ERK3 Is a Constitutively Nuclear Protein Kinase*

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The ERK3 cDNA predicts a protein of 62,000 in size with a C-terminal domain that extends 180 amino acids beyond the conserved core of ERK family protein kinases. Immunoblotting with antibodies raised to recombinant protein and to peptides from the catalytic core and three regions of the C-terminal tail revealed that ERK3 is the expected size and is ubiquitously expressed in a variety of cell lines and tissues. ERK3, unlike the MAP kinases ERK1 and ERK2, is localized in the nucleus in exponentially growing, quiescent, and growth factor-stimulated cells. If the 180 amino acids at its C terminus are deleted, the resulting ERK3 fragment of 45 kDa is still found primarily in the nucleus, indicating that the C terminus is not required for its localization. Recombinant ERK3 expressed in mammalian cells or in bacteria is a protein kinase, as deduced from its capacity to autophosphorylate. Mutation of a conserved residue (Asp171) expected to be involved in catalysis eliminated its catalytic domain. Its cDNA from rat predicts a protein of 62 kDa with a C-terminal domain that extends 180 amino acids beyond the conserved ERK core. Given the important roles of other members of this subfamily of protein kinases in cellular regulation, it is likely that ERK3 will also have important, albeit unknown, functions.

A human ERK3-related enzyme was cloned (11) that is 72% identical to rat ERK3 in the kinase domain. The tail domains of ERK3 and the ERK3-related enzyme are less similar (only 28% identical) and are unrelated over the last 65 amino acids. More recently, Flier and colleagues have found the human ERK3 homolog. It is nearly identical to rat ERK3 throughout the catalytic core as well as the tail, and appears to be alternatively spliced, generating a form that contains an additional 178 residues at the C terminus (12). Southern analysis suggests at least three ERK3-related genes, supporting the idea that there are multiple ERK3-like proteins (10). One unusual feature of ERK3 and the related kinase is that they contain an arginine in place of the subdomain VIII glutamate that is highly conserved among the protein kinases (10–13). Analysis of crystal structures of several protein kinases including ERK2 suggests that this residue is involved not in catalysis but in stabilizing the structure of the C-terminal fold of the protein kinase (14, 15). Members of the caspase family also lack this glutamate (16). The three-dimensional structure of a yeast casein kinase I indicates a different mechanism to stabilize the protein structure (17), suggesting that this arginine in ERK3 is compatible with its protein kinase activity. To test this directly, we expressed and immunopurified ERK3 to measure its catalytic activity.

In addition, we wished to confirm the existence of ERK3 protein by examining its expression and distribution in cells and tissues. ERK3-specific antibodies demonstrated that ERK3 was widely distributed and, unlike ERK1 and ERK2, ERK3 was localized to the nucleus in the absence of serum factors.

**EXPERIMENTAL PROCEDURES**

Cdl Culture—REF52, 293, COS7, and PC12 cells were cultured at 37 °C in a 10% CO2 atmosphere in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine. The Jurkat T cell line was cultured in RPMI 1640 with the same supplements. For mitogen stimulation, cdi were plated in medium without serum for 18–20 h and then treated with either epidermal growth factor (100 ng/ml), phorbol myristate acetate (10 ng/ml, Sigma) or FBS (10%) for various times.

Bacterial Expression and Purification of Histidinyl-tagged ERK3 and Glutathione S-Transferase (GST)-ERK3—To express histidyl-tagged ERK3 without the tail domain (ERK3ΔCt, Fig. 1A), the first 1092 bp of ERK3 was amplified by polymerase chain reaction (PCR) and subcloned into NdeI and EcoRI sites of pET-16B (Novagen Inc., Madison, WI) which encodes 10 histidines 5' to the NdeI site. The vector harboring tagged ERK3ΔCt was transformed into Echerichia coli strain BL21-DE3. Bacteria were grown and induced to express protein as described (18). Recombinant protein was purified by adsorption to Ni2+-NTA agarose (Qiagen, Chatsworth, CA) and eluted with a gradient of 80–250 mM imidazole in ERK purification buffer (19). The fractions were collected into tubes containing dithiothreitol (DTT), EGTA, EDTA, and benzamidine to give final concentrations of 1 mM, 1 mM, and 50 mM.

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1The abbreviations used are: ERK, extracellular signal-regulated protein kinases; MAP, mitogen-activated protein; FBS, fetal bovine serum; DTT, dithiothreitol; PCR, polymerase chain reaction; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis.
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mM, respectively. Fractions were dialyzed overnight against ERK purification buffer and stored at −80 °C.

To express ERK3 as a GST fusion protein, oligonucleotides were used for PCR amplification of either ERK3 C-terminal domain (C-Term) or full-length ERK3 and the PCR products were subcloned into EcoRI and SalI sites of pGEX-KG (20). Expression was as described except that cultures were grown in large scale (21) to an OD600 of 0.5-1 and then induced with 0.4 mM isopropyl-β-D-thiogalactoside for 12-14 h. Cells were washed once with 50 mM Tris, pH 8.0, 25% sucrose, and 10 mM EDTA and then lysed in 40 ml of 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 0.15 M NaCl, 1% Triton X-100, 1% phenylmethylsulfonyl fluoride (PMSF), 1 µM leupeptin, 1 µM pepstatin, 2 µM aprotinin, and 3 mM lysozyme. The lysates were sonicated for 2 min to shear DNA and clarified by ultracentrifugation at 25,000 rpm for 30 min in a Beckman Ti45 rotor. The supernatants were mixed with glutathione-agarose beads for 2 h, and the beads were washed four times with 20 mM HEPES, pH 7.6, 0.1 M KCl, 20% glycerol, 1 mM DTT, 1 mM PMSF. GST-ERK3 and GST-ERK3 C-Term were eluted with 5 mM reduced glutathione (Sigma), dialyzed against ERK purification buffer, and stored at −80 °C.

Generation of Anti-ERK3 Antibodies—Rabbits were immunized with hemocyanin-conjugated, synthetic peptides from the C-terminal tail or protein kinase subdomain XI of ERK3 (Fig. 1A; peptides synthesized by Clive Slaughter, University of Texas Southwestern Medical Center) as described (22). The peptide from the kinase domain, DRLAEEALSH-DEEEVQVDPR, produced antibodies in rabbits A54 and C354. The peptide from the C-terminal subdomain XI, YPDHHENKYCDLE, produced antibodies in rabbits D175 and D176. Two other peptides from the C terminus, DNSVLWRESENHYYEP and PRLASDVT-DEEEVVQDPRKY, were also used to raise antibodies in pairs of rabbits D173, D174, and A248 and Z273, respectively. Histidine-tagged ERK3 C-Term was also used as antigen (23) to raise antibodies in rabbits F324 and F325. Immunospecificity was determined by preincubating the peptide antibodies with 500 µM peptide or recombinant ERK3 as appropriate. Immunoblots were developed with the Amersham Corp. ECL kit.

Subcellular Fractionation—Minced rat tissues were resuspended in 25 ml of 10 mM HEPES, pH 7.6, 25 mM KCl, 25% glycerol, 1 mM DTT, 1 mM PMSF, and 1 µM leupeptin, and 2 mM benzamidine, disrupted by sonication, and homogenized with 3-5 strokes of a Teflon-glass motorized homogenizer. Lysates were filtered through cheesecloth, layered over 10-mL cushions of the above buffer, and sedimented at 24,000 rpm in a Beckman SW28 rotor for 30 min at 4 °C. Nuclear extracts were prepared by solubilizing the nuclear pellet in 1 ml of nuclear extraction buffer (see below) for 30 min, and sedimenting insoluble material at 60,000 rpm in a Beckman 70Ti rotor for 1 h.

COS7 and Jurkat T cells were fractionated into cytosolic and nuclear fractions as described (24, 25). Nuclear proteins were extracted with nuclear extraction buffer (0.42 M NaCl, 20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 1 mM p-nitrophenylphosphate, 1 mM PMSF, 1 µM leupeptin, 1 µM pepstatin, 2 µM aprotinin, and 0.1% SDS) for 30 min at 4 °C. After centrifugation for 30 min at 4 °C, the supernatants were mixed with glutathione-agarose beads for 2 h, and the beads were washed four times with 20 mM HEPES, pH 7.6, 0.1 M KCl, 20% glycerol, 1 mM DTT, 1 mM PMSF. GST-ERK3 and GST-ERK3 C-Term were eluted with 5 mM reduced glutathione (Sigma), dialyzed against ERK purification buffer, and stored at −80 °C.

Immunoblotting of anti-ERK3 antibodies was performed as described (26). Protein Kinase Assays—Autophosphorylation of ERK3 was performed in 30 ml of 10 mM HEPES, pH 8.0, 50 µM ATP ([γ-32P]ATP to achieve 5–15 cpm/fmol), 10 mM MgCl2, 1 mM benzamidine, and 1 mM EDTA at 30 °C for 30 min. Reactions were stopped with 5 × electrophoresis sample buffer and analyzed by SDS-PAGE and autoradiography (26). Phosphorylation of potential substrates by ERK3 was assayed as described (18, 26).

Immunofluorescence—REF52 and COS7 cells cultured on coverslips in 24-well culture plates were analyzed by immunofluorescence essentially as described (27). Cells were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline, permeabilized with 0.1% Triton X-100, and then incubated with anti-Myc antibody 9E10 (5 µg/ml, American Type Culture Collection) for 1 h. Coverslips were washed with 0.15 M NaCl, 1% Triton X-100, 0.3% deoxycholate, 3 mM MgCl2, 5 mM EGTA, 1 mM DTT, and 0.1% SDS for 30 min at 4 °C. After centrifugation for 30 min in an Eppendorf microcentrifuge, the cell lysates were incubated with 2 µl anti-HA monoclonal antibody 12CA5 (BabCo) and 30 µl of protein A-Sepharose 4B (Sigma) for 2 h at 4 °C. The immunoprecipitates were washed three times with 20 mM Tris·HCl, pH 7.5, 50 mM NaF, 1 mM sodium vanadate, 1 mM EGTA, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS; twice with 0.5 M LiCl, 0.1 mM Tris·HCl, pH 7.5; and once with 50 mM Tris·HCl, pH 7.6, 1 mM EGTA, 0.1 M NaCl. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

Other Methods—Protein concentration was assayed using the method of Lowry (30). Phosphoamino acid analysis and peptide mapping were performed as described (31).

RESULTS

Detection of ERK3 by Immunoblotting—to identify ERK3, we generated antibodies to one peptide from subdomain XI of the catalytic core, three peptides within the 22 kDa of the enzyme that lies C-terminal to the catalytic domain (Fig. 1A), as well as to recombinant protein. All of the resulting antisera specifically recognized a protein of the same size, 62 kDa, in whole cell extracts from 293, PC12, Jurkat, 3T3, COS, Rat1, REF52, and P19 cells (Fig. 1B and data not shown). Recognition was blocked by preincubation of the antisera with the antigenic peptides (data not shown). The peptide from subdomain XI was ~70% identical to comparable regions of ERK1 and ERK2; high concentrations of these 43- and 41-kDa proteins were sometimes detected. Although two of the peptide antigens from the C-terminal domain are also contained in the
ERK3-related kinase, one of these peptide antigens is from a unique region present only in ERK3 (see Fig. 1A). Thus, ERK3 was distinguished from the related kinase, because of differences in sequence of the C-terminal domains that extend past the catalytic core. The two ERK3-specific antisera, D175 and D176, recognized a protein of the same size as the other ERK3 antibodies, indicating that the immunoblotting signal is derived from ERK3, not the related kinase.

We examined the distribution and relative abundance of ERK3 in rat tissues using antisera D175 and A654, the latter an anti-ERK3 peptide antibody to subdomain XI. All tissues contained an immunoreactive band of 62 kDa, corresponding to ERK3 that were recognized by both antibodies (Fig. 2). A654 also revealed a significant band slightly smaller than ERK3 with a distinct tissue distribution, as well as minor bands of 43 and 41 kDa, corresponding to ERK1 and ERK2, consistent with the similarity of their sequences to ERK3 in subdomain XI. Both A654 and D175 also recognized proteins of 160 and 95 kDa in rat tissues, suggesting the existence of ERK3-related proteins in these tissues. The 95-kDa protein may be the alternatively spliced form of ERK3 reported by Zhu et al. (12) because it is found most highly in muscle.

Localization of ERK3—We examined the subcellular distribution of ERK3 by immunoblotting analysis of J urkat T cells separated into nuclear and cytosolic fractions. Immunoblotting with the anti-ERK3 peptide antibody A654 showed that ERK3 was predominantly in the nuclear fraction of J urkat cells with little detected in the cytosolic fraction (Fig. 3). In contrast, immunoblotting with the anti-ERK1 peptide antibody Y691 showed the expected result that ERK1 and ERK2 were most concentrated in the cytosol with small amounts in the nuclear fraction (22). Similar results were found for COS7 and PC12 cells (not shown).

The nuclear localization of ERK3 was confirmed by indirect immunofluorescence with the specific anti-ERK3 peptide antibody D175. ERK3 was found in the nuclei of log phase REF52 cells (Fig. 4, A and B). The punctate, nuclear staining was blocked by preabsorbing the antibody with antigenic peptide (Fig. 4, C and D) or recombinant GST-ERK3 (data not shown). Preimmune serum detected no signal in these cells (data not shown). Similar results were also obtained with several other cell lines, including COS, NIH3T3, Rat1, J urkat, and P19 cells. In quiescent cells and cells stimulated to activate MAP kinase activity, ERK3 was also largely in the nucleus (Fig. 4, E and F).

For comparison, the subcellular localization of ERK1 and ERK2 was examined with antibody Y691. In quiescent cells, ERK1 and ERK2 were predominantly in the cytoplasm (Fig. 4G), after 10 min of serum stimulation, they accumulated in and around the nucleus (Fig. 4H) (24) although they were also clearly visible in the cytoplasm.

The C-terminal tail of ERK3 has several standard nuclear localization sequences that might account for its constitutive presence in the nucleus. To determine if the C-terminal domain was responsible for the nuclear localization of ERK3, both the full-length protein and ERK3 lacking the C-terminal 180 amino acids, ERK3ΔCt, were expressed in COS7 cells. The transfected cells were examined by immunofluorescent staining with the anti-Myc peptide monoclonal antibody 9E10 and immunoblotting with anti-ERK3 antibody A654 after cell fractionation. Immunostaining showed that, like the endogenous protein, Myc-ERK3 was found primarily in the nucleus of the transfected cells (Fig. 5A); Myc-ERK3ΔCt was also detected in the nucleus as well as scattered in the cytoplasm (Fig. 5B). Immunoblotting of subcellular fractions also showed that endogenous ERK3 and both transfected Myc-ERK3 and Myc-ERK3ΔCt were predominantly in the nuclear fractions (Fig. 5C).

Protein Kinase Activity of ERK3—Recombinant GST-ERK3 expressed in bacteria, endogenous ERK3 immunoprecipitated by anti-ERK3 antibodies, and transfected HA-ERK3 immunoprecipitated with the anti-HA antibody were used to test the ability of ERK3 to phosphorylate in vitro substrates of ERK1 and ERK2. ERK3 phosphorylated none of these candidate substrates, including myelin basic protein, microtubule-associated protein-2, c-j un, Tal1, MyoD, c-EIk, and papilloma virus pro-
Fig. 4. Immunofluorescent localization of ERK3 in REF52 cells. A, cells stained with anti-ERK3 peptide antibody D175 (1:100 dilution); B, the same cells stained with 4,6 diamidino-2-phenylindole (27). C, cells stained with D175 preincubated with 500 μM antigenic peptide; D, the same cells stained with 4,6 diamidino-2-phenylindole. E, ERK3 in REF52 cells serum-starved for 18 h. F, same as E, except starved cells were
tein E7, to a significant extent (data not shown). It is possible that ERK3 may require phosphorylation to display quantifiable protein kinase activity. However, lack of phosphorylation is unlikely to account for the undetectable activity of ERK3, because immunoprecipitated ERK3 was at least partly in the phosphorylated form (see below) and its activity was indistinguishable from protein expressed in bacteria. ERK3ΔCt was also expressed in bacteria and 293 cells to test a potential regulatory role of the C terminus of ERK3 on its activity. This truncated form of ERK3 also had no detectable activity with these substrates.

In the absence of substrates, we tested the ability of ERK3 to autophosphorylate. Both ERK3 and ERK3ΔCt underwent autophosphorylation (Fig. 6A), confirming that ERK3 is a protein kinase. A conserved aspartate (Asp171) important for catalytic activity (32) was mutated to alanine to create D171A ERK3. This mutant did not autophosphorylate (Fig. 6D), ruling out the possibility that an associated kinase phosphorylated ERK3.

Three kinds of results suggest that ERK3 autophosphorylation was an intramolecular reaction. First, ERK3 autophosphorylated in a concentration-independent manner (data not shown). Second, ERK3 did not phosphorylate D171A ERK3. In this experiment, GST-D171A ERK3 was mixed with histidine-tagged wild type ERK3 and histidine-tagged D171A ERK3 was mixed with GST-ERK3. In neither case was autophosphorylation detected (data not shown). Third, ERK3 and D171A ERK3 were not phosphorylated by ERK3 immunoprecipitated from 293 cells with anti-ERK3 antibody D175. Phosphoamino acid analysis showed that both the ERK3 and ERK3ΔCt autophosphorylated on serine (Fig. 6B) and in both cases, the stoichiometry was less than 0.05 mol of phosphate/mol of ERK3 even after 8 h.

**ERK3 Autophosphorylation Sites**—The major site of autophosphorylation of ERK2 is Tyr185 (18, 31, 33, 34). Tyr185 and the nearby residue Thr183 are phosphorylated in the activated form of ERK2. These sites lie in the phosphorylation lip between subdomains VII and VIII of the protein kinases, a region of near identity between ERK1 and ERK2, but different in ERK3. The residue corresponding to Tyr185 is Gly191 in ERK3, while that corresponding to Thr183 is Ser189 (Fig. 6C). Considering that ERK3 autophosphorylated on serine, we tested the possibility that Ser189 is the major phosphorylation site. Ser189A and Ser189E ERK3 autophosphorylated at a significantly reduced rate compared to wild type ERK3; Ser189T,G191Y ERK3 behaved like wild type ERK3, but the residue autophosphorylated was tyrosine (Fig. 6, D and E).

Peptide mapping indicated one major phosphopeptide in either autophosphorylated full-length ERK3 or ERK3ΔCt (Fig. 7, A and B). This major phosphopeptide was absent from autophosphorylated S189A ERK3 (Fig. 7C) but was restored by mixing peptides from wild type and S189A ERK3 (Fig. 7D). These data strongly suggest that Ser189 is the major autophosphorylation site of ERK3.

**ERK3 Is Phosphorylated on Ser189 in Intact Cells**—We next examined if ERK3 is phosphorylated in intact cells, and if so, if Ser189 is the major phosphorylation site. HA-ERK3 and HA-S189A ERK3 were expressed in 293 cells, labeled with [32P]orthophosphate, and immunoprecipitated with the an-

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**Fig. 5.** The nuclear localization of ERK3 is independent of its C-terminal region. Both pCMV5-Myc-ERK3 and pCMV5-Myc-ERK3ΔCt plasmids were transiently transfected into COS7 cells. A, Myc-ERK3; B, Myc-ERK3ΔCt detected in transfected cells with anti-Myc monoclonal antibody 9E10. Exposures for panels A and B were 15 s, the same as for panels E–H of Fig. 4. C, COS 7 cells transfected as indicated, fractionated into nuclear (N) and cytosolic (S) fractions, and immunoblotted with A654 (1:2000 dilution). Myc-ERK3 and Myc-ERK3ΔCt are indicated. The prominent band at 62 kDa in the nuclear fractions is endogenous ERK3.

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restimulated with 10% FBS for 10 min. G, ERK1 and ERK2 detected with antibody Y691 in REF52 cells serum-starved for 18 h. H, same as G, except starved cells were restimulated with 10% FBS for 10 min. Exposures for panels A and C were 30 s. Exposures for panels B, D, and E–H were 15 s.
ti-HA antibody. ERK3 was phosphorylated on serine residue(s) (Fig. 8, A and B). The phosphorylation of S189A ERK3 was significantly decreased compared with wild type (Fig. 8C), suggesting that Ser189 is the major phosphorylation site of ERK3 in intact cells.

**DISCUSSION**

ERK3 is a 62-kDa protein, ubiquitously expressed in a variety of rat tissues and mammalian cell lines. Two human ERK3 isoforms have been cloned, one of which appears to be the homolog of rat ERK3 (11, 12). The human homolog is alternatively spliced resulting in ERK3 proteins of 62 and 97 kDa, the latter of which has an additional 178 residues at its C terminus. In a variety of cultured cell lines, the 62-kDa form was the only form detected by immunoblotting with antibodies to different epitopes. Zhu et al. (12) also found only the 62-kDa form in cultured cells by immunoblotting. However, in rat tissues two larger species, one of ~97 kDa, were also found, indicating that multiple ERK3-related proteins are expressed.

ERK3 is categorized as a member of the ERK subfamily based on characteristic features of its sequence. For example, subdomain V of ERK3 is 83% identical to ERK1 and subdomain IX is 72% identical. In contrast, subdomain V of cAMP-dependent protein kinase is only 17% identical to ERK1. Also striking is the similarity in lengths of inserts between conserved subdomains. On the other hand, ERK3 appears to lie on a distinct evolutionary branch from the rest of the ERK/MAP kinase family. Several properties of ERK3 distinguish it from all other known ERK homologs. ERK3 does not retain the activating tyrosine phosphorylation site, a hallmark of this family. It does not phosphorylate substrates recognized by other MAP kinases. ERK3 is constitutively nuclear; it is not translocated in response to a stimulus. However, it retains a much greater similarity to ERK1 and ERK2, particularly in the phosphorylation lip, than other known family members.

ERK3 does not phosphorylate MAP kinase substrates or substrates of other MAP kinase homologs, including nuclear
proteins such as c-jun. Songyang and Cantley examined the specificity of ERK3 by screening peptide libraries as they have for other protein kinases (35). ERK3 phosphorylated peptides in the library, further verifying that it has protein kinase activity. However, the phosphopeptides represented too small a fraction to be purified, preventing a determination of the phosphorylated sequences. These findings suggest that ERK3 has a very restricted substrate specificity. Mutation of Tyr185 of ERK2 to glycine, as exists normally in that position of ERK3, greatly impairs the ability of ERK2 to phosphorylate its substrates, suggesting that the tyrosine residue may be a key factor in protein substrate interactions in this kinase family.

In contrast to our results, the 97-kDa form of ERK3 isolated in immune complexes, was reported to phosphorylate histone H1 and myelin basic protein (12). One explanation for this apparent discrepancy is that the 97-kDa form of ERK3 may be a more active protein kinase. A second possibility is that the ERK3 immunoprecipitates were contaminated with another protein kinase. We have found at least one protein kinase that binds ERK3 very tightly and phosphorylates not only ERK3 but also myelin basic protein.

ERK3, unlike ERK1 and ERK2, is localized to the nucleus in the absence of serum factors. Similar results were obtained by immunofluorescence and by subcellular fractionation. The latter method allowed us to confirm that the protein detected was ERK3 based on its size, because almost all of it remained with nuclei during subcellular fractionation and required salt or detergent for extraction. It appears that the C-terminal 180 residues stabilize but are not required for its nuclear localization. ERK3 lacking these C-terminal residues retains a primarily nuclear distribution, although some of the protein is found in the cytoplasm. This may be due to a reduction in its size, as the truncated protein is only 45 kDa and may diffuse more readily from the nucleus than the intact protein.

Peptide mapping indicated a single major phosphopeptide in autophosphorylated ERK3. The presence of a comigrating phosphopeptide in ERK3ΔCt indicates that this site is within the catalytic domain. Ser189 is the deduced phosphorylation site, based on the loss of the phosphopeptide in an ERK3 mutant lacking this site. Ser189 lies in the phosphorylation lip between subdomains VII and VIII and corresponds to the threonine regulatory phosphorylation site in the MAP kinases. This suggests that ERK3 may be regulated in a manner analogous to other MAP kinase family members. ERK3 is poorly phosphorylated by MEK1 or MEK2, but is phosphorylated by a partially purified activity present in both soluble and nuclear extracts of cells. ERK3 autophosphorylates on this site as well but very weakly; thus, phosphorylation of this site in intact cells is likely to be mediated by a regulatory enzyme such as the one whose activity we have detected. Differences in both upstream regulators and substrates suggest that ERK3 is not in the MAP kinase cascade but in a distinct pathway. Because no ERK3 substrates have yet been identified, it is not possible to test effects of Ser189 phosphorylation on ERK3 activity. Thus, we have been unable to ascertain if this phosphorylation regulates its activity. Current efforts are directed toward the identification of ERK3 substrates that will allow us to understand more about the function and regulation of this unusual protein kinase.

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2 Z. Songyang and L. C. Cantley, personal communication.
3 M. Cheng, E. Zhen, M. Robinson, D. Ebert, E. Goldsmith, and M. H. Cobb, manuscript in preparation.
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