that the MAP2/MBP kinase lies at an early step in a protein kinase cascade. The tyrosine-phosphorylated kinase is stimulated by a variety of growth factors and other extracellular signals, including insulin (Ray and Sturgill, 1987; Boulton *et al.,* 1990a, 1991a), epidermal growth factor (Hoshi *et al.,* 1988; Ahn *et al.,* 1990), nerve growth factor (Gómez *et al.,* 1990; Miyasaka *et al.,* 1990; Tsao *et al.,* 1990; Boulton *et al.,* 1991b) and interaction of T cell surface receptors with presented antigens (Nel *et al.,* 1990a).

Chromatographic evidence from several laboratories (Cicirelli *et al.,* 1988; Hoshi, *et al.,* 1988; Ahn *et al.,* 1990; Gómez *et al.,* 1990; Nel *et al.,* 1990b; Boulton *et al.,* 1991b) suggests that the MAP2/MBP kinase activity may be derived from two or more distinct enzymes. This activity is believed to correspond to a group of 42- to 45-kDa spots originally identified 10 years ago by two-dimensional gel electrophoresis as growth factor-stimulated phosphotyrosine-containing proteins (Martinez *et al.,* 1982; Cooper *et al.,* 1984; Rossomando *et al.,* 1989). The spots have not been assigned to either multiple unique but related kinases or to different phosphorylated or otherwise modified states of a single enzyme. Recently we have purified (Boulton *et al.,* 1991a) and cloned a rat MAP2/MBP kinase (Boulton *et al.,* 1990b) that we named extracellular signal-regulated kinase 1 (ERK1). Using probes derived from ERK1, we have isolated cDNAs encoding two related kinases. Analysis of Southern blots with probes specific for each of the three kinases suggests the existence of even more members of this family (Boulton *et al.,* 1991b). Of these related enzymes, a second ERK, ERK2, is 85% identical to ERK1 and only slightly smaller (predicted size 41.2 kDa). Bacterially expressed ERK2 has a similar substrate specificity in that it phosphorylates both MAP2 and MBP. Thus, we have identified two candidates that may account for the MAP2/MBP kinase activity that has been detected after stimulation by growth factors.

Antipeptide antibodies have been generated to dissect the processes by which ERK1 is activated and to distinguish it from other ERK proteins. In this report we document the tissue and cellular distribution of ERK1 and the highly related ERK2, we identify a novel ERK, and we begin to determine which of these ERK proteinsarerespondingtothenumerousstimuli capable of increasing MAP2/MBP kinase activity.

Results

Characterization of antipeptide antibodies

Antipeptide antibodies were generated to three peptides predicted by the ERK1 cDNA (Figure 1): 1) a peptide corresponding to the C-terminal 16 amino acids, 2) a 23-residue peptide from protein kinase subdomain XI (nomenclature of Hanks *et al.,* 1988), and 3) a 16-residue peptide near the N-terminus just before the highly conserved GXGXXG of subdomain I. All of the peptides are similar to sequences in ERK2 (Figure 1). The C-terminal peptide elicited antibodies in two rabbits with distinct specificities. One antiserum (956) recognized a band the size of ERK1 (~43 kDa) both in partially purified preparations and in cell extracts (Figures 1–3), almost exclusively. With antiserum 837, 25 ng of recombinant ERK2 was visible and bands the size of ERK2 (41 kDa) and ERK1 were detected not only in tissues extracts but also in partially purified MAP2/MBP kinase preparations (Figures 2 and 6). Antiserum 691, generated to the peptide from subdomain XI, and antiserum 692, generated to the peptide near the N-terminus, also recognized the 43- and 41-kDa proteins in cell extracts (Figure 1). We conclude that the 43-kDa protein is ERK1. On the basis of the recognition of a protein of ~41 kDa (the size predicted for ERK2 from its cDNA) by three antibodies made to different ERK1 peptides, the correspondence in the tissue distributions of the 41-kDa protein (Figure 2) with the ERK2 mRNA (Figure 5 and Boulton *et al.,* 1991b), the ability of the antibodies to recognize recombinant ERK2, and the ability of antibodies produced to recombinant ERK2 to recognize the same size protein in tissues and cell extracts (data not shown), we conclude that the 41-kDa ERK protein detected in tissues and cell extracts is ERK2.

Antiserum 837 immunoprecipitated ERK1 but was unable to immunoprecipitate significant amounts of ERK2 under nondenaturing conditions; this conclusion is based on lack of activity recovered in immunoprecipitates of ERK2 (Figure 9), on the inability to recover a 41-kDa ^{32}P-labeled band from labeled cells (Boulton *et al.,* 1991b; Robbins, Boulton, and Cobb, unpublished observations), and on the inability to immunoblot a 41-kDa band (although a 43-kDa band was detected in immunoprecipitates from both stimulated and unstimulated cells) in immunoprecipitates from cell supernatants (Boulton, Robbins, and Cobb, unpublished observations).

Antiserum 956, as well as antiserum 691, recognized a third ERK that was a larger protein

SPECIFICITY OF ANTIPEPTIDE ANTIBODIES

Serum	Peptide	Subdomain	Specificity
956/837	ERK1 I F Q E T A R F Q P G A P E A P 367 ERK2 • • E • • • • • • • • Y R S	C-terminus	956 ERK1, 45 kDa 837 ERK1, ERK2
691	ERK1 K R I T V E E A L A H P Y L E Q Y Y D P T D E 327 ERK2 • • • E • • Q • • • • • • • • • • • • • S • •	XI	ERK1, ERK2 45 kDa
692	ERK1 K G Q P F D V G P R Y T Q L Q Y 35 ERK2 R • • V • • • • • • • • N • S •	I	ERK1, ERK2

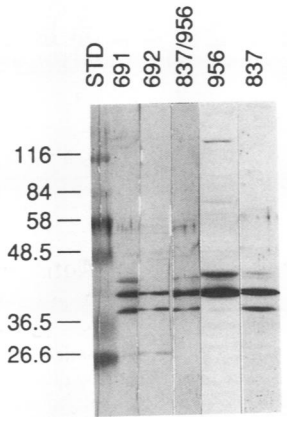

Figure 1. **Specificity of the antipeptide antibodies.** The peptide sequences used to elicit antibodies are shown in the top panel. Amino acid sequence identities of ERK1 and ERK2 are noted with (•). The numbering system for ERK1 is as described (Boulton *et al.,* 1990b). Soluble protein (100 μg) from PC12 cells was separated by electrophoresis in a 10% polyacrylamide (37.5:1, acrylamide:bisacrylamide) gel in sodium dodecyl sulfate (SDS) and transferred to nitrocellulose for Western blotting. Antisera 691 and 692 were diluted 100-fold and 956/837 is a mixture of these two antisera, each diluted 200-fold. Prestained molecular weight markers are indicated.

of ~45 kDa. The immunoreactivity of this 45-kDa protein is blocked with the peptide antigens (Figure 4). Because it is identified by antibodies to different (but not all) peptides from ERK1, the 45-kDa protein is probably a novel ERK, for which a cDNA has not yet been found.

Expression of ERKs in rat tissues

We examined the distribution and relative abundance of ERK1 and ERK2 in rat tissues using antisera 956 and 837. By determining the relative abundance of these kinases, it may be possible to determine if one or multiple of the enzymes contribute to the increase in MAP2/MBP protein kinase activity elicited by stimuli in a given tissue.

Equal amounts (100 μg except as noted in legend to Figure 2) of protein from soluble and particulate fractions of various rat tissues were immunoblotted (Figure 2) with the two antisera: 956, which has selectivity for ERK1, and 837, which recognizes both ERK1 and ERK2. All tissues examined contained immunoreactive ERK1 as deduced by reactivity of a 43-kDa band that comigrates with a standard of partially purified ERK1 (Boulton *et al.,* 1991a). ERK1 is most concentrated in spinal cord and brain, followed by lung and testes. Immunoreactive ERK1 is predominantly soluble except in spinal cord, lung, testes, and brain, where there is considerable enzyme associated with the particulate fraction. The largest ERK (~45 kDa) in spinal cord and testes is similar in size to a protein seen in several rat cell lines (see Figure 3). Kidney and intestine contain several faintly immunoreactive bands with a greater mobility (from 39–42 kDa) that react with the ERK1-selective antiserum. The smaller proteins may be distinct ERKs. Alternatively, proteolysis in these tissues might account for bands of increased mobility; however, cleavage would have to occur at the N-terminus of ERK1 because the antibodies that detected them were elicited to the C-terminus of the enzyme. Recognition of the bands of 43

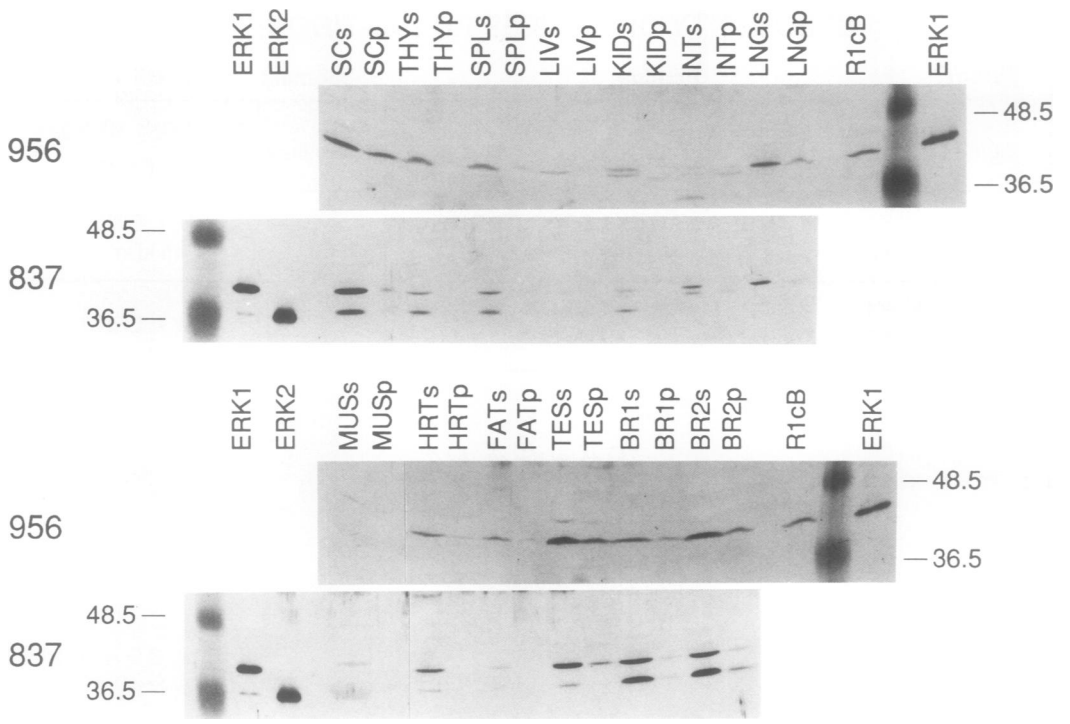

Figure 2. **Distribution of ERK1 and ERK2 in rat tissues.** One hundred micrograms (except for SCs, FATs, and BR1s probed with 956 only in which 50 μg were loaded) of protein were separated by electrophoresis as described in legend to Figure 1, then transferred to nitrocellulose for Western blotting. The antibodies used are indicated on the left of each blot. Prestained molecular weight markers indicate ~48.5 and 36.5 kDa. Partially purified ERK1 (a QAE-Sepharose pool; see Boulton *et al.*, 1991) migrated as 43 kDa and recombinant ERK2 (see Methods) as 39 kDa. Abbreviations: s, soluble fraction; p, particulate fraction; SC, spinal cord; THY, thymus; SPL, spleen; LIV, liver; KID, kidney; INT, intestine; LNG, lung; R1cB, 40 μg of Rat1 HIRc B supernatant; MUS, muscle; HRT, heart; FAT, epididymal fat; TES, testes; BR1, cerebellum; BR2, cerebrum.

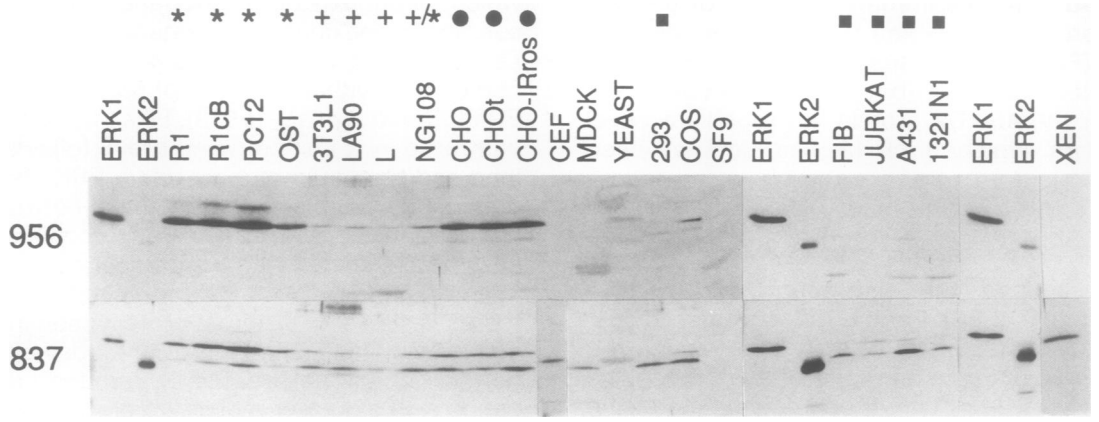

Figure 3. **Distribution of ERKs in selected cell lines.** Protein (100 μg) from the soluble fraction of various cell lines was analyzed as described in the legend to Figure 1. The upper panel shows supernatants probed with antiserum 956 and the lower panel with 837. Symbols denote the same species: *, rat; +, mouse; +/*, mouse/rat hybrid; ●, hamster; and ■, human. Cell line abbreviations: R1, Rat 1 fibroblasts; R1cB, Rat 1 HIRc B; PC12, pheochromocytoma; OST, osteosarcoma; 3T-3L1, preadipocytes; LA90, a 3T3-L1 line (see Methods); L, L cells; NG108, neuroblastoma × glioma; CHO, chinese hamster ovary; CHOt, overexpresses human insulin receptor; CHO-IRros, overexpresses insulin receptor/v-*ros* hybrid; CEF, chicken embryo fibroblasts; MDCK, Madin-Darby canine kidney; Yeast, *Saccharomyces cerevisiae* extracts; SF9, Spodoptera frugiperda; FIB, human foreskin fibroblasts; A431, human epidermoid carcinoma; Jurkat, differentiated T-cell; 1321N1, human astrocytoma; XEN, *Xenopus laevis* MBP kinase (Ferrell *et al.*, 1991).

and 45 kDa (Figure 4, e.g., testes pellet), as well as the smaller bands in kidney and intestine, is blocked by preincubation of the antibody with the peptide antigen, demonstrating the specificity of the interaction of the antibody with these bands (Figure 4).

When these same tissues are blotted with antiserum 837, a band of a smaller size (~41 kDa) corresponding to ERK2 is also detected in all rat tissues examined. As with ERK1, ERK2 is present in greatest amounts in brain and spinal cord, but is barely detectable in liver, lung, muscle, and intestine. Like ERK1, ERK2 also appears to be predominantly soluble. Very little ERK2 is in the particulate fraction, notably in tissues such as testes, in which considerable ERK1 is associated with the particulate fraction. A few of the weakly reacting bands (especially in muscle), of similar size to ERK2, appear to interact nonspecifically, because their signal is not blocked by the peptide antigen (Figure 4).

Using these antibody probes, we found no striking differences in the distributions of ERK1 and ERK2. The proteins are expressed in tissues roughly in proportion to the expression of their mRNAs (Figure 5 and Boulton *et al.,* 1990b; Boulton, *et al.,* 1991b). Northern analysis of ERK1 and ERK2 demonstrated that the messages for these two enzymes are present in all tissues examined although in very low amounts in some tissues. ERK1 mRNA was most highly expressed in spinal cord and then brain, followed by intestine and lung. ERK2 mRNA was highly expressed in brain and then spinal cord, followed by muscle and heart. The parallel between mRNA and protein is weakest in intestine for ERK1 and in muscle for ERK2; in both cases less protein is present than expected.

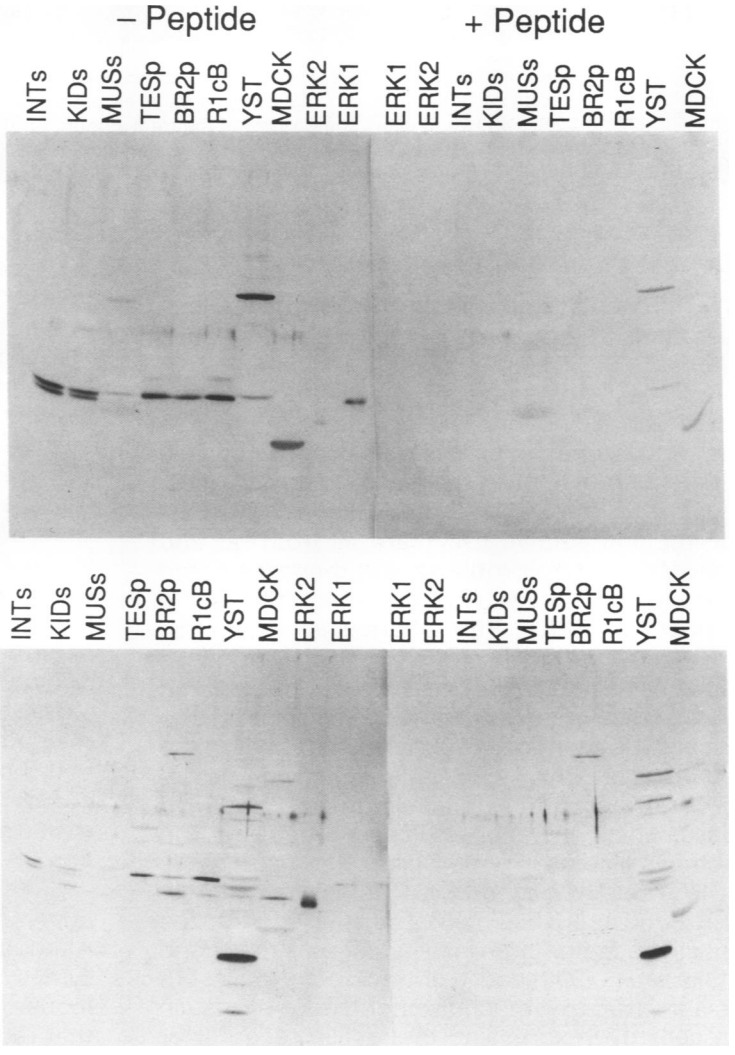

Figure 4. **Effect of peptide antigen on immunoreactivity of ERK proteins.** Indicated samples (100 μg) from tissues or cell lines were analyzed in duplicate as described in the legend to Figure 1. One set was probed as in Figure 1, whereas the duplicate was probed with antibody that had been preincubated with peptide. The upper panel shows the blot probed with 956 antiserum without (left) and with (right) 500 μM peptide. The lower blots are using 837 without (left) and with (right) 250 μM peptide.

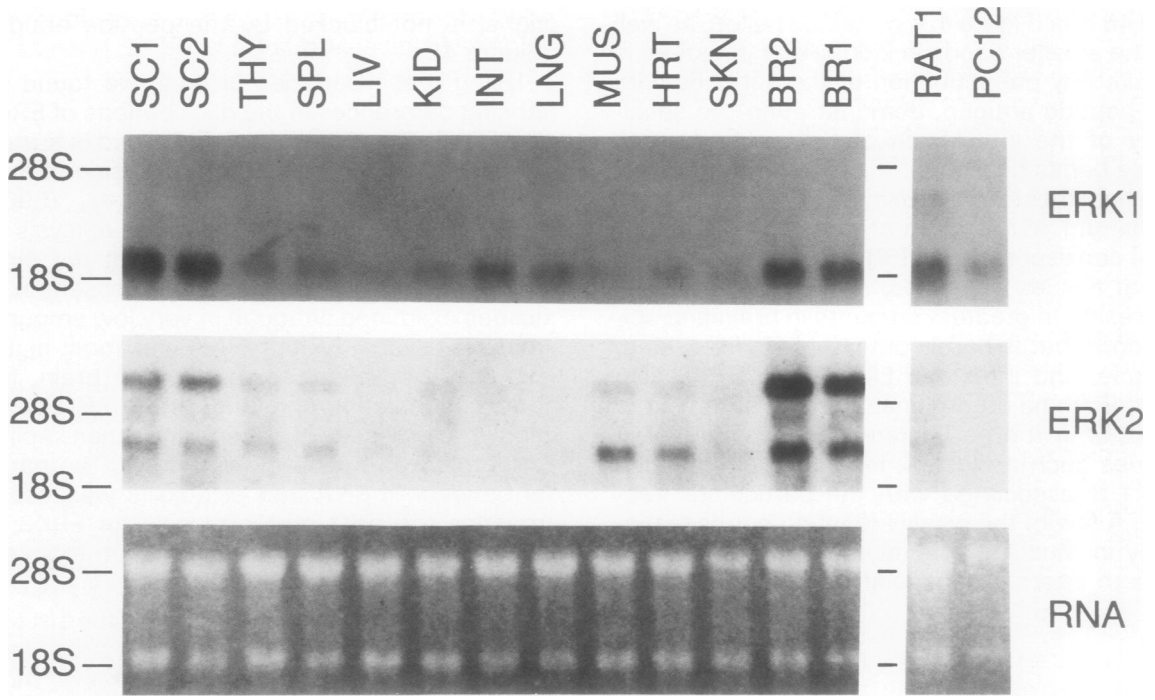

Figure 5. **Expression of ERK1 and ERK2 transcripts in rat tissue and cell lines.** ERK1- or ERK2-specific DNA fragments as described in Methods or Boulton *et al.* (1990b) were used as probes in the analysis of mRNA expression. The ethidium bromide-stained gel is shown to allow comparison of the total amount of RNA per sample. SC1, postnatal day 19 spinal cord; SC2, adult spinal cord; Thy, thymus; Spl, spleen; Liv, liver; Kid, kidney; Int, intestine; Lng, lung; Mus, muscle; Hrt, heart; Skn, skin; BR2, adult brain; BR1, postnatal day 10 brain; Rat 1, rat 1 fibroblast; PC12, pheochromocytoma cells.

Expression of ERKs in cell lines

We immunoblotted soluble protein from various cell lines (Figure 3) to compare the relative amounts of ERK1 and ERK2 and to identify cell lines that express large amounts of either or both of the two kinases for functional studies as well as ones expressing little that might be useful as transfection recipients. Of the cell lines evaluated, those that express highest amounts of immunoreactive ERK1 are all from rat and hamster. Rat 1 fibroblasts and the transfected line HIRc B have approximately the same amount of ERK1. This addresses a question raised by our earlier observation that insulin does not activate the enzyme in untransfected Rat 1 cells but does activate the enzyme in HIRc B cells (Boulton *et al.*, 1991a). Differences in the amount of ERK1 cannot account for its increased activation by insulin in Rat 1 HIRc B cells, which overexpress insulin receptors. The same is also true in the Chinese hamster ovary (CHO) cell lines examined. The enzyme is present equally in CHO cells transfected with the insulin receptor or an insulin receptor-ros hybrid (Ellis *et al.*, 1987) and in untransfected cells, but is activated to a much greater extent by insulin in cells overexpressing the insulin receptor or the receptor hybrid (Boulton *et al.*, 1990a). The greater extent of activation of ERK1 by insulin in the transfected cell lines must be due to increased amounts of the receptor, rather than to an increase in mass of ERK1 that might have resulted from receptor overexpression.

The CHO cell lines contain similar amounts of ERK2, although the IRros line appears to express slightly more ERK2 than the other two lines. Both ERK1 and ERK2 are easily detectable in the PC12 cell line. These cells express the most ERK1 of the cell lines examined. The rat osteosarcoma cell line has detectable amounts of both ERK1 and ERK2 but less than the other rat lines.

The largest (~45 kDa) of the three ERKs is recognized only in the rat cell lines. The amount of this protein is greater in the Rat 1 HIRc B line compared with the parent Rat 1 line. Even more of this protein is found in the PC12 soluble fraction.

ERK1 is detectable in mouse cell lines but in apparently smaller amounts than in the rat lines. Although it is likely that the mouse and rat enzymes are nearly identical, it is possible that mouse ERK1 contains sequence differences that reduce its recognition by both of the anti-

bodies. This might account for the finding that cells from rat and hamster appear to contain more of the ERKs than cells from other species. Thus, it is feasible to compare relative amounts only in cells and tissues from the same species. Among the mouse lines examined (3T3-L1, LA90, L, NG108), approximately equal amounts of ERK1 protein were recognized by 956 in all except NG108 (a mouse × rat hybrid), which contained slightly more immunoreactive protein. ERK2 may be present in these mouse lines at higher concentrations than ERK1.

Human cell lines were also examined with these two antibodies. Antiserum 837 recognized both proteins in all of the lines, with the predominant protein being ERK2, especially in A431 cells. A third band that was smaller (~30 kDa) than either ERK1 or ERK2 was recognized by 956. Antiserum 691 recognized the human proteins more effectively than antisera 956 or 837 (data not shown), suggesting that the peptide for the subdomain used to generate 691 may be more highly conserved across species than the C-terminus. The sequence of the human ERK1 cDNA (Geppert, Whitehurst, Owaki, Duby, Boulton, and Cobb, unpublished observation) confirms this prediction.

Only antiserum 837 recognizes an ERK protein in soluble extracts of chicken embryo fibroblasts. This protein is slightly larger than the rat, mouse, or hamster ERK2. Similarly, when dog kidney cells are blotted with the two antisera, only 837 recognizes a protein, which is of the same size as rodent ERK2. Interestingly, numerous bands in extracts of *Saccharomyces cerevisiae* are weakly recognized by both antisera, but the detection of only one faint band of ~35 kDa is blocked with peptide antigen (Figure 4).

Extracts from Cos, 293, and SF9, cell lines that are commonly used for expression of heterologous proteins, were also blotted. Only 293 cells expressed detectable ERK1, whereas both Cos and 293 contained significant amounts of ERK2. No bands were recognized in SF9 cells. Although the antibodies may not recognize ERKs from an insect, there is an immunoreactive protein detected in frog. A MAP2 kinase partially purified from *Xenopus* (Ferrell *et al.,* 1991; Ferrell, unpublished data) was recognized by antiserum 837 but not by 956, suggesting that it was more similar to ERK2 than ERK1. This protein appeared to be intermediate between ERK1 and ERK2 in size. Subsequent cloning of the *Xenopus pp42* demonstrates that it has greater sequence identity to ERK2 than ERK1, although it is very similar to both enzymes (Posada *et al.,* 1991). Whether or not pp42 recognized in other contexts is also either identical to or most closely related to ERK2 has yet to be determined.

Subcellular distribution of ERKs after insulin treatment

Activation of MAP2/MBP kinase proceeds rapidly and begins to decay after 5 min in Rat 1 HIRc B cells (Figure 6). To determine whether the loss of stimulated kinase activity from supernatants with time was due to an activation-dependent redistribution of ERK1 and/or ERK2 within the cell, we measured MAP2 kinase activity in soluble and particulate fractions from Rat 1 HIRc B cells treated with insulin for different times. We also probed these fractions with antiserum 837. MAP2 kinase activity in the soluble fraction increased >8-fold with 5 min of exposure to insulin (Figure 6, upper panel), then slowly declined. A small (~50%) increase in MAP2 phosphorylating activity was found in the particulate fraction. This increase was less pronounced in other experiments. Immunoblotting these two subcellular fractions indicated that there was no correlation between the amount of stimulated activity and changes in the amount of ERK1 or ERK2 in the soluble fraction. There may have been a slight decrease in the soluble ERK1 and ERK2 recognized by the antibody after the cells were treated with insulin for 15 min. However, blottable protein mass was at the unstimulated level after 30 min. The particulate fraction contained predominantly ERK1, but both ERK1 and ERK2 protein appeared to increase slightly in the particulate fraction after 15 min of treatment with insulin. This increase might account in part for the small stimulation of particulate MAP2 kinase activity at this time. The amounts of ERK1 and ERK2 in this fraction returned to unstimulated levels by 30 min. There was little evidence for a significant redistribution of ERKs; therefore, loss of MAP2 kinase activity from the cytosol was not due to an activation-induced association of the enzyme with membrane-bound organelles, consistent with previous findings (Anderson *et al.,* 1990; Gómez *et al.,* 1990; Ahn *et al.,* 1991) that loss of activity required desphosphorylation.

Chromatographic resolution of ERKs from cell extracts on Mono Q

Supernatants (prepared according to Methods) of insulin-stimulated and unstimulated Rat 1 HIRc B cells have been chromatographed on Mono Q. Analysis of activities on Mono Q was used to obtain information regarding the following questions. First, does insulin stimulate both

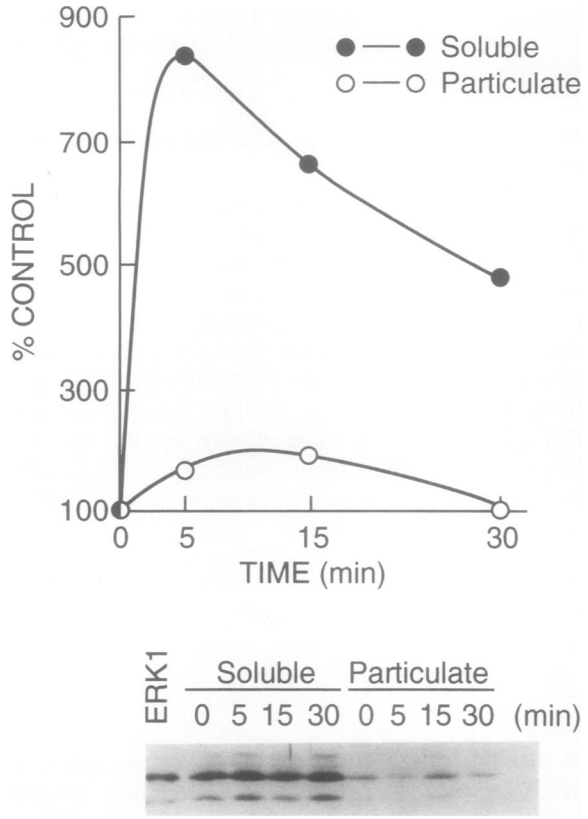

Figure 6. **Subcellular distribution of ERK1 and ERK2 after treatment with insulin.** Rat 1 HIRc B cells were treated with 10^{-8} M insulin for the indicated times. Soluble and particulate fractions (100 μg) were electrophoresed on a 10% polyacrylamide gel in sodium dodecyl sulfate, transferred to nitrocellulose, and probed with antiserum 837 (lower panel). These fractions were also assayed for MAP2 kinase activity, indicated in the upper panel as percentage of control. Basal activities were 7 pmol · min^{-1} · mg^{-1} protein for the supernatant fraction and 69 pmol · min^{-1} · mg^{-1} protein in the particulate fraction.

ERK1 and ERK2? Second, are the two major peaks of stimulated MBP kinase activity due to multiple forms of ERK1 or to distinct kinases or both? Third, where do inactive ERK1 and ERK2 elute? Fourth, does stimulation by insulin alter the chromatographic behavior of ERK1 and/or ERK2? And finally, can ERK2 be resolved from ERK1 on Mono Q for futher enzymatic characterization?

As with MAP2/MBP kinases in other cells (Hoshi *et al.,* 1988; Ahn *et al.,* 1990; Gómez *et al.,* 1990; Nel *et al.,* 1990b), the MAP2/MBP protein kinases activated by insulin in Rat 1 HIRc B cells can be resolved into two distinct peaks of kinase activity on Mono Q (Figure 7). By the use of a shallow gradient, the two peaks can be separated by several fractions, with the first peak of stimulated kinase activity eluting in fractions 33–38 (conductivities of 11.25–13 mS) and the second peak of stimulated activity eluting in fractions 45–51 (conductivities of 14–16 mS). There was little or no MBP kinase activity in the Mono Q profile of soluble proteins from unstimulated cells.

Immunoreactive ERK1 from insulin-treated cells, detected using a mixture of antisera 837 and 956, also eluted from the column in two peaks, the first in fractions 30–34 and the second in fractions 44–52 of the stimulated profile (Figure 7). The second peak of ERK1 clearly comigrated with the second peak of stimulated kinase activity. However, the first peak of ERK1 eluted slightly earlier (fractions 30–34) than the first peak of protein kinase activity (fractions 33–38) but eluted in the same fractions as the protein (fractions 30–38) from the unstimulated cells. Thus, from this analysis alone, it was not clear whether ERK1 contributed to the first stimulated peak of kinase activity. Blotting similar Mono Q profiles with antiserum 956, the ERK1-selective antibody, showed that the 43-kDa band was the only immunoreactive protein in fractions 30–34 and 44–52, supporting the claim that the 43-kDa band is ERK1. In the profile from unstimulated cells all of the ERK1 eluted in fractions 30–36, indicating that on activation the portion of ERK1 corresponding to the second protein peak bound more tightly to Mono Q. Together the two peaks of ERK1 in the stimulated profile contained roughly the same amount of immunoreactive ERK1 as in the single peak in the unstimulated profile, suggesting that the majority of the immunoreactive protein is in these fractions and not elsewhere in the column profile.

ERK2 also eluted as one peak just ahead of ERK1 in the unstimulated profile (conductivities of 8–11.7 mS) in fractions 22–32, with fractions 28–30 having the most immunoreactive ERK2 (Figure 7). On stimulation of the cells with insulin, the elution of a portion of the ERK2 protein was altered, appearing in fractions 26–36 (8.5–13 mS), with the majority of immunoreactivity now in fractions 32–34. On the basis of its elution properties, ERK2 did not contribute to the second peak of kinase activity; however, it eluted with and, therefore, may have accounted for the majority of enzymatic activity in the first stimulated peak. Although it cannot be excluded that the MBP kinase activity in peak 1 is due to an unidentified enzyme, ERK2 is an MBP kinase based on data with the recombinant protein (Boulton *et al.,* 1991b). The fact that a portion of the 41-kDa ERK2 protein shifts its elution after insulin treatment suggests that ERK2 is modified in a manner similar to ERK1. Further-

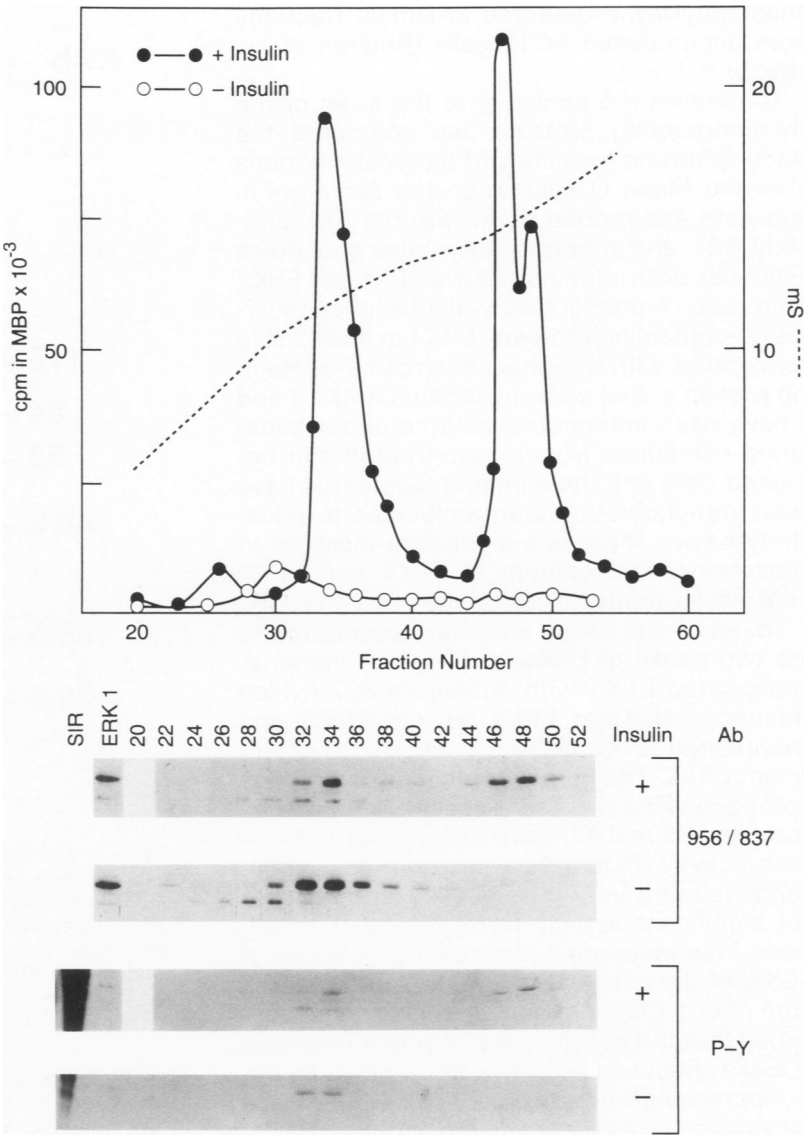

Figure 7. **Chromatography of Rat 1 HIRc B supernatants on Mono Q.** Soluble protein from cells with (+) or without (–) treatment with 10^{-8} M insulin were chromatographed on Mono Q and eluted with a gradient of 0–0.225 M NaCl in buffer (see Methods). MBP kinase activity is plotted in the upper panel. Even-numbered fractions between 20 and 60 plus fractions 33, 35, 37, 45, 47, 49, and 51 were assayed in the stimulated profile. The indicated fractions were precipitated with trichloroacetic acid and resuspended in electrophoresis sample buffer. One-third of the precipitated protein was subjected to electrophoresis for subsequent Western blotting, with antisera 956 plus 837. The remainder was electrophoresed on a second gel and then transferred to nitrocellulose for blotting with antibodies to phosphotyrosine (P-Y). SIR indicates an aliquot of an insulin receptor fragment that has been autophosphorylated and used as a positive control for tyrosine phosphate containing protein (Cobb *et al.*, 1989).

more, the fact that the ERK2 protein that is shifted comigrates with the peak of activity is additional evidence that this peak is due to active ERK2.

Immunoblotting of these fractions with antibodies to phosphotyrosine revealed that, in the insulin-stimulated profile, proteins that comigrated with the second peak of ERK1 protein (fractions 46–50) and the later eluting ERK2 protein (fractions 32–35) contained phosphotyrosine, suggesting that this modification is in part responsible for their retarded elution from Mono Q and the resulting increase in activity. Thus, phosphorylation of the enzymes caused by insulin increased their binding to Mono Q, and apparently the phosphorylated forms of ERK1 and ERK2 accounted individually for the two peaks of kinase activity. However, there was phosphotyrosine on a portion of ERK1 in the profile from stimulated cells (fraction 34) that overlapped in elution with the first peak of kinase activity, making it difficult to determine whether one or both ERKs contributed to this peak of activity. When the profile from unstimulated cells was blotted with antibodies to phosphotyrosine, a phosphotyrosine-containing, 43-kDa protein, which comigrated with the ERK1 standard, was present in fractions 32–34. These same fractions contained the majority of inactive ERK1 as detected with antisera 956 and 837. The basal phosphotyrosine on the 43-kDa protein may be a consequence of the overexpression of the insulin receptor (~10^6/cell) in the HIRc B line. There was very little or no

phosphotyrosine detected in similar fractions from unstimulated PC12 cells (Boulton *et al.*, 1991b).

To confirm the similarity in the sizes of the immunoreacting proteins, we compared the electrophoretic mobilities of the peak fractions from the Mono Q column on the same gel in duplicate and probed them with the ERK antibody, 691, and antiphosphotyrosine antibodies (Figure 8). Both immunoreactive ERK1 and ERK2 from peak 1 comigrated with the phosphotyrosine-containing proteins. ERK1 in peak 2 also comigrated with the phosphotyrosine-containing protein in that peak. In addition, ERKs 1 and 2 have been immunoprecipitated under denaturing conditions from insulin-treated and untreated cells and the immunoprecipitates have been immunoblotted with antibodies to phosphotyrosine. There is a significant increase in phosphotyrosine content in ERK1 and ERK2 from insulin-treated cells (Boulton *et al.*, 1991b).

To determine which enzymes contributed to the two peaks of kinase activity, we immunoprecipitated ERK1 with antiserum 837 (which immunoprecipitates ERK1 but not ERK2) and precipitated ERK2 with an antibody to recombinant ERK2. The immunoprecipitated proteins from each of the two peaks of stimulated activity (fractions 35 and 47) were assayed for MBP kinase activity (Figure 9). Antiserum 837 immunoprecipitated very little activity from peak 1 but significant activity from peak 2. Furthermore, immunoprecipitation resulted in a loss of >85% of the activity from peak 2 but little loss from peak 1 (data not shown). This finding confirmed that the second peak of activity was due to ERK1. Because no active ERK1 could be immunoprecipitated from peak 1, the MBP kinase activity was probably not due to activated ERK1. Antibody to recombinant ERK2 immunoprecipitated a small amount of MBP kinase activity from the first peak but none from the second peak. The efficiency of immunoprecipitation of this antibody is poor as determined from its ability to precipitate only a small fraction of autophosphorylated recombinant ERK2 protein. Thus, it cannot be used to ascertain whether removal of ERK2 from peak 1 removes all MBP kinase activity.

Apparently only a portion of the ERK1 is activated by insulin under these conditions. The delayed elution of activated ERK1 may require further modifications, perhaps phosphorylation on serine/threonine and/or additional tyrosine residues (see Figure 10). Because the first peak contained tyrosine-phosphorylated ERK1 but very little precipitable ERK1 activity, it may be that the ERK1 in this peak lacked serine/threonine phosphate. These other phosphorylations may activate the protein on stimulation of cells with insulin. Because ERK1 contained phosphotyrosine but was inactive in untreated cells, phosphorylation on serine/threonine, not tyrosine residues, may be limiting in the activation of ERK1 in Rat 1 cells.

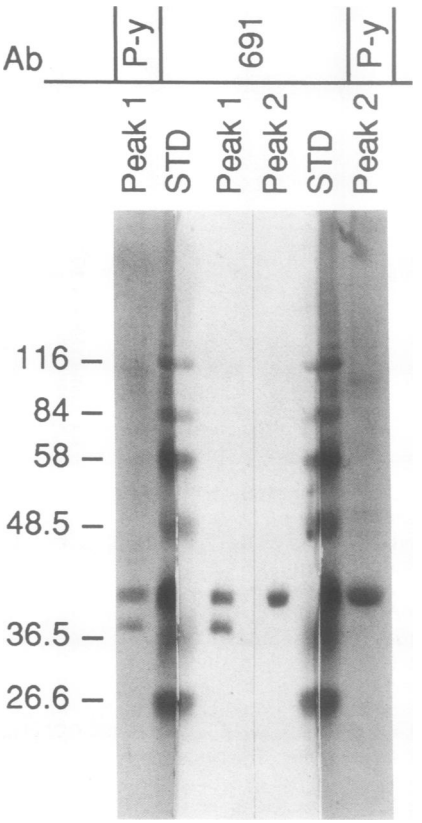

Figure 8. **Comparison of electrophoretic mobility of immunoreacting proteins.** Two fractions from each peak of MBP kinase activity were pooled and precipitated as in Figure 7. One-fourth of the protein was probed with antiserum 691 and the remainder was probed with antiphosphotyrosine antibodies (P-Y).

Altogether five observations lead us to suggest that the first peak of activity is derived from active ERK2. First, the later eluting ERK1 protein comigrates with the second peak of activity and, similarly, the later eluting portion of the ERK2 protein comigrates with the first peak of activity. Second, the first peak of ERK1 from stimulated cells does not exactly comigrate with the first peak of activity. In fact, the first peak of ERK1 protein in the stimulated profile and the peak of ERK1 protein in the unstimulated profile elute in nearly the same fractions. Third, phosphotyrosine is present in the later eluting fractions of ERK1 and ERK2, those that coelute with the peaks of activity. Fourth, on the basis of im-

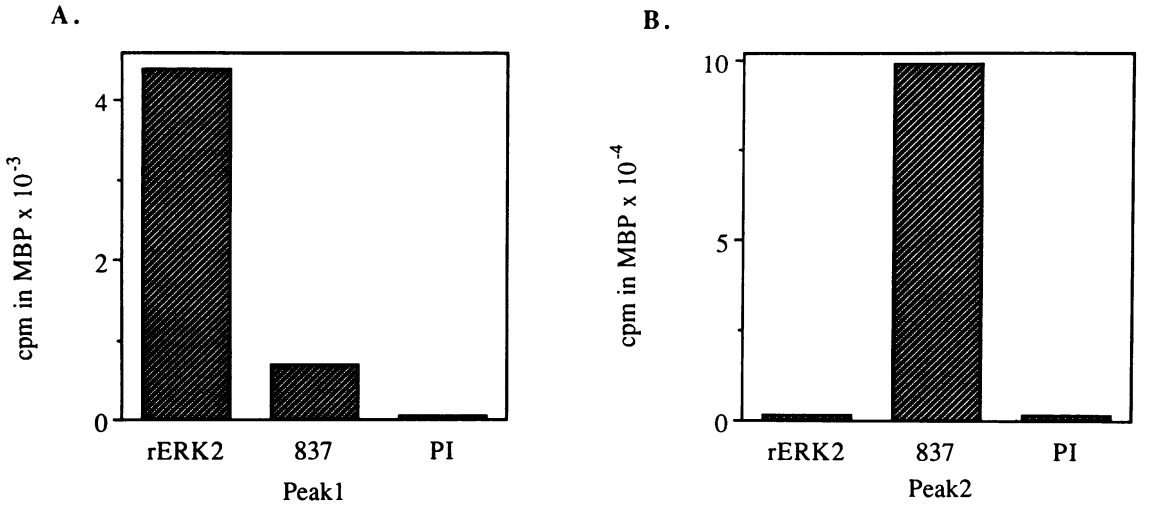

Figure 9. **Immunoprecipitation of MBP kinase activity from Mono Q fractions.** Aliquots (50 μl) of each peak fraction from the insulin-treated profile described in Figure 7 were immunoprecipitated and assayed for MBP kinase activity. Counts per minute in MBP phosphorylated by proteins in the immune complexes using preimmune serum (PI), 837, or antibody to recombinant ERK2 (rERK2) as the precipitating antibody. (A) Activity immunoprecipitated from peak 1. (B) Activity immunoprecipitated from peak 2.

munoprecipitation, ERK1 does not contribute to the first peak of activity but some activity can be immunoprecipitated with ERK2 antibodies. Fifth, recombinant ERK2 has a substrate specificity similar to ERK1 (Boulton *et al.,* 1991b). Thus, on the basis of indirect evidence, the immune complex data, activity of recombinant ERK2, and the correlations of protein mass, phosphotyrosine content, and kinase activity noted above, it is likely that peak 1 represents the active form of ERK2. Proof will await antibodies that immunoprecipitate this kinase more efficiently.

Properties of ERK1 and ERK2

We characterized certain properties of the two peaks of activity to make an initial comparison of the two enzymes. The activity in peak 1, which is probably due to ERK2, phosphorylated MBP 2.8-fold better than MAP2, unlike recombinant ERK2, which phosphorylates both substrates at roughly equal rates. ERK1 (peak 2) phosphorylated MBP 2.2-fold better than MAP2 (Figure 10A), consistent with data for the purified protein (Boulton *et al.,* 1991a). Ahn *et al.* (1990) have found two peaks of EGF-stimulated MBP kinase, chromatographically similar to the insulin-stimulated peaks identified here, which contain proteins that immunoreact with our antibodies (Ahn *et al.,* 1991; and data not shown). On the basis of chromatographic behavior and sizes of crossreacting bands, Peak E3 in that report is most likely ERK2 (our peak 1) and peak E4 is most likely ERK1 (our peak 2). Both peaks of MBP kinase activate an S6 peptide kinase (Ahn and Krebs, 1990), also demonstrating the similarity in substrate specificities of the two enzymes. An unusual property of ERK1 is its ability to use either Mg^{2+} or Mn^{2+} as a source of divalent cation (Boulton *et al.,* 1991a). Fractions containing ERK1 and ERK2 from the Mono Q profile were assayed in the presence of increasing concentrations of either Mg^{2+} or Mn^{2+}. Like ERK1, the other peak of kinase activity had the ability to use either cation; the concentration dependence for both ions was virtually identical for both enzymes; maximum activity was exhibited at 10–15 mM (Figure 10B).

To compare the regulation of the two enzymes, each was treated with specific phosphatases. Both peaks of activity were inactivated by treatment with the serine/threonine-selective phosphatase 2a (>85%; Figure 10C) and to a lesser extent with the tyrosine-specific phosphatase, CD45 (~70%; Figure 10C). This has been noted for other partially purified preparations of MAP2 kinases (Anderson *et al.,* 1990; Gómez *et al.,* 1990; Ahn *et al.,* 1991). These results suggest that, for each enzyme, phosphorylation on both tyrosine and threonine and/or serine is required for full activity and that the phosphotyrosine-containing 41-kDa protein is the kinase that accounts for the MBP kinase activity in the first peak. On the basis of these three properties, substrate specificity, cation dependence, and regulation by multiple phosphorylations, these two peaks

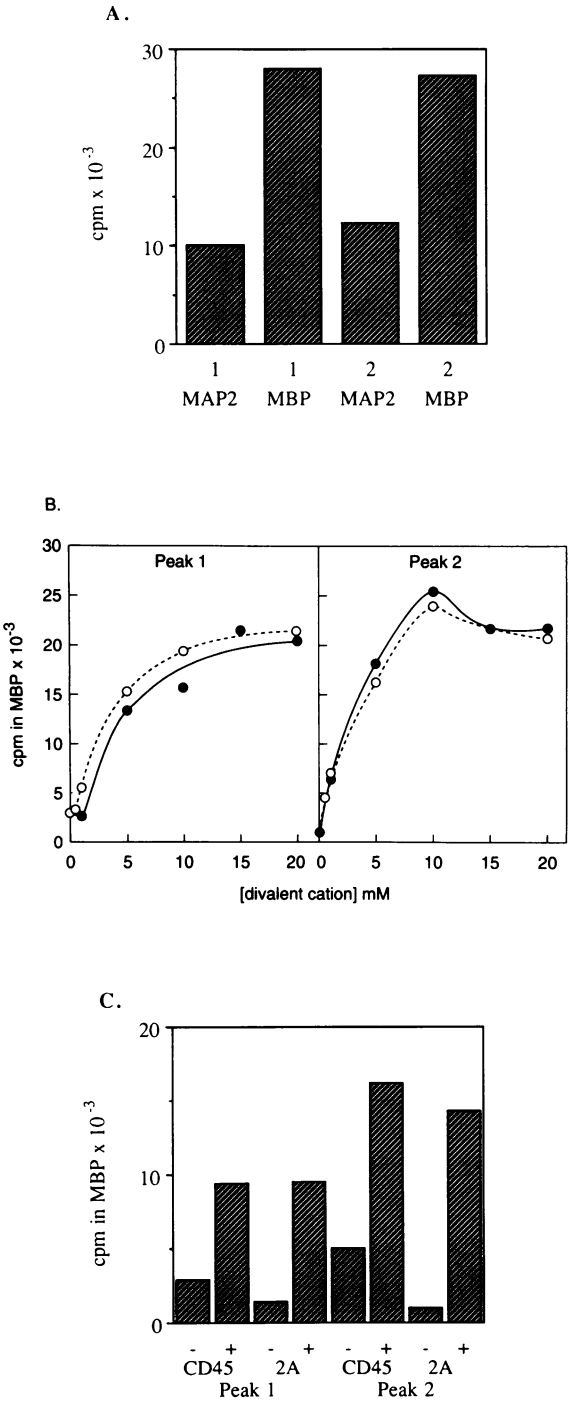

Figure 10. **Comparison of enzymatic properties of enzymes in peak 1 and peak 2.** (A) Peak 1 (1) or peak 2 (2) were assayed for their ability to phosphorylate either MAP2 or MBP, both at a final concentration of 0.3 mg/ml. (B) Peak 1 and peak 2 were assayed with increasing concentrations of the divalent cations Mg^{2+} (●) or Mn^{2+} (○). Phosphorylation of MBP is shown. (C) Peak 1 or peak 2 was incubated with the indicated phosphatase, CD45 or 2A, 60 min in the absence (–) or presence (+) of inhibitor. One millimolar sodium vanadate was used to inhibit CD45 and 10 mM sodium phosphate for 2A. Activity with MBP after treatment as above is shown.

of MBP kinase activity most likely represent the highly related enzymes, ERK1 and ERK2.

Discussion

The results of these studies indicate that both ERK1 and the related ERK2 are widely distributed in cells and tissues. They are conserved sufficiently well across species to be recognized by antibodies to small peptide epitopes. At least two of the ERKs are activated by both insulin in Rat 1 HIRc B cells and nerve growth factor in PC12 cells (Boulton *et al.*, 1991b), indicating that neither of these is regulated exclusively by a single extracellular signal in a single cell type. Thus, at least two enzymes, ERK1 and ERK2, appear to contribute to the observed stimulation of MAP2/MBP kinase and the increases in phosphotyrosine in proteins in the size range of 40–45 kDa elicited by growth factors.

Stimulation shifts the elution of only a portion of each protein. Because not all of each protein in the activated profile migrates with the activity, not all the ERK1 or the ERK2 is activated under the conditions used. The shift in elution of ERK1 on activation is consistent with the findings of Ahn *et al.* (1990) that activation of a 42-kDa MAP2/MBP kinase alters its elution from Mono Q. We have made similar observations as to the migration and antiphosphotyrosine immunoreactivity of ERK1 and putative ERK2 in PC12 cells treated with nerve growth factor (Boulton *et al.*, 1991b).

Phosphorylation of these ERKs on tyrosine is required, but not sufficient, for maximum activity. ERK1 appears to exist in stimulated cells in at least three forms, two of which are inactive: unphosphorylated (by which we mean lacking the activating phosphorylations, some of which must occur on serine/threonine residues) and inactive, tyrosine phosphorylated and inactive, and tyrosine phosphorylated and active, presumably due to the acquisition of critical serine/threonine and perhaps additional tyrosine phosphorylations. In unstimulated Rat 1 HIRc B cells, but not in unstimulated PC12 cells, there is also a major component of inactive ERK1 that is tyrosine phosphorylated. Because a population of ERK1 is tyrosine phosphorylated but inactive, this form of ERK1 either lacks the serine/threonine phosphate that activates it, has accumulated phosphate on tyrosines that are functionally silent, or both. Direct evidence that both ERK1 and ERK2 are regulated by phosphorylation on both tyrosine and serine/threonine comes from the demonstration that both are inactivated with phosphatases that remove either serine/threonine or tyrosine phosphates

and the ERK1 immunoprecipitated from nerve growth factor- or insulin-treated cells contains phosphotyrosine, phosphothreonine, and phosphoserine (Boulton *et al.,* 1991b).

A further implication of the results of immunoblotting the chromatographic profiles is that serine/threonine phosphorylation is the rate-limiting step in the activation of ERK1 and perhaps ERK2 by extracellular signals. Both insulin and nerve growth factor (Boulton *et al.,* 1991b) cause an increase in phosphotyrosine content of ERK1 protein that is not active. And, in fact, in Rat 1 HIRc B cells, some ERK1 contains phosphotyrosine in the absence of the stimulus. The large increase in MBP kinase activity induced in certain cell types by okadaic acid (Haystead *et al.,* 1990), an inhibitor of serine-threonine phosphatases (Haystead *et al.,* 1989), is consistent with this notion. Additional studies of the time courses of phosphorylation of these proteins and their responses to a variety of pharmacological agents may further elucidate the mechanisms of their activation.

Methods

Cells and tissues

Rat tissues and cultured cells (treated or not with growth factors as described in Boulton *et al.,* 1991a) were homogenized by douncing (cells) or using a polytron sonifier (tissues) in 20 mM p-nitrophenylphosphate, 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 50 mM sodium fluoride, 5 mM benzamidine, 50 μM sodium vanadate, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10^{-7} M pepstatin. After sedimentation at 2000 × *g* for 10 min, supernatants were further sedimented for 1 h at 100 000 × *g* to yield soluble and particulate fractions. Rat 1 HIRcB cells were obtained from D. McClain (University of Alabama, Birmingham, AL); LA90 cells expressing a temperature-sensitive form of pp60 v-*src* were obtained from O. Rosen (Sloan-Kettering Memorial Cancer Center, New York, NY); and the OST and 1321N1 lines were obtained from J. Perkins (University of Texas Southwestern, Dallas, TX). A preparation from *Xenopus laevis* enriched in MAP2 kinase was obtained from J. Ferrell (University of Wisconsin, Madison, WI)..

Northern analysis

RNA isolation, Northern blotting, ^{32}P-radiolabeling by polymerase chain reaction (PCR) and hybridization to labeled probes were performed as described in Maisonpierre *et al.* (1990a). The DNA probe used for ERK2 was a 300-bp fragment from nucleotide 1122 to 1422 generated by amplification using specific primers and the cDNA as the template in a PCR reaction (Boulton *et al.,* 1991b). The ERK1 probe was as described in Boulton *et al.* (1990b).

Antibodies and immunoblotting

The peptide IFQETARFQPGAPEAP was synthesized by Lynn DeOgny and Clive Slaughter and consisted of one peptide species as determined by mass spectrometry. The other two peptides (see Figure 1) were the kind gifts of Natalie Ahn and Edwin Krebs (University of Washington, Seattle, WA) and were >90% pure by HPLC analysis. The peptides, coupled to hemocyanin from *Limulus polyphemus* with glutaraldehyde, were used to immunize rabbits at 2- to 4-wk intervals. Immunospecificity was determined by preincubating the peptide antibodies with 250 (for 837) and 500 (for 956) μM peptide. Proteins immunoreacting with antisera 837 and 956 were detected using peroxidase-conjugated goat antirabbit IgG (Cappel, Durham, NC). Immunodection of proteins containing phosphotyrosine employed a rabbit polyclonal antibody described by Boulton *et al.* (1991a) and immunoreacting proteins were visualized by the use of peroxidase-conjugated or alkaline phosphatase-conjugated secondary antibody. Prestained protein standards were from Sigma (St. Louis, MO). A polyclonal antiserum to recombinant ERK2 was the kind gift of Regeneron Pharmaceuticals (Tarrytown, NY) and will be described more fully elsewhere.

Immune complex kinase assay

Aliquots (50 μl) from the Mono Q fractions were incubated with 4 μl of antiserum 837, antibody to recombinant ERK2, or preimmune serum for 2 h on ice. Prewashed Pansorbin (Calbiochem, San Diego, CA) was then added for 1 h on ice. Immunoprecipitated protein was recovered by sedimentation in a microcentrifuge for 2 min. The material not precipitated was set aside for later assay. The immunoprecipitates were washed two times with 0.25 M Tris, pH 7.6, and 0.1 M NaCl and finally resuspended in 50 μl of 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 8. The immunoprecipitated kinase and the unprecipitated material were assayed by adding an equal volume of a 2× reaction mixture giving final concentrations of 50 μM [γ-^{32}P]ATP (4400 cpm/pmol), 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 0.3 mg/ml MBP, and 25 mM HEPES, pH 8.0, and incubated at 30°C for 30 min. The assays were terminated by removing the supernatants from the pelleted immune complexes and precipitating MBP in the assays with trichloroacetic acid.

Chromatography

Supernatants (~10 mg protein) were diluted threefold with water and applied to a Mono Q HR 5/5 column in 50 mM β-glycerol phosphate, pH 7.3, 0.1 mM sodium vanadate, 1 mM EGTA, and 0.1 μM pepstatin. Protein was eluted with a 60-ml gradient of 0–0.225 M NaCl in the buffer. One-milliliter fractions were collected. Fractions used for immunoblotting were precipitated with trichloroacetic acid as described in Boulton *et al.* (1991a).

Inactivation with phosphatases

A fraction from each of the two stimulated peaks of kinase activity from a Mono Q profile was exchanged into 25 mM HEPES, pH 7.5, 1 mM DTT, and 1 mM EDTA by chromatography on Sephadex G-50. Ten microliters of the exchanged kinases were incubated in 0.1% β-mercaptoethanol, 7.7 mg/ml bovine serum albumin, and either 98 U/ml CD45 (kindly provided by E. Fischer and N. Tonks, University of Washington, Seattle, WA) or 13 μg/ml of the catalytic subunit of phosphatase 2A (kindly provided by M. Mumby, University of Texas Southwestern, Dallas, TX). The dephosphorylation reactions were performed for 60 min at 30°C in the absence or presence of the appropriate phosphatase inhibitor; for CD45 1 mM sodium vanadate was added and for the reactions with phosphatase 2A 10 mM sodium phosphate was added. After incubation with the phosphatases, these amounts of inhibitors were added to stop the dephosphorylation reactions. The ability to phosphorylate

MBP was then measured (Boulton *et al.,* 1991a) in 50-μl reactions containing an additional 5 mM sodium phosphate.

Other methods

Assay for MAP2/MBP kinase activity was described previously (Boulton *et al.,* 1991a). ERK2 used as a standard was the recombinant protein expressed in *E. coli* using the second in-frame methionine to initiate as described in (Boulton *et al.,* 1991b). Other reagents and methods were as described (Boulton *et al.,* 1991a).

Acknowledgments

We thank Natalie Ahn and Edwin Krebs (University of Washington, Seattle, WA) for synthesis of two peptides; Natalie Ahn, Rony Seger, Edwin Krebs (University of Washington), James Ferrell (University of Wisconsin, Madison, WI), and Jonathan Cooper (Hutchinson Cancer Research Center, Seattle, WA) for communication of data before publication; Nikos Panayotatos (Regeneron Pharmaceuticals, Tarrytown, NY) for bacterial extracts containing ERK2; Steven Nye (Regeneron Pharmaceuticals) for antibody to recombinant ERK2; Leonardo Belluscio (Regeneron Pharmaceuticals) for preparing RNA; David Robbins for critical reading of the manuscript; Colleen Vanderbilt for technical assistance; and Jo Hicks for preparation of the manuscript.

Received: January 22, 1991.
Revised and accepted: March 27, 1991.

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