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Extracellular Signal-Regulated Kinases 2 Autophosphorylates on a Subset of Peptides Phosphorylated in Intact Cells in Response to Insulin and Nerve Growth Factor: Analysis by Peptide Mapping

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The phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) in response to insulin in Rat 1 HIRc B cells and in response to nerve growth factor (NGF) in PC12 cells has been examined. ERK1 and ERK2 are phosphorylated on serine in the absence of the stimuli and additionally on tyrosine and threonine residues after exposure to NGF and insulin. NGF stimulates tyrosine phosphorylation of ERK1 more rapidly than threonine phosphorylation. Two-dimensional phosphopeptide maps of both ERK1 and ERK2 phosphorylated in intact cells treated with NGF or with insulin display the same three predominant phosphopeptides that comigrate when digests of ERK1 and ERK2 are mixed. As many as five additional phosphopeptides are detected under certain conditions. Autophosphorylated recombinant ERK2 also contains the three tryptic phosphopeptides found in ERKs labeled in intact cells. These experiments demonstrate that ERK1 and ERK2 are phosphorylated on related sites in response to two distinct extracellular signals. The data also support the possibility that autophosphorylation may be involved in the activation of the ERKs.

INTRODUCTION

Increased phosphorylation of ribosomal protein S6 is one of the best documented consequences of insulin and growth factor action. As such, it has proven a valuable system in which to search for protein kinases that mediate the effects of growth factors and cytokines and has provided a paradigm for the study of mechanisms underlying the early steps in signal transduction (Rosen *et al.*, 1981; Erikson, 1991). Two protein kinases that act sequentially in this pathway, an S6 kinase and an S6 kinase kinase, have been identified (Sturgill *et al.*, 1988; Gregory *et al.*, 1989; Ahn and Krebs, 1990). The S6 kinase kinase is an insulin- and growth factor-stimulated enzyme distinguished by its ability to phosphorylate microtubule-associated protein-2 (MAP2) and myelin basic protein (MBP) in vitro (Ray and Sturgill, 1987; Cicirelli *et al.*, 1988; Hoshi *et al.*, 1988; Boulton *et al.*, 1991a). We have cloned two such enzymes, extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), that phosphorylate MAP2 and MBP and act as S6 kinase kinases in vitro (Gregory *et al.*, 1989; Ahn and Krebs, 1990; Boulton *et al.*, 1990). The activities of these enzymes are regulated by insulin, nerve growth factor (NGF), and many other agents (Cobb *et al.*, 1991a, and references therein).

The parallels between signal-transducing mechanisms utilized by NGF and those of growth factors, such as insulin and epidermal growth factor, have emerged, beginning with studies showing that NGF induced the tyrosine phosphorylation of a number of unidentified proteins (Maher, 1988) and continuing with the recent discovery that Trk is an NGF receptor (Kaplan *et al.*, 1991). Serine/threonine kinases are also activated in common by these factors. Blenis and Erikson (1986) and Mutoh and coworkers (1988) demonstrated activation of S6 kinases upon NGF treatment. Greene and asso-

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ciates have found that the activities of multiple kinases, one of which may be an S6 kinase, are increased (Volonte *et al.*, 1989). Subsequently, several groups have shown the activation and tyrosine phosphorylation of at least two MAP2/MBP kinases (Gómez *et al.*, 1990; Gotoh *et al.*, 1990; Ahn *et al.*, 1991b; Boulton *et al.*, 1991b), ERK1 and ERK2, that are candidates for the role of S6 kinase kinases.

ERK1 and ERK2 are phosphorylated and activated (Boulton and Cobb, 1991; Boulton *et al.*, 1991b) in response to both NGF and insulin. To improve our understanding of the mechanisms of activation of these kinases, we have examined the amino acids phosphorylated in ERK1 and ERK2 and the tryptic phosphopeptides generated from the enzymes in response to each of these stimuli. In addition, we have compared the phosphopeptides from ERKs immunoprecipitated from stimulated cells with those that arise after autophosphorylation of ERK2 in vitro.

MATERIALS AND METHODS

^{32}P-Labeling, Immunoprecipitation, and Phosphoamino Acid Analysis of ERKs

Rat 1 HIRc B or PC12 cells were grown to confluence in 100-mm dishes, changed to serum-free Krebs-Ringer-bicarbonate solution containing 2% bovine serum albumin for 60 min, and then labeled with ^{32}P (2 mCi/ml) in the above solution (Boulton *et al.*, 1991b) for 4 h with or without the addition of insulin (0.18 μM) or NGF (Upstate Biotechnology, Lake Placid, New York; 75 ng/ml) for the last 5 min or other time as indicated. The cells were washed in cold homogenization buffer (20 mM tris(hydroxymethyl)aminomethane, pH 7.5, 20 mM p-nitrophenyl phosphate, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 50 mM sodium fluoride, 50 μM sodium orthovanadate, and 5 mM benzamidine) containing 2 mM phenylmethylsulfonyl fluoride and 0.1 μM pepstatin. Cells were harvested in 1 ml of this buffer per dish and homogenized by douncing. After centrifugation at 100 000 × *g* for 1 h at 4°C, the supernatants were adjusted to final concentrations of 0.5% sodium dodecyl sulfate (SDS) and 1 mM dithiothreitol (DTT), heated to 100°C for 1–2 min, and diluted fourfold with homogenization buffer containing 1.25% sodium deoxycholate, 1.25% Triton X-100, and 1 mM DTT. The samples were first treated with preimmune serum and Pansorbin (Calbiochem, San Diego, CA) (which were removed by sedimentation) and then incubated with 7.5 μl of antiserum 837 per dish for 1 h on ice. Immune complexes were collected with Pansorbin, and the collected pellets were washed in homogenization buffer plus 0.2% Triton X-100 containing first 2 M NaCl plus 10 mg/ml bovine serum albumin, second 0.15 M NaCl plus 0.01% SDS, and finally 0.15 M NaCl. The pellets were resuspended in 35 μl of 2.5-fold concentrated electrophoresis sample buffer, heated for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using 10% polyacrylamide gels. For phosphoamino acid analysis, individual ERK1 or ERK2 bands were excised from dried gels and hydrolyzed in 6 N HCl for 60 min. Phosphoamino acids were analyzed as described (Boulton *et al.*, 1991b).

Kinetics of NGF Action

^{32}P-labeling was as described above and cells were lysed with boiling 0.5% SDS, 1 mM DTT in homogenization buffer after the times of NGF treatment specified in the legend to Figure 2. The lysates were heated for 1–2 min and centrifuged for 10 min at 4°C in a microcentrifuge. The supernatants were diluted and ERKs were immunoprecipitated as above. The immunoprecipitates were either analyzed on 10% polyacrylamide gels in SDS or reimmunoprecipitated as follows. Antigen-antibody complexes were denatured by adding 0.5% SDS, 1 mM DTT in homogenization buffer followed by boiling for 1–2 min. The solutions were then diluted fourfold with 1.25% Triton X-100, 1.25% sodium deoxycholate, and 1 mM DTT in the same buffer. Immune complexes were collected a second time and washed as described above. Phosphoamino acid analysis was performed on ERKs immunoprecipitated once from cells after various times of NGF treatment.

ERK2 Autophosphorylation

Purified recombinant ERK2 (Boulton *et al.*, 1991b) was autophosphorylated in the presence of 10 mM $MgCl_2$, 1 mM DTT, 1 mM benzamidine, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 8.0, and [γ-^{32}P]ATP at 30°C overnight. ERK2 was isolated by SDS-PAGE on a 10% polyacrylamide gel, electroeluted, and subjected to tryptic digestion as described below.

Two-Dimensional Peptide Mapping

Two-dimensional mapping was essentially as described by Haycock (1990). The labeled ERKs were individually electroeluted from wet gel slices in 15 mM ammonium bicarbonate and 0.1% SDS. The electroeluted material was dried, washed three times with ice-cold acetone: water (9:1), and dissolved in 110 μl of 50 mM ammonium bicarbonate containing 0.09 mg/ml TPCK-treated trypsin (Sigma, St. Louis, MO). After 16 h at 30°C, an additional 10 μl of 1 mg/ml trypsin was added for 5 h further incubation at 30°C. The digest was heated to >90°C for 2–3 min, dried, and dissolved in 50 μl of water. After dissolving and drying the digested peptides three to four times, the peptides were finally dissolved in 5–10 μl of water and applied 5 cm from the edge and 2 cm from the bottom of a 20 × 20-cm cellulose plate (Kodak 13255, Rochester, NY). Each plate was saturated with 2% ammonium carbonate (pH 8.9) and subjected to electrophoresis on a Pharmacia (Piscataway, NJ) FBE-3000 apparatus at 800 V for 60 min at 0–4°C. The plates were dried at room temperature, and 1 μl of 10 mg/ml DNP-lysine was applied to each origin as a standard for chromatography in the second dimension. The second dimension was developed in butanol:pyridine:acetic acid:water (37.5:25:7.5:30). Plates were exposed to XAR5 (Kodak) or hyperfilm (Amersham, Arlington Heights, IL) at −80°C with intensifying screens for up to 2 wk.

Immune Complex Assay

Immune complex kinase assays were performed essentially as described (Boulton and Cobb, 1991), except that 10 mg/ml bovine serum albumin was included in all solutions.

Protein Kinase Assay

Protein kinase assays were performed with MAP2 as substrate essentially as described (Boulton *et al.*, 1991a) on 100 000 × *g* supernatants of untreated cells or cells treated with NGF as noted above.

RESULTS

The activities of ERK1 and ERK2 are regulated by phosphorylation (Cobb *et al.*, 1991a, and references therein). As a prelude to studying the regulation of the phosphorylation of the MAP2 kinases ERK1 and ERK2 by NGF, we characterized the concentration dependence and kinetics of MAP2 kinase activation by NGF. Half-maximal activation of soluble MAP2 kinase activity occurred at ~26 ng/ml NGF; maximum activation, resulting in as much as a 30-fold increase in specific MAP2 kinase activity in supernatants, was achieved at 260 ng/ml NGF (Figure 1A). The concentration dependence,

although steep, is similar to that reported by others (Miyasaka *et al.*, 1990; Tsao *et al.*, 1990). The onset of the response was rapid; within 2 min NGF consistently caused a ~10-fold increase in MAP2 kinase specific activity, from 6.5 to 55 pmol · min^{-1} · mg^{-1}, (Figure 1B). The specific activity increased further to 15- to 20-fold above the unstimulated activity between 5 and 30 min of NGF treatment. Thereafter, activity declined to 8- to 10-fold above basal activity, remaining at approximately this specific activity for the subsequent 2 h (unpublished observation). This pattern is similar to that reported by Tsao *et al.* (1990), Gotoh *et al.* (1990), and Schanan-King *et al.* (1991) (although see also Miyasaka *et al.*, 1990).

After 5 and 30 min of exposure to NGF, this stimulated MAP2 kinase activity is comprised primarily of the two MAP2/MBP kinases, ERK1 and ERK2 (Boulton *et al.*, 1991b). To verify that the overall time course of

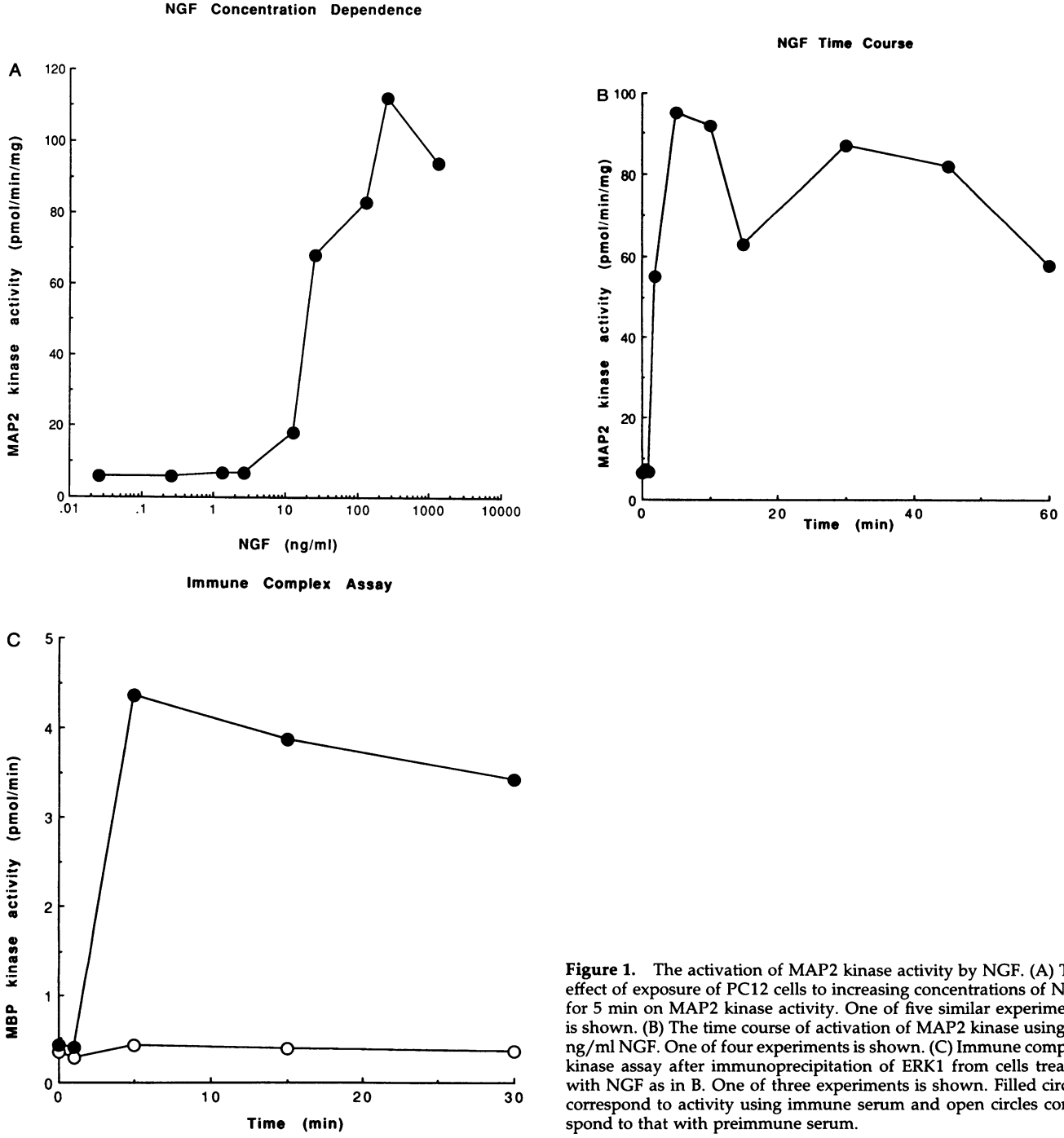

Figure 1. The activation of MAP2 kinase activity by NGF. (A) The effect of exposure of PC12 cells to increasing concentrations of NGF for 5 min on MAP2 kinase activity. One of five similar experiments is shown. (B) The time course of activation of MAP2 kinase using 75 ng/ml NGF. One of four experiments is shown. (C) Immune complex kinase assay after immunoprecipitation of ERK1 from cells treated with NGF as in B. One of three experiments is shown. Filled circles correspond to activity using immune serum and open circles correspond to that with preimmune serum.

MAP2 kinase activity specifically reflects ERK activity, we immunoprecipitated ERK1 using an antibody specific for ERK1 under nondenaturing conditions and measured MBP kinase activity in the immune complexes. The antiserum is able to extract as much as 85% of the ERK1 activity from Mono Q fractions but does not precipitate ERK2 unless it has first been denatured (Boulton and Cobb, 1991). As shown in Figure 1C, the increased activity in extracts with time was paralleled by the increased activity of ERK1 detected in immune complexes.

To examine the temporal relationship between NGF-dependent ERK phosphorylation and NGF-stimulated MAP2 kinase activity, ERK1 was immunoprecipitated from ^{32}P-labeled PC12 cells after various times of treatment with NGF (Figure 2A). NGF treatment increased ^{32}P-incorporation into both ERK1 and ERK2 within 1–2 min of treatment; the ^{32}P-content of both enzymes reached a plateau within 5–10 min of exposure to NGF (data not shown for ERK2). The differentially phosphorylated forms of the enzymes are immunoprecipitated equally well because immunoblotting of ERK immunoprecipitates from treated and untreated cells demonstrates equal recoveries of the kinases (Boulton *et al.*, 1991b). To determine which amino acids were phosphorylated in the proteins, phosphoamino acid analysis was performed on ERK1 (Figure 2B) from unstimulated and NGF-stimulated PC12 cells. Before exposure to NGF, ERK1 contains predominantly phosphoserine. After 5 min of treatment with NGF, the phosphoserine content changed relatively little, but phosphothreonine and phosphotyrosine appeared in both kinases. In NGF-stimulated ERK1, tyrosine phosphorylation was discerned easily at 2 min of stimulation. Phosphothreonine, however, was easily detected only at 5 min. Because recovery of phosphoamino acids is not quantitative, the molar ratios of the phosphoamino acids cannot be inferred from these data.

We wished to compare insulin-stimulated ERKs to NGF-stimulated ERKs to discern similarities and differences in their phosphorylation. However, in our hands insulin did not stimulate MAP2 kinase activity in PC12 cells. Therefore, to evaluate the effects of insulin on ERK phosphorylation, we chose to examine the insulin-responsive Rat 1 HIRc B cell line. We previously showed that after 5 min of insulin treatment MAP2 kinase activity in Rat 1 HIRc B cells was highly stimu-

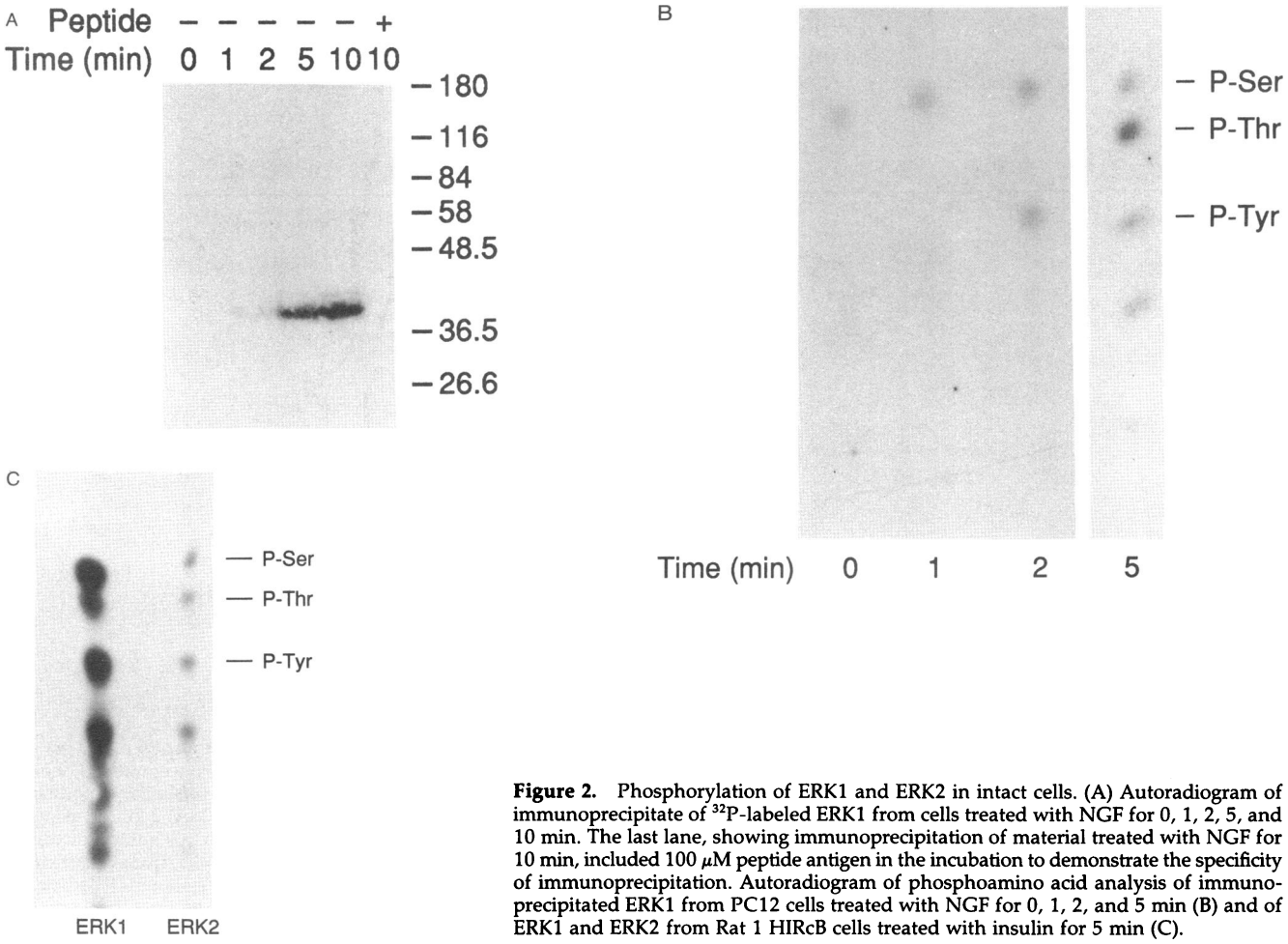

Figure 2. Phosphorylation of ERK1 and ERK2 in intact cells. (A) Autoradiogram of immunoprecipitate of ^{32}P-labeled ERK1 from cells treated with NGF for 0, 1, 2, 5, and 10 min. The last lane, showing immunoprecipitation of material treated with NGF for 10 min, included 100 μM peptide antigen in the incubation to demonstrate the specificity of immunoprecipitation. Autoradiogram of phosphoamino acid analysis of immunoprecipitated ERK1 from PC12 cells treated with NGF for 0, 1, 2, and 5 min (B) and of ERK1 and ERK2 from Rat 1 HIRcB cells treated with insulin for 5 min (C).

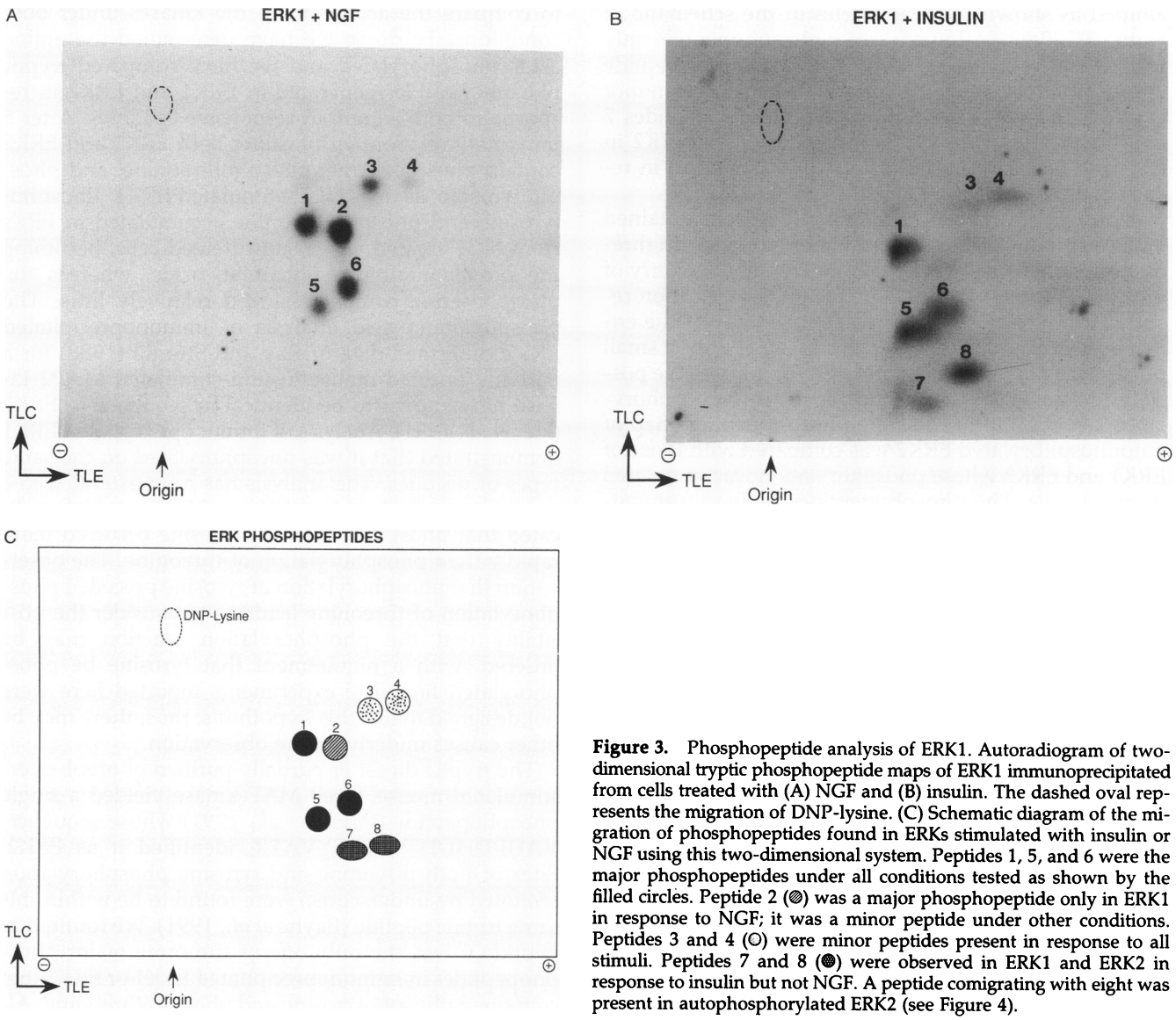

Figure 3. Phosphopeptide analysis of ERK1. Autoradiogram of two-dimensional tryptic phosphopeptide maps of ERK1 immunoprecipitated from cells treated with (A) NGF and (B) insulin. The dashed oval represents the migration of DNP-lysine. (C) Schematic diagram of the migration of phosphopeptides found in ERKs stimulated with insulin or NGF using this two-dimensional system. Peptides 1, 5, and 6 were the major phosphopeptides under all conditions tested as shown by the filled circles. Peptide 2 (◍) was a major phosphopeptide only in ERK1 in response to NGF; it was a minor peptide under other conditions. Peptides 3 and 4 (○) were minor peptides present in response to all stimuli. Peptides 7 and 8 (◉) were observed in ERK1 and ERK2 in response to insulin but not NGF. A peptide comigrating with eight was present in autophosphorylated ERK2 (see Figure 4).

lated (Boulton *et al.*, 1991a) and that both ERK1 and ERK2 were phosphorylated (Boulton *et al.*, 1991b). In this study we found phosphotyrosine and phosphothreonine were present in ERK1 and ERK2 after 5 min of insulin treatment of Rat 1 HIRc B cells (Figure 2C).

To examine the phosphorylation sites of ERK1 and ERK2, we prepared two-dimensional tryptic maps of the two ERKS immunoprecipitated from PC12 cells stimulated with NGF and from Rat 1 HIRc B cells stimulated with insulin. The purposes were as follows: 1) to determine whether ERK1 and ERK2 were phosphorylated on the same or different sites, 2) to determine whether two distinct extracellular signals used the same or different sites to regulate the activity of each ERK, and 3) to establish a method to allow comparison of ERKs phosphorylated in intact cells to ERKs phosphorylated in vitro. As can be seen in Figure 3 (A [NGF], and B [insulin]), as many as eight phosphopeptides could be distinguished in ERK1 from NGF- and insulin-treated cells. The peptides have been assigned numbers for ease of comparison (Figure 3C). Phosphopeptides that comigrated with those in ERK1 (see below) were also present in ERK2 from NGF- and insulin-treated cells (Figure 4, A and B). It was confirmed that the peptides had the same mobilities by mixing the tryptic hydrolysate of ERK1 with that of ERK2, each derived from NGF-stimulated cells, and then subjecting the mixture to electrophoresis and chromatography (Figure 4C). Again, the same phosphopeptide pattern was present; peptides 1, 2, 5, and 6 were apparent, although peptide 2 was of reduced intensity compared with the map of ERK1 alone. The consensus of numerous experiments indicated that peptides 1, 5, and 6 were the major phosphopeptides in both ERKs under all the conditions ex-

amined as shown by filled circles in the schematic of Figure 3C. Peptide 2 was a major phosphopeptide only in ERK1 in response to NGF; it was a minor peptide under other conditions. Peptides 3 and 4 were minor peptides present in response to all stimuli. Peptides 7 and 8 were observed primarily in ERK1 and ERK2 in response to insulin in Rat 1 HIRcB cells but not in response to NGF in PC12 cells.

Autophosphorylated recombinant ERK2 contained ^{32}P incorporated predominantly into tyrosine and threonine residues (Seger *et al.*, 1991). The stoichiometry of phosphorylation was low, but the phosphorylation resulted in up to a 10-fold increase in activity of the enzyme (Seger *et al.*, 1991), suggesting that only a small percentage of it had been activated. To address the possible physiological significance of the autophosphorylation of ERK1 and ERK2, the phosphopeptide map of autophosphorylated ERK2 was compared with those of ERK1 and ERK2 whose phosphorylation was stimulated in intact cells. The phosphopeptides derived from autophosphorylated ERK2 (Figure 4D) appeared to comigrate with peptides 1, 5, 6, and 8, a subset of the tryptic phosphopeptides found on ERK1 and ERK2 phosphorylated in intact cells (Figures 3, A and B and 4, A and B). As noted above, three of these phosphopeptides (1, 5, and 6) are found in all of the maps of ERKs labeled in intact cells. To confirm that these peptides have the same mobilities as those phosphorylated in intact cells, peptides 1, 5, and 6 were individually eluted from a two-dimensional map of ERK1 immunoprecipitated from NGF-stimulated cells and from a map of autophosphorylated ERK2. Each ERK1 peptide was mixed with the peptide presumed to be the same from autophosphorylated ERK2 (1 with 1, 5 with 5, and 6 with 6), and the three mixtures were each subjected to two-dimensional analysis. Peptides from ERK1 from intact cells and those from autophosphorylated ERK2 each contributed half of the counts per minute loaded on the plates. As anticipated, each peptide mixture yielded only one phosphopeptide on the map (Figure 5, A–C). By overlaying these three maps, the pattern of the original tryptic digest was reproduced (Figure 5D).

To determine more about the major phosphopeptides, 1, 5, and 6, the phosphoamino acid content of the peptides from ERK1 labeled in intact PC12 cells was determined (Figure 6). Peptides 1 and 5 contained only phosphotyrosine, whereas peptide 6 contained both phosphotyrosine and phosphothreonine. Phosphoserine was not detected in these peptides. For comparison, the phosphoamino acids in peptides derived from autophosphorylated recombinant ERK2 were also determined (unpublished observations). Once again, peptides 1 and 5 contained only phosphotyrosine, whereas peptide 6 contained both phosphotyrosine and phosphothreonine.

DISCUSSION

Because ERK1 and ERK2 are phosphorylated and activated in response to both NGF and insulin, we wished to compare the activation of the kinases under both conditions. To do so we have determined the amino acids phosphorylated and we have compared tryptic phosphopeptides generated in ERK1 and ERK2 in response to both stimuli in responsive cell lines. After 5 min of stimulation with insulin, both ERK1 and ERK2 contain phosphoserine, phosphothreonine, and phosphotyrosine, as does NGF-stimulated ERK1. Phosphotyrosine and phosphothreonine accumulated in ERKs from NGF-treated and insulin-treated cells, becoming the predominant phosphoamino acids, whereas the phosphoserine content changed relatively little. The phosphoamino acid analysis of immunoprecipitated ERK2 confirmed data of Ray and Sturgill (1988) for a partially purified mouse insulin-stimulated MAP2 kinase now known to be identical in sequence to ERK2 (Her *et al.*, 1991). Analysis of immunoprecipitated ERK1 demonstrated that it was phosphorylated on the same types of residues. The analysis has been extended to a time course of phosphorylation of ERK1, which indicated that phosphorylation of tyrosine occurred more rapidly than phosphorylation of threonine. The observation that phosphorylation of tyrosine preceded phosphorylation of threonine leads us to consider the possibility that the phosphorylation reaction may be ordered, with a requirement that tyrosine be phosphorylated first. The experiments reported here were not designed to test this hypothesis; thus, there may be other causes underlying the observation.

The tryptic digest of partially purified phorbol-ester-stimulated mouse T cell MAP kinase yielded a single phosphopeptide (Payne *et al.*, 1991) whose sequence, VADPDHDHTGFLTEYVATR, identified it as ERK2. Sites of both threonine and tyrosine phosphorylation (denoted by underscores) were found to be within this same tryptic peptide (Payne *et al.*, 1991). We found that peptide 6 was the only one of the three major phosphopeptides in immunoprecipitated ERK1 or ERK2 that contained phosphotyrosine and phosphothreonine. Although the exact ratios of phosphate on the two amino acids cannot be determined from this sort of analysis, the fact that more phosphothreonine than phosphotyrosine was recovered is consistent with an equimolar ratio of the two in this peptide. Thus, peptide 6 may correspond to the peptide found to be phosphorylated in mouse ERK2.

The schematic diagram of ERK phosphopeptides shows the number and relative positions of eight tryptic peptides present under various conditions described in this paper. On NGF stimulation, we find phosphopeptides 1, 2, 5, and 6 predominate in ERK1 with less phosphate associated with peptides 3 and 4. In ERK2, peptides 1, 5, and 6 are the major phosphopeptides, although 2, 3, and 4 were apparent following longer autoradiography. On insulin stimulation, we find peptides 1, 5, and 6 predominate in both ERK1 and ERK2. There are several reasons we expected to detect multiple phosphopeptides. First, electrophoresis at the

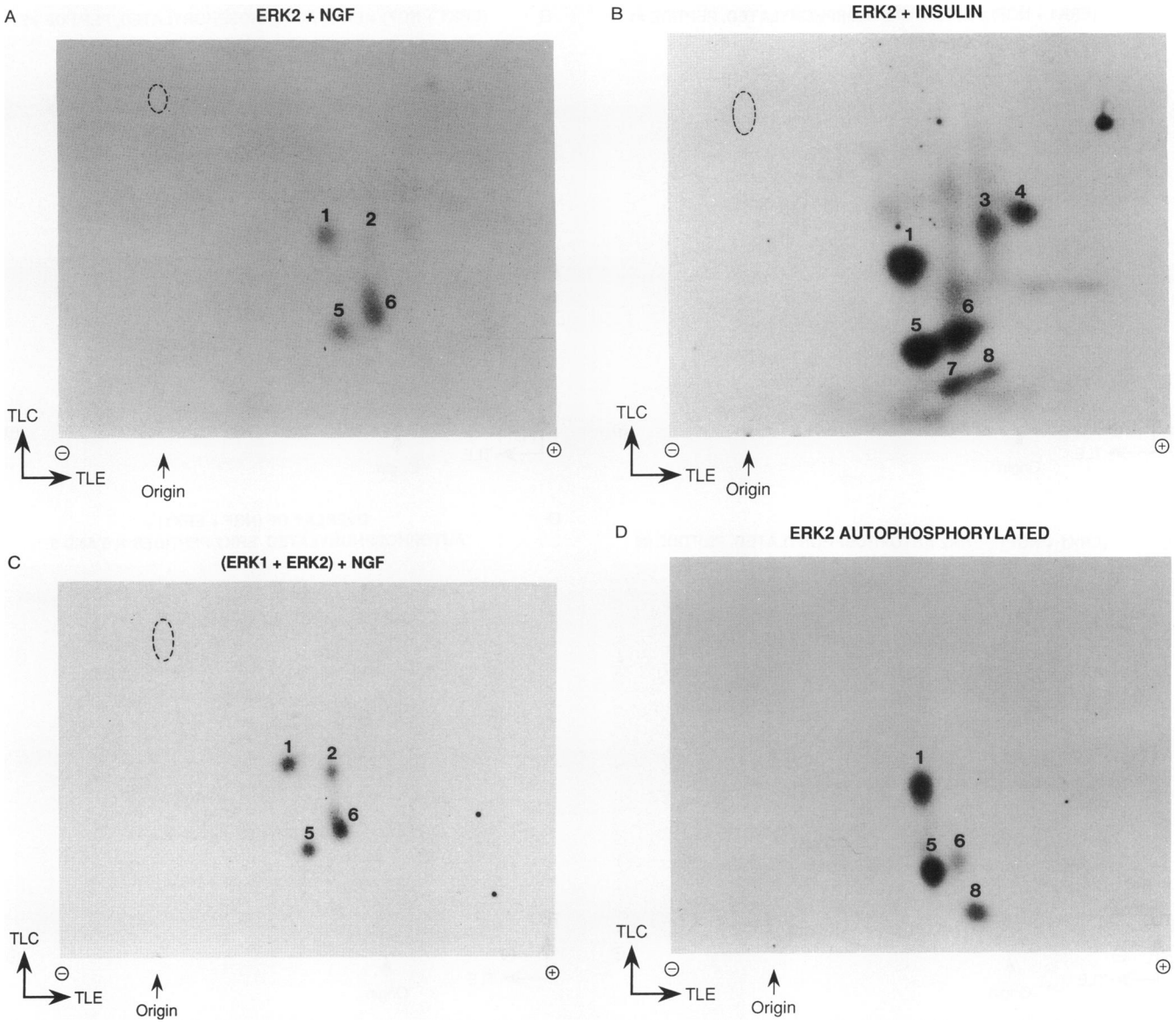

Figure 4. Phosphopeptide analysis of ERK2. Autoradiogram of two-dimensional tryptic phosphopeptide maps of ERK2 immunoprecipitated from cells treated with (A) NGF and (B) insulin. (C) two-dimensional tryptic phosphopeptide map of a mixture of ERK1 and ERK2 from NGF-stimulated cells. Equal cpm from each ERK were applied. (D) tryptic phosphopeptide map of autophosphorylated ERK2.

pH we used (8.9) is more likely to resolve multiple related peptides and peptides of similar phosphorylation state than electrophoresis at a lower pH (Haycock, 1990). Second, we are digesting and mapping ERK2 and ERK1 from immunoprecipitates containing enzymes in both active and inactive forms. Inactive and active forms of ERKs are resolved during purification (Boulton *et al.*, 1991b; Boulton and Cobb, 1991). The method used to isolate the MAP kinase from which one phosphopeptide was identified (Payne *et al.*, 1991) was partial purification detected by kinase activity, not immunoprecipitation. Third, although phosphorylation on tyrosine and threonine appears to be critical for maximum activity of these kinases, phosphoserine is detected in ERK1 before and after NGF or insulin treatment. This suggests that among the five minor phosphopeptides (2, 3, 4, 7, and 8) will be sites of serine phosphorylation, some of which might be ligand stimulated.

Gotoh *et al.* (1990) have shown that NGF has a slower more prolonged effect than EGF on MAP2 kinase activity in PC12 cells. We confirmed this result (Robbins and Cobb, unpublished data) and found that upon two-dimensional mapping of ERKs phosphorylated in response to NGF, peptide 2 was a major tryptic phosphopeptide, whereas in response to EGF, peptide 2 was

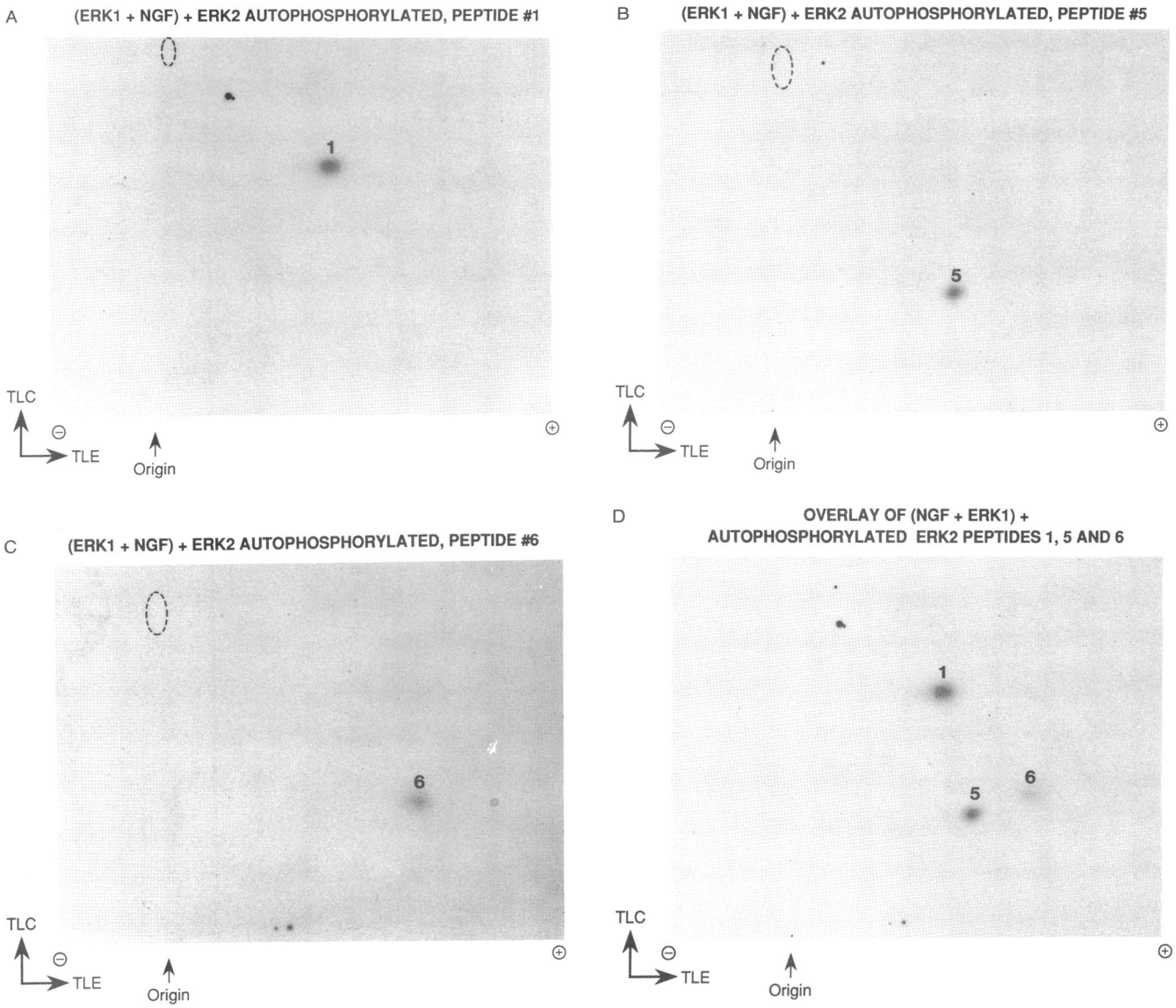

Figure 5. Comparison of phosphopeptides from autophosphorylated ERK2 to those from ERK1 immunoprecipitated from NGF-stimulated cells. Autoradiogram of mixtures of peptide 1 (A), peptide 5 (B), and peptide 6 (C) from a map of autophosphorylated ERK2 with those from a map of ERK1 immunoprecipitated from NGF-stimulated cells (D). Overlay of autoradiograms shown in A–C using the origin as the reference point.

absent (Seger and Robbins, unpublished data). The more prolonged phase of MAP2 kinase activation in PC12 cells was found to be protein kinase C-dependent by Miyasaka *et al.* (1990). Peptide 2 is a minor component of insulin-stimulated ERK1 and ERK2 after digestion, yet upon treatment of fibroblasts with insulin plus phorbol ester, its proportion is greatly increased (Robbins and Cobb, unpublished data). The peptide is also absent from autophosphorylated and in vitro activated ERK2 (Ahn *et al.*, 1991a). Although the sequence of the phosphorylation site on peptide 2 and its functions are not known, its appearance is associated with prolonged activation of ERKs. Interestingly, although ERK1 and ERK2 seem to be activated in concert in all cells examined and their tryptic phosphopeptides comigrate, peptide 2 is less apparent in NGF-stimulated ERK2 than ERK1.

The appearance of multiple tryptic phosphopeptides suggests that phosphorylation of sites in addition to those recently proposed may be involved in ERK regulation (Payne *et al.*, 1991). In addition to our peptide mapping, work from other laboratories raises the possibility that there may be more than two sites of phosphorylation regulating the ERKs. Gotoh *et al.* (1991b) found that activation of *Xenopus* MAP kinase was accompanied by phosphorylation of the enzyme on tryosine and serine residues, even though the threonine and tyrosine residues reported to be phosphorylated in

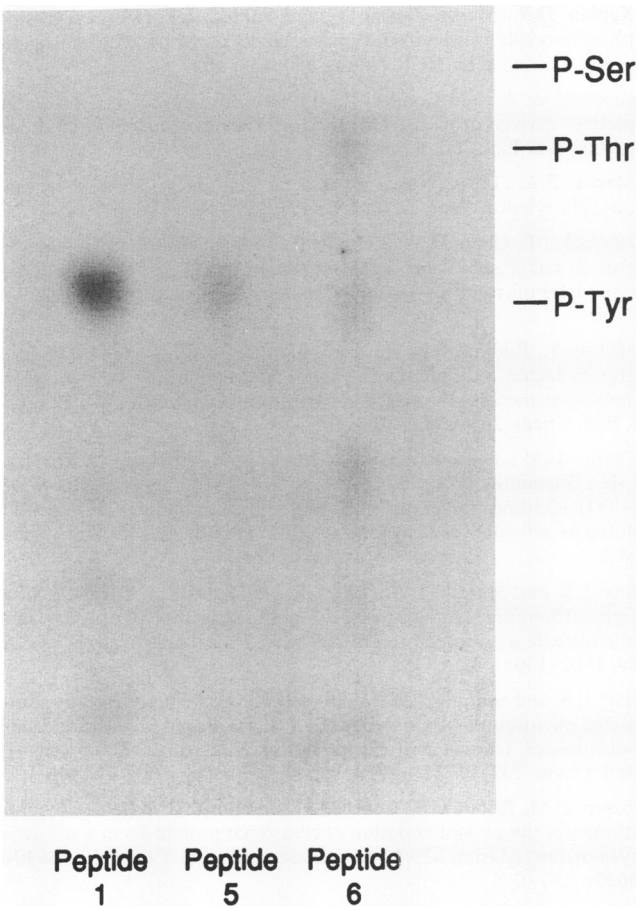

Figure 6. Phosphoamino acid analysis of tryptic phosphopeptides. Autoradiogram of phosphoamino acid analysis of peptides 1, 5, and 6 from ERK1 labeled in intact cells.

mouse ERK2 are conserved in the *Xenopus* enzyme (Gotoh *et al.*, 1991a). Sanghera *et al.* (1990) found that the sea star MBP kinase autophosphorylated on serine residues and that this phosphorylation resulted in a modest increase in activity. Tsao and Greene (1991) recently presented evidence suggesting that phosphorylation as well as dephosphorylation may be involved in shutting off ERK activity. Work done by Cooper and colleagues on pp42, which is believed to be MAP kinase, suggested that there may be additional tyrosine phosphorylation sites (Kazlauskas and Cooper, 1988). The locations and roles of such additional sites remain to be determined.

We recently have shown that recombinant ERK2 is capable of autophosphorylating tyrosine and threonine (Seger *et al.*, 1991). From the present data, the autophosphorylated protein contains tryptic phosphopeptides that are a subset of the phosphopeptides found on the protein in intact cells. Furthermore, the three phosphopeptides consistently found in ERKs labeled in intact cells are present in the autophosphorylated protein, supporting the notion that the ability of ERK2 to autophosphorylate, although limited in vitro, may be physiologically relevant. However, the distribution of phosphate among the peptides is different from that found in intact cells. Peptide 6 from autophosphorylated ERK2 contains a smaller proportion of the phosphate than it does in intact cells. Nevertheless, stimulation of ERK2 activity by autophosphorylation may contribute to the activation of ERKs in intact cells; autophosphorylation may be a component of the mechanism utilized by the MAP2/MBP kinase activator described by Ahn *et al.* (1991a,b). The MAP2 kinase activator is capable of vastly raising the stoichiometry of ERK2 phosphorylation, by causing phosphorylation of peptides comigrating with peptides found in vivo (Ahn *et al.*, 1991b).

The ability of these kinases to catalyze their own tyrosine phosphorylation may be a function of a partially formed active site. Once the active site is stabilized by phosphotyrosine, the molecule may take on the specificity (for Ser/Thr) predicted by its sequence and no longer phosphorylate tyrosine residues. Thus far, no other ERK 1 or 2 substrates have been found that are phosphorylated on tyrosine. The ERK activator (Ahn *et al.*, 1991a) may be a protein kinase or it may allow ERK1 or 2 to assume a conformation more favorable for autophosphorylation. These possibilities have been discussed in more detail elsewhere (Cobb *et al.*, 1991b).

In summary, we have shown that 1) the three major tryptic phosphopeptides (1, 5, and 6) of both ERK1 and ERK2 are seen under all conditions examined here; 2) other phosphopeptides are also found consistently but to a lesser extent and may be specific to the type of extracellular signal, cell, or ERK; and 3) the three major phosphopeptides are the same as those seen in autophosphorylated ERK2, implying that ERK2 autophosphorylation may be a physiologically relevant event.

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