

# Identification of Novel ERK2 Substrates through Use of an Engineered Kinase and ATP Analogs\*

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The mitogen-activated protein kinases are key regulators of cellular organization and function. To understand the mechanism(s) by which these ubiquitous kinases affect specific cellular changes, it is necessary to identify their diverse and numerous substrates in different cell contexts and compartments. As a first step in achieving this goal, we engineered a mutant ERK2 in which a bulky amino acid residue in the ATP binding site (glutamine 103) is changed to glycine, allowing this mutant to utilize an analog of ATP (cyclopentyl ATP) that cannot be used by wild-type ERK2 or other cellular kinases. The mutation did not inhibit ERK2 kinase activity or substrate specificity *in vitro* or *in vivo*. This method allowed us to detect only ERK2-specific phosphorylations within a mixture of proteins. Using this ERK2 mutant/analog pair to phosphorylate ERK2-associated proteins in COS-1 cells, we identified the ubiquitin ligase EDD (E3 identified by differential display) and the nucleoporin Tpr (translocated promoter region) as two novel substrates of ERK2, in addition to the known ERK2 substrate Rsk1. To further validate the method, we present data that confirm that ERK2 phosphorylates EDD *in vitro* and *in vivo*. These results not only identify two novel ERK2 substrates but also provide a framework for the future identification of numerous cellular targets of this important signaling cascade.

The mitogen-activated protein kinase (MAPK)<sup>1</sup> or extracellular signal-regulated kinase (ERK) pathway is an evolutionarily conserved signaling pathway that regulates many cellular processes, including proliferation, differentiation, gene tran-

scription, and cellular migration (1). Activation of the Ras oncogene stimulates the membrane recruitment and activation of the Raf protein kinases (2–4), which in turn phosphorylate and activate MAPK kinase or ERK kinases (MEK) 1 and 2. These in turn phosphorylate and activate ERK1 and ERK2 (5). When in the inactive state, ERKs are anchored in the cytoplasm due to a basal association with the MEKs. Following activation, the ERKs migrate to various cellular locations, including the nucleus, microtubules, focal contacts, and others (6–9) where they phosphorylate a variety of substrates. These diverse phosphorylations allow the ERKs to orchestrate a complex but coordinated response to extracellular signals. The pattern and timing of substrate selections determines the specific biological outcome.

The identification and characterization of the direct targets of a particular signaling pathway is paramount to understanding the function of that pathway within the cell. The Ras to ERK pathway is a key regulator of many cellular processes. Although there are many substrates of the ERKs that have been identified (10, 11), the diverse roles of the ERKs within the cell suggest that many unknown substrates remain to be identified. Identification of the kinase that directly phosphorylates a particular substrate can be difficult due to the overlap of consensus phosphorylation sequences, redundancy of kinases that can phosphorylate a particular site, differences between *in vivo* and *in vitro* specificity, and the diversity of kinases that can be present in a reaction, even in “pure” protein preparations. Several different approaches to determine novel MAPK substrates have been used (12). These include two-hybrid analysis (13, 14), proteomic approaches under conditions of ERK activation (11), phosphorylation of column fractions from cells (15), and a solid phase phosphorylation assay (16).

To identify additional novel substrates of ERK2, we employed a method developed by Shokat and coworkers (17, 18) to specifically label direct substrates of a particular protein kinase in a mixture of cellular proteins. Protein kinases contain similar ATP binding domains, including conserved residues that come into close contact with the N-6 position of ATP. Mutation of the ATP binding site at one or both of these conserved positions to a smaller amino acid residue can allow the mutant protein kinase, but not other cellular protein kinases, to utilize analogs of ATP that contain a bulky substituent on the N-6 position. When radiolabeled ATP analog is used in a kinase reaction with the mutant kinase and a mixture of cellular proteins, only direct substrates of the mutant kinase become radiolabeled. This method has been successfully used to identify direct substrates for the Src tyrosine kinase (19) and for the MAPK JNK (20).

We used an engineered mutant of ERK2 to search for new

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<sup>1</sup> The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; JNK, c-Jun amino-terminal kinase; EDD, E3 identified by differential display; Tpr, translocated promoter region; HECT, homology to E6-AP carboxyl terminus; E3, ubiquitin-protein isopeptide ligase; cp, cyclopentyl; EGF, epidermal growth factor; CMV, cytomegalovirus; MBP, myelin basic protein; NDPK, nucleoside diphosphate kinase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GST, glutathione S-transferase.

ERK substrates that physically associate with the MAPK. As validation of our method, our assay resulted in the labeling and identification of Rsk1, a known ERK2 substrate (7). We also identified two novel ERK2 substrates, the ubiquitin ligase EDD (E3 identified by differential display) and the nucleoporin Tpr (translocated promoter region). EDD is the human homolog of the *Drosophila* gene *hyperplastic discs* and was originally identified as a gene up-regulated in response to progesterin (21). EDD is believed to serve as an E3 ubiquitin ligase due to the presence of a HECT domain (homology to E6-AP carboxyl terminus) in its carboxyl terminus that binds ubiquitin through a single cysteine residue (21). Honda *et al.* (22) have identified the topoisomerase II beta binding protein TopBP1 as a ubiquitination target of EDD. Tpr is a nucleoporin that is a component of the nuclear basket of the nuclear pore complex (23, 24). Functional studies suggest a role for Tpr in the nuclear export of proteins with a nuclear export signal (25).

In this report we describe the engineering of ERK2 to allow it to utilize an analog of ATP. Based on sequence alignment of ERK2 with other protein kinases and the crystal structure of ERK2, we generated alanine and glycine mutations of ERK2 at isoleucine 82 and glutamine 103, singly or in combination, to generate an ERK2 with a larger ATP binding pocket. Mutation of these residues did not significantly inhibit ERK2 kinase activity *in vitro* or signaling *in vivo*. ERK2 mutants with a glutamine to glycine mutation at position 103 were able to efficiently utilize several ATP analogs to phosphorylate the ERK substrate Elk1 (26) *in vitro*. Using ERK2 Q103G and [ $\gamma$ - $^{32}$ P]cyclopentyl-ATP, we were able to radiolabel EDD, Tpr, and Rsk1 as ERK2-associated substrates in COS-1 cells. We further validated these results by demonstrating that EDD was phosphorylated by ERK2 *in vitro* and in an MEK-dependent manner in cells in response to EGF. This work describes the generation of a molecular tool for the identification of many more targets of this important signaling cascade.

#### EXPERIMENTAL PROCEDURES

**Cell Culture and Plasmids**—COS-1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). COS-1 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (Invitrogen). FLAG-ERK2 has been described previously (27). FLAG-ERK2 mutants were generated using the Transformer mutagenesis kit (Clontech, Palo Alto, CA). Transfections were performed using LipofectAMINE (Invitrogen) according to the manufacturer's recommendations. A plasmid for expression of amino-terminal FLAG-tagged EDD was constructed by cloning full-length EDD from pBluescript EDD (21) into the pCMV-Tag2B vector (Stratagene, La Jolla, CA).

**Luciferase Assays**—COS-1 cells were transfected in duplicate with 1  $\mu$ g of 5 $\times$  GAL4-luciferase; 50 ng of GAL4-Elk1 (28); 100 ng of MEK1 S218D/S222D; and 5, 25, or 100 ng of ERK2 plasmid. DNA amounts were brought up to 2  $\mu$ g of total DNA with empty vector. The cells were incubated in serum-free media overnight and harvested at 24 h. Luciferase activity was determined on a Monolight 2010 Luminometer (Analytical Luminescence, Ann Arbor, MI).

**Analog Inhibition Assays and Elk1 Phosphorylation**—FLAG-ERK2 plasmids were transfected into COS-1 cells using LipofectAMINE (Invitrogen). The cells were later serum-starved for 4 h before stimulation with 10 ng/ml EGF (Upstate Biotechnology, Lake Placid, NY) for 10 min. The cells were harvested in FLAG lysis buffer (27) and FLAG-ERKs immunoprecipitated with M2 anti-FLAG-agarose (Sigma, St. Louis, MO). The immunoprecipitates were washed three times in lysis buffer and twice in kinase buffer (25 mM HEPES, pH 7.4, 10 mM magnesium acetate, and 1 mM dithiothreitol). For analog inhibition assays, duplicate reactions containing 20  $\mu$ g of myelin basic protein (MBP), 10  $\mu$ M ATP, 10  $\mu$ Ci/reaction [ $\gamma$ - $^{32}$ P]ATP (6000 Ci/mmol, PerkinElmer Life Sciences, Boston, MA), and either buffer control or 100  $\mu$ M ATP analog. The reactions were incubated at 30 °C for 10 min, resolved on a gel, and transferred to nitrocellulose. MBP bands were excised and quantitated by Cerenkov counting. Each value was compared with the mean of the control reactions (no analog), averaged with its duplicate, and graphed as a percentage of the control, which was set

to 100%. For Elk1 phosphorylation, the immunoprecipitated ERKs were mixed with 1  $\mu$ g of GST-Elk1 and either 100  $\mu$ M ATP or 100  $\mu$ M ATP analog. The reactions were incubated at 30 °C for 10 min, resolved on a gel, and immunoblotted with anti-phospho-S383 Elk1 antibodies (New England BioLabs, Beverly, MA).

**$^{32}$ P-Labeling of Cyclopentyl ADP (cpADP)**—Nucleoside diphosphate kinase (NDPK, 200 units, Sigma) and 800  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP were added in a 100-ml reaction containing HBS (150 mM NaCl, 20 mM HEPES, pH 7.4, and 5 mM MgCl<sub>2</sub>). The reaction was allowed to equilibrate at 30 °C for 5 min.  $^{32}$ P-Labeled NDPK was purified from free [ $\gamma$ - $^{32}$ P]ATP by two successive rounds of Microspin G50 columns (Amersham Biosciences, Piscataway, NJ). 1000 pmol of cpADP was added to the  $^{32}$ P-labeled NDPK in HBS, and the reaction was carried out for 20 min at 30 °C. After the reaction, contents were transferred to a Microcon-YM30 (Millipore, Bedford, MA) and centrifuged at 13,000 rpm for 15 min. With this procedure we usually obtained ~200  $\mu$ Ci of purified [ $\gamma$ - $^{32}$ P]cyclopentyl-ATP ([ $\gamma$ - $^{32}$ P]cpATP).

**Immunoprecipitations, Kinase Assays, and Two-dimensional Gel Electrophoresis**—100-mm dishes of COS-1 cells ( $3 \times 10^6$  cells) were transfected with 6  $\mu$ g of FLAG-ERK2 or FLAG-ERK2-QG and were allowed to recover overnight. Transfected cells were then serum-starved for 4–5 h and stimulated with EGF (10 ng/ml) for 10 min. Cells were washed once with cold phosphate-buffered saline and then lysed with hypotonic lysis buffer at 4 °C (20 mM HEPES, pH 7.4, 2 mM EGTA, 2 mM MgCl<sub>2</sub>, 200 mM sodium orthovanadate, 2  $\mu$ g/ml aprotinin, 0.4 mM microcystin, and 2 mM phenylmethylsulfonyl fluoride). The lysate was clarified by centrifugation at 13,000 rpm in a microcentrifuge for 20 min. ERK2 or ERK2-QG were immunoprecipitated with anti-FLAG-M2-agarose beads (Sigma). Immunoprecipitates were washed three times with hypotonic lysis buffer. Kinase assays were performed with immunoprecipitated proteins in a 40- $\mu$ l reaction volume containing 25 mM HEPES, pH 7.4, 20 mM magnesium acetate; 1 mM dithiothreitol, 1 mM ATP, or cpATP; and 10  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP or [ $\gamma$ - $^{32}$ P]cpATP for 15 min at 30 °C. Reactions were stopped by addition of an equal volume of 2 $\times$  SDS-sample buffer and followed by boiling for 5 min. 20  $\mu$ l of these reactions was analyzed on a 10% SDS-PAGE gel. The resolved proteins were visualized by the Vorum silver staining protocol (29), and the gel was autoradiographed by exposing to Kodak Biomax MS film (Eastman Kodak, Rochester, NY). Remaining samples were clarified of M2-agarose beads by passing through wizard columns (Promega, Madison, WI), and the proteins were precipitated with 5 volumes of cold acetone. Precipitated proteins were resuspended in 260  $\mu$ l of two-dimensional sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% dithiothreitol, and 1% IPG buffer (pH 4–7, Amersham Biosciences)) and resolved on Immobiline Dry Strips (Amersham Biosciences, pH 4–7 linear (L), 13 cm) for 28,000 V-h. The proteins were resolved on 10% SDS-PAGE gels in the second dimension. Silver staining and autoradiography were then performed on the gels.

**Identification of Novel ERK2 Substrates**—Four 150-mm dishes of COS-1 cells ( $6 \times 10^6$  cells/dish) transfected with pCDNA3 or FLAG-ERK2-QG were used. Lysis and immunoprecipitation was performed as described above. Immunoprecipitated ERK2 and associated proteins were eluted from the M2-agarose beads with FLAG peptide and analyzed on 8% SDS-PAGE. Labeled proteins were excised from the gel and microsequenced using a Finnigan LCQ ion trap mass spectrometer with a protana nanospray ion source (W. M. Keck Biomedical Mass Spectrometry Laboratory, University of Virginia).

**In Vitro Kinase Reaction and Phosphoamino Acid Analysis**—COS-1 cells were transfected with either FLAG-ERK2, FLAG-ERK2-QG, pCMV-Tag-EDD (FLAG-EDD), or pCMV-Tag2B. After overnight recovery, cells were serum-starved for 5 h. The cells were stimulated with EGF (10 ng/ml) for 10 min and lysed in hypotonic lysis buffer, and FLAG-ERKs were immunoprecipitated with anti-FLAG-M2-agarose beads. Immunoprecipitated proteins were washed three times with hypotonic lysis buffer. Immunoprecipitated FLAG-EDD or pCMV-Tag2B (control) was mixed with immunoprecipitated ERK2 or ERK2-QG, and kinase reactions were carried out as described above with [ $\gamma$ - $^{32}$ P]cpATP for 3 min. For time course kinase reactions, immunoprecipitated proteins were eluted from the M2-agarose beads with FLAG peptide prior to mixing them. Kinase reactions were performed as mentioned above with [ $\gamma$ - $^{32}$ P]cpATP. Samples were resolved on 8% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes followed by autoradiography. Bands corresponding to labeled EDD were excised, and phosphoamino acid analysis was performed as described (30).

**Metabolic Labeling and Phosphopeptide Analysis**—100-mm dishes of COS-1 cells ( $\sim 3 \times 10^6$  cells) were transfected with 7  $\mu$ g of FLAG-EDD and 1  $\mu$ g of HA-ERK2 and were allowed to recover overnight. Cells were

serum-starved for 1 h in Dulbecco's modified Eagle's medium followed by phosphate-free RPMI (Invitrogen) for 1 h. Cultures were then labeled in phosphate-free RPMI containing 3 mCi/ml carrier-free  $^{32}\text{P}$  (PerkinElmer Life Sciences) for 3 h followed by stimulation with EGF (10 ng/ml) for 10 min. Cultures treated with the MEK inhibitor UO126 were incubated with 50  $\mu\text{M}$  inhibitor during the 3-h labeling period and during EGF stimulation. Cells were lysed in M2 lysis buffer (27) followed by immunoprecipitation with anti FLAG-M2-agarose beads. Immunoprecipitates were washed five times with M2 lysis buffer, resolved on 8% SDS-PAGE gels, and transferred to nitrocellulose membranes followed by autoradiography. Bands corresponding to the labeled EDD were excised, and tryptic peptides were analyzed by two-dimensional separation on thin-layer cellulose plates (30).

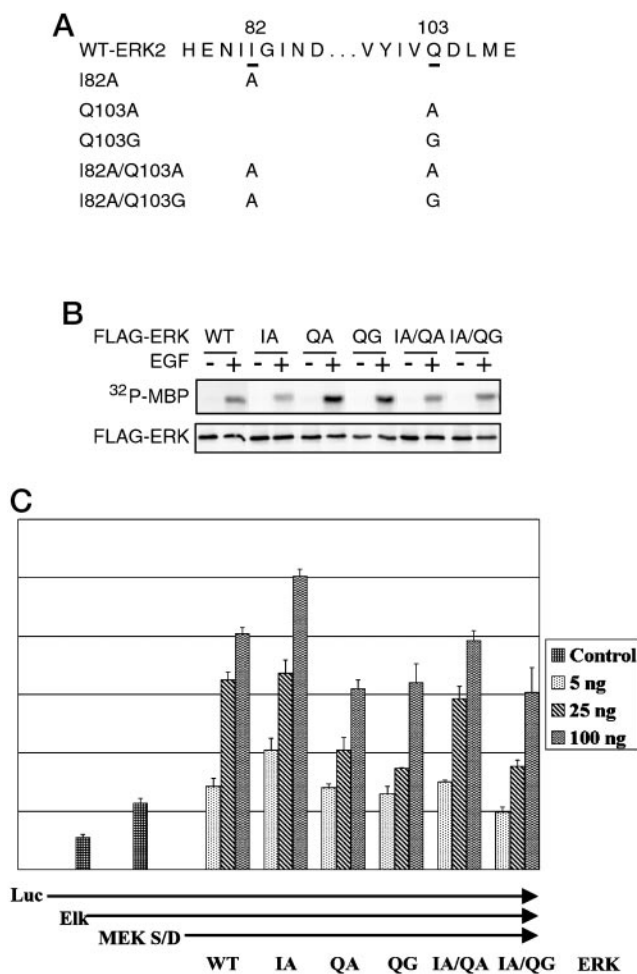
## RESULTS

Based on the crystal structure of ERK2 bound to ATP (31) and sequence alignment of the ATP binding site of several protein kinases (18), we observed that both isoleucine 82 and glutamine 103 are at conserved positions that come into close contact with the N-6 position of adenine in ATP. We hypothesized that mutation of one or both of these residues to either alanine or glycine would generate a larger ATP binding pocket enabling the mutant kinase to accept and utilize ATP analogs with bulky side groups at the N-6 position. We therefore generated five ERK2 mutants that contained either single or double mutations of these residues to either alanine or glycine. The five mutants generated were: ERK2 I82A; ERK2 Q103A; ERK2 Q103G; ERK2 I82A/Q103A; and ERK2 I82A/Q103G (Fig. 1A). All ERK2 constructs encoded an amino-terminal FLAG tag.

We first examined whether mutation of these residues affected the ability of ERK2 to phosphorylate its substrates *in vitro* and *in vivo*. ERK2 or an ERK2 mutant was transfected into COS-1 cells, and the cells were stimulated with EGF. *In vitro* kinase assays were performed with immunoprecipitated ERK2 and the ERK2 mutants using myelin basic protein (MBP) and [ $\gamma$ - $^{32}\text{P}$ ]ATP as substrates. ERK2 and the mutants had roughly equivalent levels of kinase activity (Fig. 1B), although alanine mutations at isoleucine 82 somewhat inhibited ERK2 kinase activity toward MBP. Similar experiments using serum stimulation or co-transfected mutationally activated MEK1 (MEK1 S218D/S222D), demonstrated that these mutants could be phosphorylated and activated by activated MEK1 (data not shown).

To determine if the ERK2 mutants could signal to a physiological ERK2 substrate *in vivo*, GAL4-Elk1 luciferase assays were performed. This system utilizes a plasmid containing a luciferase gene with five GAL4 binding sites in the promoter and a plasmid encoding a fusion of the DNA-binding domain of GAL4 and the ERK-responsive transactivation domain of Elk1 (28). MEK1 S218D/S222D was co-transfected to activate both endogenous and transfected ERK. Co-transfection of ERK2 or an ERK2 mutant stimulated luciferase expression in a dose-dependent manner (Fig. 1C). These data demonstrate that mutation of one or both residues in the ATP binding site of ERK2 did not substantially affect the localization, substrate recognition, or kinase activity of ERK2.

We next needed to identify the optimal pairing between an ATP binding site mutant and an ATP analog. To do this, we screened each of our ERK mutants with a panel of seven ATP analogs. We first determined the ability of each analog to inhibit the capacity of ERK2 or an ERK2 mutant to phosphorylate MBP with [ $\gamma$ - $^{32}\text{P}$ ]ATP. If an ATP analog was able to compete with [ $\gamma$ - $^{32}\text{P}$ ]ATP for an ERK2 mutant, incorporation of radiolabeled phosphate into MBP would be inhibited. ERK2 or an ERK2 mutant was immunoprecipitated from transfected COS-1 cells stimulated for 10 min with EGF. The FLAG immunoprecipitates were aliquoted equally and used in a kinase reaction containing MBP, 10  $\mu\text{M}$  ATP, and 10  $\mu\text{Ci}$  of [ $\gamma$ - $^{32}\text{P}$ ]ATP, with or without 100  $\mu\text{M}$  ATP analog. The reactions



**FIG. 1. ERK2 ATP binding site mutants.** A, ERK2 ATP binding site mutants containing an amino-terminal FLAG tag and mutations at isoleucine 82 and glutamine 103. B, COS-1 cells were transfected with ERK2 or an ERK2 mutant. The cells were serum-starved for 5 h before stimulation with 10 ng/ml EGF for 10 min. FLAG-ERKs were immunoprecipitated and tested for their kinase activity toward myelin basic protein (MBP). C, ERK2 mutants can phosphorylate and activate Elk1 *in vivo*. COS-1 cells were transfected with 5X Gal4-luciferase, Gal4-Elk1, MEK1 S218D/S222D, and ERK2 or an ERK2 mutant. ERK2 plasmids were transfected at 5, 25, and 100 ng. The cells were serum-starved after the transfection and harvested for luciferase assays 24 h post transfection.

were resolved on a gel and transferred, and incorporation of  $^{32}\text{P}$  into MBP was quantified by Cerenkov counting. Comparison of reactions containing an ATP analog (Fig. 2, lanes 2–8 in each panel) to control reactions without analog (Fig. 2, lane 1 in each panel) demonstrated that none of the analogs inhibited the ability of wild-type ERK2 to utilize ATP. By contrast, many of the ATP analogs had varied abilities to compete with [ $\gamma$ - $^{32}\text{P}$ ]ATP for phosphate incorporation into MBP, suggesting that some of the ERK2 mutants were able to utilize or at least bind to the ATP analogs. Mutation of isoleucine 82 alone had little effect on the susceptibility of ERK2 to inhibition by an analog. Mutation of glutamine 103 to alanine increased the susceptibility of ERK2 to inhibition by an analog, whereas mutation of this residue to glycine had the greatest effect on the ability of an analog to inhibit  $^{32}\text{P}$  incorporation into MBP. The Q103G mutation (either by itself or in conjunction with the I82A mutation) demonstrated a significant reduction of  $^{32}\text{P}$  incorporation into MBP when kinase reactions were performed in the presence of analog. These results suggested that residue 103 of ERK2 was the critical residue in controlling the capacity of ERK2 to accept an ATP analog with a bulky N-6 substituent.

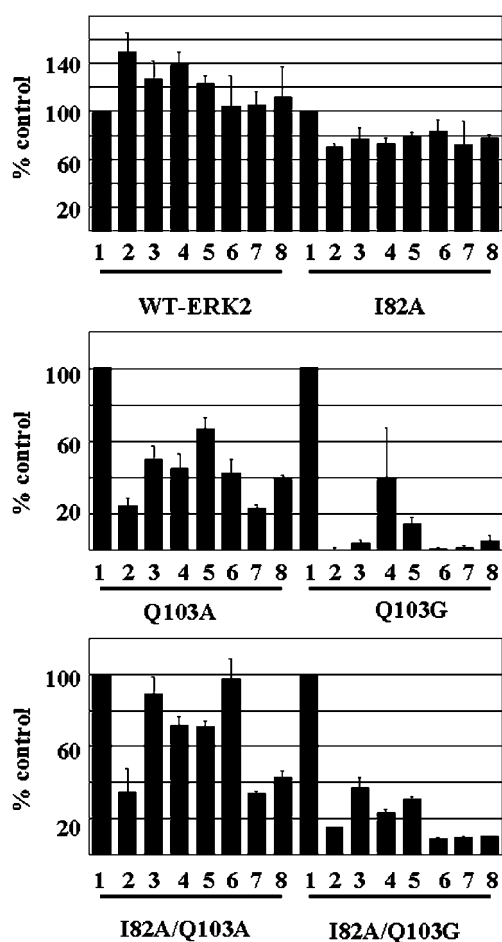


FIG. 2. **Analog inhibition assay.** FLAG-ERK2 plasmids were transfected into COS-1 cells. The cells were serum-starved and stimulated with 10 ng/ml EGF for 10 min. FLAG-ERKs were immunoprecipitated, and each was aliquoted into eight sets of duplicate kinase reactions. Kinase reactions were performed with MBP, 10  $\mu$ M ATP, 10  $\mu$ Ci/reaction [ $\gamma$ - $^{32}$ P]ATP, without (lane 1) or with (lanes 2–8) 100  $\mu$ M ATP analog. ATP analogs were: *N*-6-benzyl ATP (lane 2), *N*-6-cyclopentyl-ATP (lane 3), *N*-6-(*p*-methyl)benzyl-ATP (lane 4), *N*-6-(1,3-dimethyl)butyl-ATP (lane 5), *N*-6-(3,3-dimethyl)butyl-ATP (lane 6), *N*-6-(2-phenethyl)-ATP (lane 7), and *N*-6-(1-methyl)butyl-ATP (lane 8). The reactions were performed at 30 °C for 10 min, run on a gel, and transferred to nitrocellulose. MBP bands were excised and quantitated by Cerenkov counting. The values were normalized to a reaction without ATP analog (lane 1), which was set at 100%.

In particular, a substantial inhibition of  $^{32}$ P incorporation into MBP by ERK2 Q103G was observed with addition of either *N*-6-benzyl-ATP (98.8% inhibition, lane 2, middle panel, right side), *N*-6-cyclopentyl-ATP (95.2% inhibition, lane 3, middle panel, right side), *N*-6-(3,3-dimethyl)butyl-ATP (98.4% inhibition, lane 6, middle panel, right side), *N*-6-(2-phenethyl)-ATP (97.8% inhibition, lane 7, middle panel, right side), and *N*-6-(1-methyl)butyl-ATP (94.1% inhibition, lane 8, middle panel, right side).

There are two likely explanations for the inhibition of  $^{32}$ P incorporation into MBP by the addition of excess unlabeled ATP analog. First, the ATP analog could be utilized as an ATP source by the mutant ERK2 kinase to phosphorylate MBP, thus effectively competing with the [ $\gamma$ - $^{32}$ P]ATP for usage by the mutant ERK2. Alternatively, the kinase could bind the analog but not be able to utilize it, thus effectively blocking [ $\gamma$ - $^{32}$ P]ATP from the ATP binding site of the kinase. To differentiate between these two possibilities, we screened ERK2, ERK2-Q103G, and ERK2-I82A/Q103G for their ability to phosphorylate GST-Elk1 with either ATP or an ATP analog. The reactions

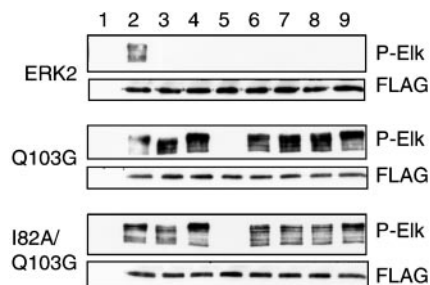
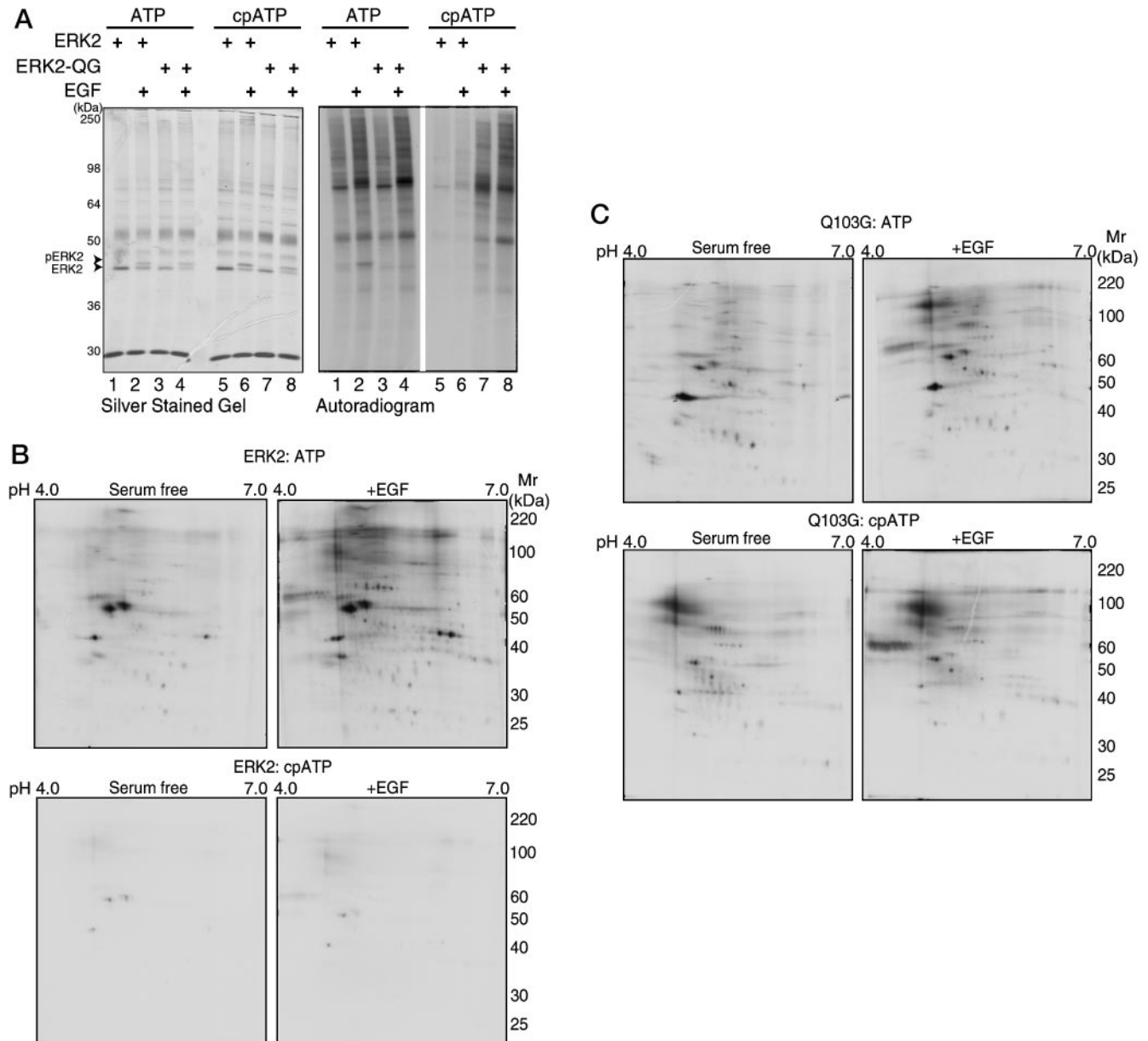


FIG. 3. **ERK2 phosphorylation of Elk1 with ATP analogs.** FLAG-ERK2 plasmids were transfected into COS-1 cells, and the cells were serum-starved before stimulation with 10 ng/ml EGF for 10 min. FLAG-ERKs were immunoprecipitated, aliquoted, and used in kinase reactions with GST-Elk1 and either 100 mM unlabeled ATP (lane 2) or 100 mM unlabeled ATP analog (lanes 3–9). The reaction in lane 1 contained GST-Elk1 and ATP but no ERK. ATP analogs were: *N*-6-benzyl-ATP (lane 3), *N*-6-cyclopentyl-ATP (lane 4), *N*-6-(*p*-methyl)benzyl-ATP (lane 5), *N*-6-(1,3-dimethyl)butyl-ATP (lane 6), *N*-6-(3,3-dimethyl)butyl-ATP (lane 7), *N*-6-(2-phenethyl)-ATP (lane 8), and *N*-6-(1-methyl)butyl-ATP (lane 9). The reactions were incubated at 30 °C for 10 min, resolved on a gel, and transferred to nitrocellulose. The membranes were then immunoblotted with antibodies to phospho-S383 Elk1.

were resolved on a gel and immunoblotted with an antibody that specifically recognizes phospho-S383 Elk1, the major site of phosphorylation by ERK2. Incubation of GST-Elk1 in the absence of ERK2 did not result in S383 phosphorylation (lane 1). All three ERK2 proteins were able to phosphorylate GST-Elk1 with normal ATP (Fig. 3, lane 2 in all *p*-Elk1 panels). However, wild-type ERK2 could not use any of the ATP analogs to phosphorylate GST-Elk1 (Fig. 3, top panel, lanes 3–9). These data support the previous result that none of the analogs could inhibit wild-type ERK2 from using [ $\gamma$ - $^{32}$ P]ATP to phosphorylate MBP.

Conversely, both ERK2-Q103G and ERK2-I82A/Q103G were able to effectively use several ATP analogs to phosphorylate GST-Elk1. Overall, ERK2-Q103G appeared to use the analogs more effectively than ERK2-I82A/Q103G, correlating with the inhibition assay in Fig. 2. ERK2-Q103G effectively used all of the ATP analogs tested with the exception of *N*-6-(*p*-methyl)benzyl-ATP. Although this analog appeared somewhat effective in the inhibition assay, it was the least efficient of the seven and was most likely an example of an analog that simply blocks the ERK2 ATP binding site. Based on the inhibition assay and the Elk1 phosphorylation assay, we decided to use ERK2-Q103G (henceforth designated ERK2-QG) along with cyclopentyl ATP (henceforth designated cpATP) (Fig. 3, middle panel, lane 4) in our attempts to identify novel ERK2 substrates.

**Detection of Direct Substrates of ERK2 with [ $\gamma$ - $^{32}$ P]cpATP—**To identify relevant substrates of ERK2, one would like to introduce labeled ATP analog into the live cells. However, cells are impermeable to ATP, and addition of labeled ATP analog to digitonin-permeabilized cells results in hydrolysis of the analog ATP within 1 min (32). Furthermore, the intracellular concentration of ATP is 3 mM (33), making it difficult to achieve analog concentrations sufficient to compete with ATP for kinases. Therefore, it was necessary to apply this technique to cell lysates or fractions. We chose to express the ERK2 and ERK2-QG (mutant ERK2) in COS-1 cells and then immunoprecipitate ERKs under non-stringent conditions where we would expect many ERK-binding proteins to remain associated. The immunoprecipitates were then used in a kinase reaction with either [ $\gamma$ - $^{32}$ P]ATP or [ $\gamma$ - $^{32}$ P]cpATP. In addition to ERK2 and ERK2-QG, other co-immunoprecipitated kinases would be capable of utilizing [ $\gamma$ - $^{32}$ P]ATP to phosphorylate co-immunoprecipitated substrates. However, reactions with



**FIG. 4. Detection of ERK2 substrates.** FLAG-ERK2 or FLAG-ERK2-QG plasmids were transfected into COS-1 cells. Cells were serum-starved for 4–5 h followed by stimulation with EGF (10 ng/ml) for 10 min. ERK2 and ERK2-QG were immunoprecipitated, and kinase reactions were performed with [ $\gamma$ - $^{32}$ P]ATP or [ $\gamma$ - $^{32}$ P]cpATP. **A**, kinase reactions were resolved on 10% SDS-PAGE followed by silver staining and autoradiography. ATP or cpATP labels on the top indicate kinase reactions carried out with [ $\gamma$ - $^{32}$ P]ATP or [ $\gamma$ - $^{32}$ P]cpATP, respectively. ERK2 and dually phosphorylated ERK2 (pERK2) are marked by arrows. **B**, ERK2 *in vitro* kinase reactions with [ $\gamma$ - $^{32}$ P]ATP or [ $\gamma$ - $^{32}$ P]cpATP analyzed on pH 4–7 linear (L) two-dimensional gels. **C**, ERK2-QG kinase reactions with [ $\gamma$ - $^{32}$ P]ATP or [ $\gamma$ - $^{32}$ P]cpATP analyzed on pH 4–7 L two-dimensional gels.

ERK2-QG and [ $\gamma$ - $^{32}$ P]cpATP should result in the phosphorylation of only those proteins that are direct ERK substrates. Therefore, we should detect fewer radiolabeled substrates in the reactions with [ $\gamma$ - $^{32}$ P]cpATP as compared with reactions with [ $\gamma$ - $^{32}$ P]ATP.

ERK2 or ERK2-QG from unstimulated or EGF-stimulated cells were immunoprecipitated, and associated proteins were labeled in an *in vitro* kinase reaction using either [ $\gamma$ - $^{32}$ P]ATP or [ $\gamma$ - $^{32}$ P]cpATP. The reactions were resolved on SDS-PAGE, silver-stained, and subjected to autoradiography (Fig. 4A). ERK2 and ERK2-QG proteins were expressed at similar levels and were activated efficiently after EGF stimulation (Fig. 4A, silver stain). Both ERK2 and ERK2-QG used [ $\gamma$ - $^{32}$ P]ATP very efficiently, generating similar patterns of phosphorylated substrates on SDS-PAGE (Fig. 4A, autoradiogram). Phosphorylation of substrates in the absence of EGF stimulation was most

likely due to the basal activity of ERK2. There were noticeable differences in the substrates recognized by ERK2 prior to and after EGF stimulation (Fig. 4A, autoradiogram; compare lane 1 with 2, lane 3 with 4, and lane 7 with 8). These differences are probably due to (i) differences in the association of substrates with ERK2 depending on the phosphorylation status of ERK2; (ii) dissociation of phosphorylated substrates from ERK2 after EGF stimulation *in vivo*; or (iii) prior phosphorylation of the substrate *in vivo* during the 10-min EGF stimulation.

When the kinase reactions were performed with [ $\gamma$ - $^{32}$ P]cpATP, there was a marked difference in the pattern of labeled substrates between reactions with ERK2 and ERK2-QG. Neither wild-type ERK2 nor its associated proteins could efficiently utilize [ $\gamma$ - $^{32}$ P]cpATP. The background phosphorylation with wild-type ERK2 that was detected with [ $\gamma$ - $^{32}$ P]cpATP was most likely due to trace contamination with [ $\gamma$ - $^{32}$ P]ATP

(~0.1%, data not shown) that occurred when generating [ $\gamma$ - $^{32}$ P]cpATP. At this level of electrophoretic resolution, few differences were detected in the substrates recognized by ERK2-QG in reactions with [ $\gamma$ - $^{32}$ P]ATP versus [ $\gamma$ - $^{32}$ P]cpATP (Fig. 4A, autoradiogram; compare lanes 3 and 4 with lanes 7 and 8). However, when the resolution of the gel systems was increased by using two-dimensional gels (Fig. 4, B and C), the advantages of the analog approach became evident.

ERK2 *in vitro* kinase reactions with [ $\gamma$ - $^{32}$ P]ATP or [ $\gamma$ - $^{32}$ P]cpATP resolved by two-dimensional gel electrophoresis are shown in Fig. 4B. These results further demonstrated that ERK2 and its associated proteins could not utilize [ $\gamma$ - $^{32}$ P]cpATP. *In vitro* kinase reactions using ERK2-QG were also analyzed on two-dimensional gels (Fig. 4C). Although the phosphorylation patterns between the [ $\gamma$ - $^{32}$ P]ATP and the [ $\gamma$ - $^{32}$ P]cpATP reactions were somewhat similar, we detected fewer substrates with [ $\gamma$ - $^{32}$ P]cpATP. The use of [ $\gamma$ - $^{32}$ P]cpATP decreased the overall level of phospho-proteins while enhancing ERK2-QG-specific phosphorylations. These results clearly demonstrate the advantage of using [ $\gamma$ - $^{32}$ P]cpATP for specific detection of the direct substrates of ERK2.

**Identification of Novel ERK2 Substrates**—After optimizing the conditions for the specific detection of direct substrates of ERK2-QG with [ $\gamma$ - $^{32}$ P]cpATP, we scaled up the procedure to detect co-immunoprecipitating substrates by mass spectrometry. Proteins co-immunoprecipitating with ERK2-QG were eluted along with ERK2 from M2 beads with FLAG peptide. Kinase reactions were then performed with [ $\gamma$ - $^{32}$ P]cpATP, and the reactions were resolved on an 8% SDS-PAGE (Fig. 5A). Two radiolabeled silver-stained bands, one at ~80 kDa and the other at ~250 kDa, were excised from the lane with a non-EGF-stimulated (serum-free) sample (Fig. 5A, lane 2) and were sequenced by mass spectrometry. Eleven peptides (16.3% coverage) corresponding to the ERK2 substrate Rsk1 (34) were detected in the 80-kDa band. The isolation of a known ERK2 substrate validated our methodology for detecting ERK2 substrates. Mass spectrometry analysis of the 250-kDa band revealed 14 peptides (7% total coverage) corresponding to the E3 ubiquitin ligase EDD (E3 identified by differential display). EDD is the human homolog of the *Drosophila* gene *hyperplastic discs* and was originally identified as a gene up-regulated in response to progesterin (21). EDD is a 300-kDa protein and contains a region that has extensive homology to HECT domain ubiquitin ligases (21).

In a similar experiment we detected a radiolabeled substrate (~250 kDa) that specifically associated with activated ERK2-QG (Fig. 5B). Mass spectrometric analysis detected 23 peptides (12.4% protein coverage) corresponding to the nucleoporin Tpr (translocated promoter region) (35, 24). Tpr is a nucleoporin that is localized within the nuclear pore complex and potentially has a role in nuclear protein export (25).

Identical samples were also analyzed on pH 3–10 linear (L) two-dimensional gels and silver-stained. Most of the radiolabeled protein spots that were obtained in kinase reactions were below the detection level, and hence we could not obtain any sequence data (data not shown). In addition to phosphorylated proteins, spots corresponding to proteins that were specifically associated with ERK2, but not phosphorylated, were core and sequenced by mass spectrometry. The substrates and ERK2-associated proteins identified are shown in Table I. Although *in vitro* phosphorylation of some of the associated proteins was not detected, they are still potential substrates in cells. To further validate our method of labeling ERK2-associated substrates, we attempted to confirm our results with one of the novel substrates we discovered.

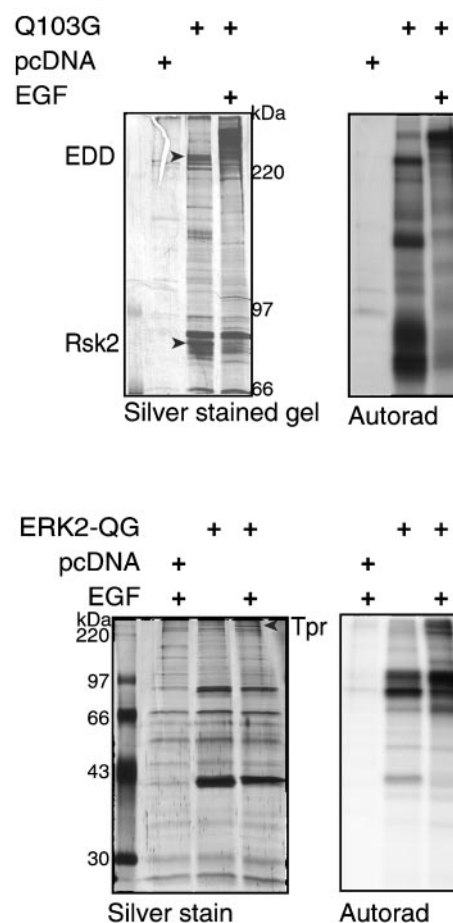


FIG. 5. **Identification of Rsk1, EDD, and Tpr.** pCDNA3.1 or ERK2-QG plasmids were transfected into COS-1 cells (four 150-mm dishes/lane,  $5\text{--}6 \times 10^6$  cells/dish at the time of transfection). Cells were serum-starved, stimulated with EGF, and lysed with hypotonic lysis buffer followed by immunoprecipitation with anti-FLAG-M2-agarose beads. Proteins were eluted with FLAG peptide and kinase reactions carried out with [ $\gamma$ - $^{32}$ P]cpATP. A, proteins were resolved on a 8% SDS-PAGE visualized by silver staining and subjected to autoradiography. Bands corresponding to 80-kDa Rsk1 and 250-kDa EDD are indicated by arrows. B, proteins were resolved on 12% SDS-PAGE, silver-stained, and subjected to autoradiography. The band corresponding to Tpr is indicated by an arrow.

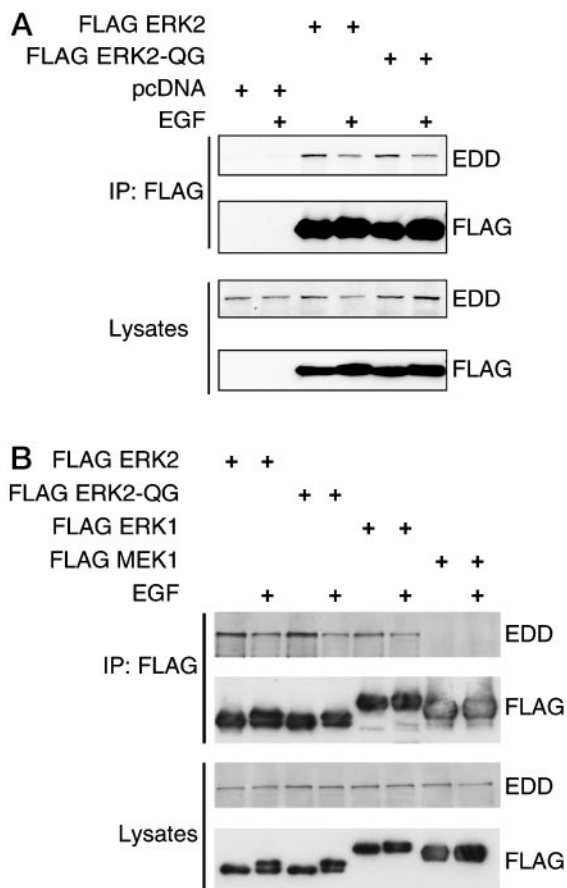
**EDD Associates Specifically with ERK2 and ERK1 but Not MEK1**—We chose to reconfirm our results with the novel substrate EDD. For this, ERK2 and ERK2-QG were immunoprecipitated from transiently transfected COS-1 cells, resolved on SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-FLAG and anti-EDD antibodies. Similar to our results in Fig. 5A, EDD associated more strongly with ERK2 and ERK2-QG in unstimulated versus EGF-stimulated cells (Fig. 6A, lane 3 versus 4 and lane 5 versus 6). To ensure that the interaction of EDD with ERK2 was specific, we immunoprecipitated ERK2, ERK2-QG, ERK1, and MEK1 from transiently transfected COS-1 cells and probed the blots with anti-FLAG and anti-EDD antibodies. ERK2, ERK2-QG, and ERK1 interacted with endogenous EDD (Fig. 6B); however, we could not detect any interaction between MEK1 and EDD. These results suggest that ERK2 and ERK1, but not MEK1, specifically associate with EDD.

**ERK2-QG Phosphorylates EDD *In Vitro***—To determine if ERK2-QG would phosphorylate EDD *in vitro*, FLAG-ERK2, FLAG-ERK2-QG, FLAG-EDD, and the vector control were immunoprecipitated from transiently transfected COS-1 cells. Immunoprecipitated ERK2 or ERK2-QG was mixed with im-

TABLE I  
Substrates and binding partners of ERK2

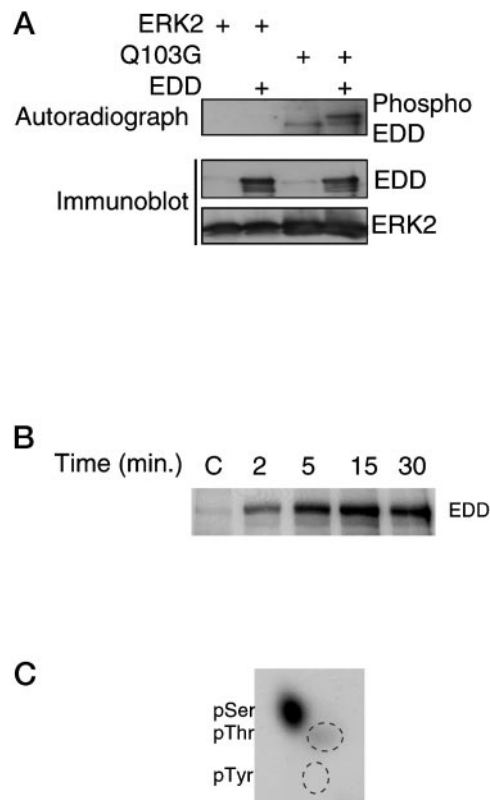
Proteins	Substrate	Binds to inactive ERK2	Binds to dually phosphorylated ERK2
Rsk1	Yes	Yes	Yes <sup>a</sup>
EDD	Yes	Yes	Yes <sup>a</sup>
Tpr	Yes	No	Yes
N-Aminoacylpeptide hydrolase	No	No	Yes
14.3.3 Epsilon	No	Yes	Yes
14.3.3 Zeta/Delta	No	Yes	Yes

<sup>a</sup> Binding was weaker when compared with that of inactive ERK2.



**FIG. 6. EDD associates specifically with ERK2, ERK1 but not MEK1.** *A*, cells transfected with pCDNA3.1, FLAG-ERK2, and FLAG-ERK2-QG were serum-starved for 4–5 h followed by stimulation with EGF. Cells were lysed and proteins were immunoprecipitated with FLAG-M2-agarose beads, resolved on 10% SDS-PAGE, and immunoblotted with anti-FLAG M2 or anti-EDD antibody (21). *B*, cells transfected with FLAG-ERK2, FLAG-ERK2-QG, FLAG-ERK1, and FLAG-MEK1 were serum-starved and stimulated with EGF. Cells were lysed with hypotonic lysis buffer, and proteins were immunoprecipitated with FLAG-M2-agarose beads, resolved on a SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with anti-EDD and anti-FLAG M2 antibodies.

munoprecipitated EDD or vector control and *in vitro* kinase reactions were carried out with [ $\gamma$ -<sup>32</sup>P]cpATP. Because ERK2 does not utilize [ $\gamma$ -<sup>32</sup>P]cpATP, we did not detect any phosphorylation with vector control or EDD (Fig. 7A; lanes 1 and 2). ERK2-QG was able to efficiently phosphorylate EDD *in vitro* using [ $\gamma$ -<sup>32</sup>P]cpATP (Fig. 7A, compare lane 4 versus 3). We then performed a time course of the *in vitro* kinase reaction with ERK2-QG and EDD. Labeling of EDD in the *in vitro* kinase reaction reached its peak by 15 min (Fig. 7B, lane 4) with a slight decrease at 30 min (Fig. 7B, lane 5). EDD has a total of ten SP and ten TP sites present in 2799 amino acids, and any of these sites can be potentially phosphorylated by ERK2. To narrow down the target sites, phosphoamino acid analysis of



**FIG. 7. *In vitro* labeling and phosphoamino acid analysis.** *A*, cells were transfected with either FLAG-ERK2, FLAG-ERK2-QG, FLAG-EDD, or CMV-Tag2B vectors. Cells were serum-starved, and ERK2 and ERK2-QG were stimulated with EGF (10 ng/ml) for 10 min. Proteins were immunoprecipitated with FLAG-M2-agarose beads, and ERK2 or ERK2-QG were mixed with EDD or vector control. Kinase reactions were carried out with [ $\gamma$ -<sup>32</sup>P]cpATP at 30 °C for 3 min. *B*, time course of *in vitro* kinase reactions. Immunoprecipitated FLAG-ERK2-QG was mixed with FLAG-EDD or vector control (control, C), and the kinase reactions were carried out for the time indicated above. In the control kinase reactions were performed for 15 min. *C*, phosphoamino acid analysis of *in vitro* labeled EDD from time course reactions. *p*, phosphorylated.

*in vitro* phosphorylated EDD was performed. Our results indicated that the bulk of the phosphorylation was on serine (Fig. 7C), although we did observe very faint phosphorylation of threonine.

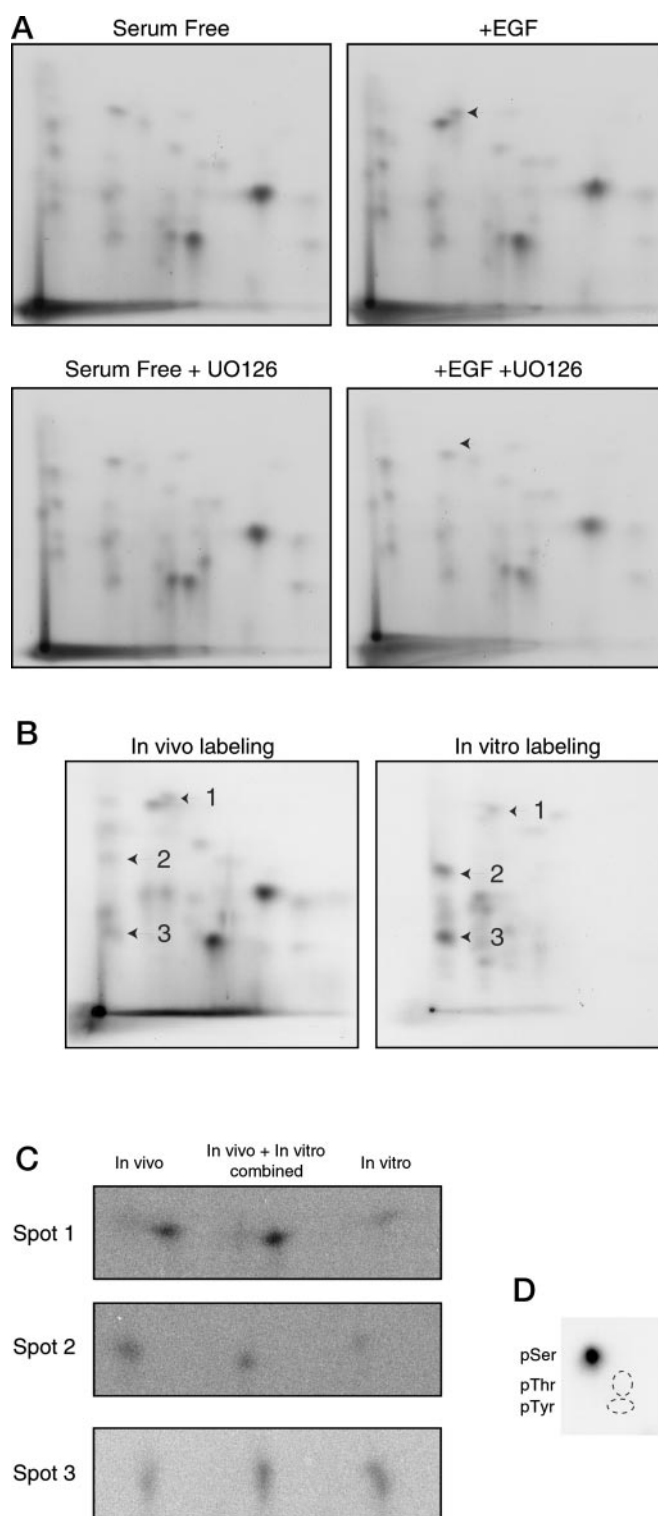
**ERK2 Phosphorylates EDD *In Vivo***—It was apparent that ERK2 phosphorylated EDD *in vitro* at one or more serine residues. However, it was important to know if ERK2 phosphorylated EDD *in vivo*. COS-1 cells transfected with FLAG-EDD and HA-ERK2 were metabolically labeled with or without EGF stimulation. In addition, these labeling reactions were carried out with or without pretreatment of the cells with the MEK1 inhibitor UO126. Two-dimensional tryptic phosphopeptide maps were analyzed to determine if there were any differences in the pattern of tryptic peptides with EGF treatment as compared with non-stimulated cells and if these changes were

reversible with UO126 pretreatment. Tryptic peptide maps obtained under various conditions are shown in Fig. 8A. These data demonstrate that EDD was phosphorylated on many sites in unstimulated COS-1 cells (serum-free, Fig. 8A). We detected the appearance of a new tryptic phospho-peptide with EGF stimulation (Fig. 8A, shown by an *arrow*). Pretreatment of cells with the MEK1 inhibitor UO126 abolished the emergence of this tryptic phospho-peptide, indicating that the phosphorylation was either by ERK2 or a downstream kinase. To determine which of these peptides were due to direct phosphorylation by ERK2, we compared the tryptic peptide maps of *in vivo* and *in vitro* labeled EDD (Fig. 8B). Our results show that only a few tryptic peptides were present in both *in vivo* and *in vitro* tryptic peptide maps. These were most likely phosphorylated directly by ERK2, whereas tryptic peptides that were present only in labeled EDD *in vivo* were probably a result of phosphorylation by other cellular kinases. Interestingly, the tryptic phospho-peptide that appeared after EGF stimulation was also detected with *in vitro* labeling (Fig. 8C, *spot 1*). Because these tryptic peptides were analyzed on different TLC plates, to be absolutely sure that the *in vitro* and *in vivo* labeled tryptic peptides, which are migrating at the same positions, were in fact the same peptides, we eluted them from the plates and re-analyzed them on the same TLC plate. Peptides 1, 2, and 3 (Fig. 8B) were eluted from *in vivo* and *in vitro* labeled EDD peptide maps and were spotted on the same TLC plate individually and in concert. Results obtained for the three different peptides are shown in Fig. 8C. It is evident from our results that peptides eluted from *in vivo* and *in vitro* labeled EDD migrate at the same position on the TLC plate, indicating that these peptides were identical. These results demonstrate that tryptic peptides 1, 2, and 3 are directly phosphorylated by ERK2, not a kinase downstream of ERK2 or any other cellular kinase. Phosphoamino acid analysis of *in vitro* labeled EDD demonstrated that the bulk of phosphorylation was on serine. The tryptic phosphopeptide, which appears following EGF stimulation (Fig. 8A, indicated by *arrow*, and Fig. 8B, *peptide 1*) also was phosphorylated on serine (Fig. 8D), which is in agreement with the phosphoamino acid analysis of *in vitro* labeled EDD (Fig. 7C).

#### DISCUSSION

To identify novel ERK2 substrates, we adopted the approach developed by Shokat and coworkers (18) that involves mutating the ATP binding site of a kinase of interest in a way that allows it to utilize modified analogs of ATP. Based on this strategy, we generated mutations in ERK2 at positions 82 and 103 in the ATP binding site. The mutations did not significantly inhibit ERK activation in response to growth factors or mutationally activated MEK. In addition, ERK2 substrate recognition and signaling were not noticeably affected by the mutations *in vitro* or *in vivo*. Our results demonstrate that glycine mutations at glutamine 103 were very effective at generating a larger ATP binding pocket, enabling this mutant, but not wild-type ERK2, to utilize several ATP analogs to phosphorylate Elk1.

To identify biologically relevant substrates, one would ideally introduce labeled ATP analogs into live or permeabilized cells. However, because cells are impermeable to ATP analogs and labeled ATP analogs are quickly hydrolyzed upon addition to digitonin-permeabilized cells (32) and because of the formidably high intracellular concentrations of ATP (33), the analog approach requires the use of lysed or fractionated cells. Heterogeneous nuclear ribonucleoprotein K was identified as a substrate of JNK using the ATP analog method in a cell lysate after the addition of recombinant modified JNK and a [ $\gamma$ - $^{32}$ P]ATP analog (20). Addition of large quantities of a kinase to a cell lysate could result in nonspecific or aberrant phospho-



**FIG. 8. Phosphopeptide maps of *in vivo* and *in vitro* labeled EDD.** A, phosphopeptide maps of *in vivo* labeled EDD. Cells were metabolically labeled with [ $^{32}$ P]orthophosphate for 3 h followed by stimulation with 10 ng/ml EGF for 10 min. For samples with the MEK inhibitor UO126, 50  $\mu$ M inhibitor was present during the 3-h labeling and during EGF stimulation. Immunoprecipitated EDD was digested with trypsin, and the resulting phospho-peptides were mapped by thin layer chromatography (30). B, comparison of phosphopeptide maps of *in vivo* and *in vitro* labeled EDD. Spots that were at similar positions on the TLC plates are indicated by *numbers* and *arrows*. C, peptides 1, 2, and 3 from *in vivo* and *in vitro* labeled EDD were eluted and loaded on the same TLC plate separately or together. These peptides were then mapped as described. D, phosphoamino acid analysis of EDD tryptic phosphopeptide that appears upon EGF stimulation (*in vivo* labeled EDD, peptide 1, Fig. 8B). *p*, phosphorylated.



rylation of substrates. Therefore, we chose to target only those substrates that were directly associated with ERK2. In our work, we expressed the mutant and wild-type ERK2 *in vivo* and then immunoprecipitated ERK2 with associated proteins, with the rationale that this subset of the proteome would more likely contain biologically relevant ERK2 substrates. These experiments allowed us to identify ERK substrates in both unstimulated and mitogen-stimulated cells. *In vitro* kinase reactions with wild-type ERK2 and ERK2-QG immunoprecipitates from serum-deprived and EGF-stimulated cells resulted in similar phosphorylation patterns when [ $\gamma$ - $^{32}$ P]ATP was used, demonstrating that the proteins had similar binding partners and substrate specificity. The use of [ $\gamma$ - $^{32}$ P]cpATP with wild-type ERK2 resulted in minimal incorporation of  $^{32}$ P into proteins, demonstrating that wild-type ERK2 and its associated proteins could not utilize [ $\gamma$ - $^{32}$ P]cpATP. However, the use of [ $\gamma$ - $^{32}$ P]cpATP with ERK2-QG resulted in robust phosphorylation of associated proteins. Interestingly, the labeling pattern obtained from ERK2-QG revealed some spots that co-migrated with spots when [ $\gamma$ - $^{32}$ P]ATP was used, as well as the disappearance of some spots and the appearance of others. The disappearance of some labeled spots suggests that these phosphorylations were carried out by associated kinases in the presence of [ $\gamma$ - $^{32}$ P]ATP. The appearance of new putative substrates in the presence of [ $\gamma$ - $^{32}$ P]cpATP demonstrates that by using an ATP analog that can only be utilized by ERK2-QG, there was an enhancement of the "signal to noise" ratio, presumably because the ERK2-QG no longer competes with co-immunoprecipitating kinases for ATP. The increased intensity of these minor spots strongly supports the advantage of using the ATP analog system to identify substrates.

Comparison of the labeling pattern of substrates by ERK2-QG in the unstimulated and stimulated states revealed differences in labeling of associated proteins. Although some proteins were labeled under both conditions, we often observed an enhanced labeling of substrates by ERK2 immunoprecipitated from unstimulated cells. Although at first counterintuitive, these results could be due either to an EGF-stimulated decrease in association of the ERK and the substrate (*e.g.* note the decreased association with Rsk1 in Fig. 5A, *left panel*) or an increased intracellular phosphorylation of the substrate after EGF stimulation.

Several ERK binding partners in unstimulated cells, including MEK1 and Rsk1, are also ERK substrates. The main ERK2 phosphorylation site on MEK1, Thr-292, is constitutively phosphorylated in cells, and phosphorylation is not stimulated by growth factor treatment (36). Moreover, ERK leaves its MEK anchor following growth factor stimulation. For both of these reasons, we are unlikely to detect a significant phosphorylation of MEK1 in our assay. However, the most prominent phosphorylated protein band from unstimulated cells was a ~90-kDa protein that was identified as Rsk1 (34). This served to validate the assay, demonstrating that the assay allowed identification of a known ERK substrate.

The nucleoporin Tpr was identified as an ERK2 substrate in EGF-stimulated cells, but not in unstimulated cells. Tpr localizes to the nuclear pore complex. Although the function of Tpr in this complex is unclear, it may play a role in the nuclear export of proteins (25). ERK proteins are known to dimerize (6) and translocate to the nucleus (7, 8) by transport in an active, Ran-dependent manner, as well as by passive diffusion (37), although the exact mechanisms remain unknown. The interaction with and phosphorylation of Tpr by ERK following growth factor stimulation raises intriguing possibilities for both Tpr function and ERK localization. ERK phosphorylation of Tpr may play a role in Tpr-regulated transport of proteins out of the

nucleus. Alternatively, ERK association with Tpr may regulate the nuclear import or export of ERK. The association of ERK2 with Tpr after EGF stimulation, but not in unstimulated cells, suggests that ERK may interact with Tpr as ERK traverses the nuclear pore.

Although the ubiquitin ligase EDD was found associated with ERK2 under both conditions, it was primarily labeled in kinase reactions from unstimulated cells. EDD was exclusively found in this phosphorylated band. The absence of phospho-labeled EDD in reactions from EGF-stimulated cells suggests that this protein was rapidly phosphorylated by ERK2 in the cells during the 10 min of EGF stimulation. Indeed, *in vivo* orthophosphate labeling experiments revealed the appearance of a MEK-dependent phospho-peptide in EDD 10 min after EGF stimulation. *In vitro* phosphorylation of EDD with ERK2-QG generated several phospho-peptides that co-migrated with those from the *in vivo* labeling. In particular, *in vitro* phosphorylation of EDD generated a phospho-peptide that co-migrated with the MEK-dependent *in vivo* labeled peptide. These *in vivo* data confirm the conclusion that EDD is a substrate for ERK2.

Both the presence of a HECT domain in the carboxyl terminus of EDD and the ability of a cysteine residue in this region to bind ubiquitin suggest that EDD acts as a ubiquitin ligase and ERK2 phosphorylation of EDD may affect this activity of EDD. Another possibility is that EDD affects the ubiquitination of ERK2. ERK2 was recently shown to be ubiquitinated and degraded in response to osmotic shock induced by sorbitol treatment (38). Although the ubiquitin ligase that induces ERK ubiquitination is unidentified, the association of EDD with ERK2 in the basal state may provide a mechanism by which ubiquitin-mediated degradation of ERK occurs.

In conclusion, we have created a mutant ERK2 kinase that can utilize an analog of ATP to specifically label ERK2 substrates. These results not only identify two novel ERK substrates, the nucleoporin Tpr and the ubiquitin ligase EDD, but also provide a framework by which many other ERK substrates can be identified.

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## Identification of Novel ERK2 Substrates through Use of an Engineered Kinase and ATP Analogs

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