The Docking Interaction of Caspase-9 with ERK2 Provides a Mechanism for the Selective Inhibitory Phosphorylation of Caspase-9 at Threonine 125

Caspase-9 plays a critical role in the initiation of apoptosis by the mitochondrial pathway. Activation of caspase-9 is inhibited by phosphorylation at Thr125 by ERK1/2 MAPKs in response to growth factors. Here, we show that phosphorylation of this site is specific for these classical MAPKs and is not strongly induced when JNK and p38α/β MAPKs are activated by anisomycin. By deletion and mutagenic analysis, we identify domains in caspase-9 and ERK2 that mediate their interaction. Binding of ERK2 to caspase-9 and subsequent phosphorylation of caspase-9 requires a basic docking domain (D domain) in the N-terminal prodomain of the caspase. Mutational analysis of ERK2 reveals a 157TTCD160 motif required for recognition of caspase-9 that acts independently of the putative common docking domain. Molecular modeling supports the conclusion that Arg10 in the D domain of caspase-9 interacts with Asp160 in the TTCD motif of ERK2. Differences in the TTCD motif in other MAPK family members could account for the selective recognition of caspase-9 by ERK1/2. This selectivity may be important for the antiapoptotic role of classical MAPKs in contrast to the proapoptotic roles of stress-activated MAPKs.

Apoptosis is an evolutionarily conserved form of cell death that plays a fundamental role in development and tissue homeostasis in multicellular organisms (1). Caspases, a family of cysteine aspartyl proteases, play a central role in the implementation of apoptosis (2). Caspases are synthesized as inactive zymogens (procaspases) that are activated by oligomerization and/or proteolytic processing to form active enzymes in response to apoptotic stimuli (2). In the intrinsic or mitochondrial pathway of apoptosis induced by a wide variety of stress or damage signals, cytochrome c is released from the mitochondria into the cytosol, where it binds to Apaf-1 (apoptotic protease-activating factor 1) and induces its oligomerization (3, 4).

This leads to the recruitment of procaspase-9, promoting its dimerization, activation, and autocatalytic processing (5, 6). Caspase-9 then initiates a proteolytic cascade, cleaving and activating downstream effector caspases, such as caspase-3 and caspase-7. These effector caspases further promote cytochrome c release in a positive feedback mechanism and directly catalyze the cleavage of key structural and regulatory proteins that results in the morphological and biochemical changes associated with apoptotic cell death (7).

Signal transduction pathways activated by extracellular and intracellular stimuli can impinge on the intrinsic apoptotic pathway and control cell fate. Abnormal or constitutive activation of cell signaling pathways may lead to tumorigenesis through inappropriate suppression of cell death (8, 9). One important control point of apoptosis targeted by cell signaling pathways downstream of cytochrome c release is at the level of the apoptosome (10). Several signal transduction pathways modulate apoptosome function through phosphorylation of caspase-9 or Apaf-1 (11–14). Notably, we have shown previously that caspase-9 is directly inhibited through phosphorylation by ERK1/2 mitogen-activated protein kinases (MAPKs)4 at Thr125 in response to epidermal growth factor (EGF) or a phorbol ester, TPA (12).

ERK1/2 are classical MAPKs that are activated mainly by growth factors or mitogenic stimuli. They form part of the larger MAPK family that also includes the c-Jun N-terminal kinases (JNKs) and p38 kinases, which are activated by a variety of cellular stresses as well as extracellular stimuli (15). Individual MAPKs have different biological functions that are determined not only by their selective activation in response to different stimuli but also by their distinct substrates (16). All MAPKs preferentially catalyze the phosphorylation of substrates containing the minimal consensus sequence Ser/Thr-Pro, which is recognized by the active site of the kinase (17). Additional interactions are mediated by docking motifs in substrates, often located distant from the site of phosphorylation, which provide high affinity and selective recognition by particular MAPKs (18, 19).

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4 The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular regulated kinase; JNK, Jun N-terminal kinase; RSK, ribosomal S6 kinase; OA, okadaic acid; CD, common docking; KIM, kinase interaction motif; GST, glutathione S-transferase; TPA, 12-0-tetradecanoyl-phorbol-13-acetate; EGF, epidermal growth factor; HA, hemagglutinin; MBP, myelin basic protein; WT, wild type.

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In general, activation of ERK MAPKs is associated with suppression of apoptosis, whereas JNK and p38 MAPKs are involved in the induction of apoptosis (20). Although these roles may depend on cell type and stimulus and are likely to be integrated with the control of cell growth and the cell division cycle, which may even have antagonistic effects on cell survival, this suggests that distinct MAPKs are likely to have different effects on proteins involved in the control of apoptosis, such as caspase-9. Here, we have investigated the recognition of caspase-9 by MAPKs. We show that Thr125 in caspase-9 is selectively phosphorylated by ERK1/2 but not JNK or p38α/β. We identify a basic docking motif (D domain) in the prodomain of caspase-9 that regulates the binding and phosphorylation of caspase-9 by ERK1/2. We also demonstrate a requirement for a TTCD motif in ERK2, which is not conserved in p38 or JNK, for mediating efficient phosphorylation of caspase-9. Molecular modeling indicates that there is a direct interaction between the D domain of caspase-9 and the TTCD motif in ERK2. These findings uncover the molecular basis for the selective phosphorylation and regulation of caspase-9 by ERK1/2, providing a mechanism by which ERK1/2 may selectively suppress apoptosis in contrast to JNK and p38α/β MAPKs.

MATERIALS AND METHODS

Plasmid Constructs and Site-directed Mutagenesis—Human caspase-9 was amplified from U2OS cells and cloned into pcDNA3 (Invitrogen). To replace serine residues in caspase-9 with alanine residues, we used the QuikChange™ site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) as indicated by the manufacturers. The mutations were verified by sequence analysis.

For expression of recombinant proteins, cDNAs were subcloned into pGEX-4T-1 (Amersham Biosciences). Expression of recombinant proteins was induced in Escherichia coli BLR (DE3) at 30 °C for 2 h by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. Glutathione S-transferase (GST)-tagged proteins were affinity-purified with glutathione-Sepharose 4B (Amersham Biosciences) before elution in buffer (10 mM Hepes-KOH at pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μg ml⁻¹ each of aprotinin, leupeptin, and pepstatin A) containing 100 mM glutathione. Glutathione was removed by filtration through a Sepharose 4B column before elution in buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM Na3VO4, 50 mM NaF, and 5 mM β-glycerophosphate) before boiling. Protein synthesis was measured as described previously (12). Secondary antibodies for Western blots were goat anti-rabbit or anti-mouse IgG coupled to horseradish peroxidase (Bio-Rad) or donkey anti-sheep IgG coupled to horseradish peroxidase (Sigma). For immunoprecipitation of HA-tagged proteins, an anti-HA-agarose conjugate was purchased from Sigma.

Other Chemicals—Okadaic acid, epidermal growth factor, UO126, and G418 sulfate were purchased from Calbiochem. SB 203580 and phorbol 12-myristate 13-acetate were obtained from LC Laboratories (Woburn, MA). Ponasterone A was obtained from AXXORA (Nottingham, UK). Zeocin was purchased from Invivogen (San Diego, CA). PD184352 and active GST-ERK2 were gifts from Prof. D. Alessi (University of Dundee). Active His-ERK2 was a gift from Prof. S. Keyse (University of Dundee).

Cell Extracts—HeLa cell cytosolic (S100) extracts were purchased from Cilbiotech (Mons, Belgium) and were supplied in 10 mM Hepes-KOH, pH 7.5, 10 mM KCl, 10 mM MgCl₂, 0.5 mM dithiothreitol at a protein concentration of 8–12 mg ml⁻¹.

Phosphorylation of Recombinant GST-Caspase-9—0.5 μg of GST-caspase-9, inactivated by mutation of the catalytic cysteine (C287A), including proteins with additional residues changed in the N-terminal region, was incubated for 15 min at 30 °C with active His-ERK2 in kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂ and 1 mM dithiothreitol) containing 100 μM [γ-³²P]ATP (specific activity, 6 × 10⁸ cpm nmol⁻¹). The reaction was terminated by the addition of reducing SDS-PAGE loading buffer. Samples were subjected to SDS-PAGE followed by autoradiography.

Peptide Competition Assay—Recombinant GST-caspase-9 (0.5 μg) was incubated with purified active His-ERK2 and [γ-³²P]ATP for 15 min at 30 °C in 15 μl of kinase buffer with the indicated concentrations of wild type peptide (ADRRRLR-RCLRRLV), the R10A peptide (ADRRLLARCLRRLV), the R10A/R11A peptide (ADRRLAAACRRLV), and an unrelated control peptide R-DIS (CPKSKKVKSHRSHT). Caspase-9 phosphorylation was analyzed by SDS-PAGE and autoradiography. Gels were analyzed by staining with Coomassie Blue for visualization of caspase-9 to verify equal amounts in the reaction. For quantification of phosphorylation, protein bands were excised and analyzed by scintillation counting. Experiments were conducted three times, and a representative data set is shown.

GST Pull-down Assay—0.5 μg of GST-caspase-9 C287A, including proteins with additional residues changed in the N-terminal region, was incubated in HeLa cytosolic S100 extract with 1 μM okadaic acid for 60 min. GST proteins were recovered on glutathione-Sepharose beads (Amersham Biosciences) by incubating for 1 h at 4 °C with rotation. Beads were pelleted by centrifugation and washed three times in buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM Na₃VO₄, 50 mM NaF, and 5 mM β-glycerophosphate) before boiling in reducing SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and immunoblotted with antibodies to ERK1/2, phospho-ThrT255 caspase-9 or GST as indicated.

Inducible Expression of Caspase-9 in Cells—cDNAs of human caspase-9 (wild type or alanine-mutated at arginine 10) were subcloned into the pIND expression plasmid before cotransfection of recombinant pIND and pVgRXR into U2OS cells, in which we have previously characterized the regulation of caspase-9 by MAPKs. Western blots were goat anti-rabbit or anti-mouse IgG coupled to horseradish peroxidase (Bio-Rad) or donkey anti-sheep IgG coupled to horseradish peroxidase (Sigma). For immunoprecipitation of HA-tagged proteins, an anti-HA-agarose conjugate was purchased from Sigma.
of caspase-9 by ERK1/2 (12). Stably transfected cells were selected and maintained in DMEM supplemented with 10% fetal bovine serum and 100 μg ml⁻¹ penicillin/streptomycin together with 500 μg ml⁻¹ zeocin and 800 ng ml⁻¹ G418 sulfate. Transcriptional expression of exogenous caspase-9 was induced with the indicated concentrations of ponasterone A (AXXORA, Nottingham, UK).

**ERK2 Kinase Assay**—Transfections of HEK293 cells were performed using 10 μg of CsCl-purified HA-ERK2 plasmid DNA and 60 μl of Superfect according to the manufacturer’s instructions (Qiagen). After 3 h, culture medium was replaced with medium containing 10% serum. 20 h after transfection, cells were serum-starved overnight before being stimulated with 50 ng ml⁻¹ EGF for 5 min. Cells were lysed in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM Na₃VO₄, 50 mM NaF, 5 mM β-glycerophosphate, 1 μg ml⁻¹ leupeptin, 1 μg ml⁻¹ aprotonin, 1 μg ml⁻¹ pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 0.1% β-mercaptoethanol, and 1 mM okadaic acid) and HA-ERK2 immunoprecipitated with 40 μl of anti-HA agarose beads (Sigma) for 1 h at 4 °C with rotation. Beads were then pelleted by centrifugation and washed three times in lysis buffer. The washed beads were mixed with substrates in a kinase buffer (50 mM Tris-HCl at pH 7.5, 10 mM MgCl₂, and 1 mM dithiothreitol) containing 100 μM [γ-³²P]ATP (specific activity 6 × 10⁶ cpm nmol⁻¹) and incubated for 15 min at 30 °C. The reaction was terminated by the addition of reducing SDS-PAGE loading buffer. Samples were then subjected to SDS-PAGE followed by autoradiography. For quantification of phosphorylation, protein bands were excised and analyzed by scintillation counting. To ensure that differences in phosphorylation of caspase-9 and RSK by mutagenesis of ERK2 were not due to differences in kinase activity caused by mutation, quantification of phosphorylation was calculated taking into account any differences in activity toward myelin basic protein (MBP).

**Molecular Modeling**—Two Protein Data Bank structures, the caspase-9 prodomain (21, 22) and phosphorylated and activated rat ERK2 (23), were run through ClusPRO (24), a fully automatic docking software program that first docked the proteins based only on the shape complementarity (no electrostatics) and retained the top 20,000 structures. The complexes generated from the docking step were then rapidly screened, and the desolvation and electrostatic energies were calculated. The top 1500 electrostatic conformations and the top 500 desolvation conformations were kept for further analysis. For each of the 2000 conformations retained in the filtering stage, the best 10 were determined using a clustering algorithm that scores them based on free energy landscape calculations. PyMOL software (DeLano Scientific) was used to render models.

**RESULTS**

**Caspase-9 Is Phosphorylated at Thr¹²⁵ in Cells by ERK1/2 but Not by JNK or p38 MAPKs**—We have previously established that ERK1/2 directly phosphorylate and inhibit caspase-9 at a single major site, Thr¹²⁵ (12). We were interested to determine if other members of the MAPK family might also phosphorylate caspase-9 in cells. We transfected HEK293 cells with caspase-9 and stimulated them for 60 min with anisomycin, a protein synthesis inhibitor that activates the JNK pathway (25) and all isoforms of p38 MAPK (26). Although anisomycin stimulated the phosphorylation of both JNK at sites associated with its activation and its downstream target c-Jun, showing that the JNK pathway was indeed activated, no increase in phosphorylation of caspase-9 at Thr¹²⁵ was detected (Fig. 1A). By contrast, stimulation by the phorbol ester TPA resulted in ERK1/2 activation and subsequent phosphorylation of caspase-9 at Thr¹²⁵, as expected. TPA-stimulated phosphorylation of ERK1/2 and caspase-9 were blocked by co-incubation with the MEK1/2...
recombinant GST-caspase-9 proteins were incubated with wild-type (WT) or GST-caspase-9 with mutation of basic residues (arginines 7, 10, and 11) or two hydrophobic residues (leucines 14 and 16). Basic residues are highlighted in boldface type, and the hydrophobic \(\phi_1-X-\phi_3\) motif is underlined. C, schematic of caspase-9 with the putative docking domain located in the N-terminal prodomain, which also contains a CARD region (underlined, boldface type). The sites of proteolytic processing are indicated by small arrows.

FIGURE 3. Identification of a docking domain required for ERK MAPK phosphorylation of caspase-9. A, phosphorylation of GST-caspase-9 (WT) or N-terminally truncated GST-caspase-9 lacking the first 79 amino acids (\(\Delta 79\)) by active ERK2 and \([\gamma-\text{32P}]\text{ATP}.\) Caspase-9 phosphorylation was analyzed by SDS-PAGE and autoradiography (top). B, sequence alignment of known D domains in selected MAPK substrates aligned with the putative docking site in caspase-9 (residues 6–16). Basic residues are highlighted in boldface type, and the hydrophobic \(\phi_1-X-\phi_3\) motif is underlined. C, schematic of caspase-9 with the putative docking domain located in the N-terminal prodomain, which also contains a CARD region (underlined, boldface type). The sites of proteolytic processing are indicated by small arrows.

Anisomycin also activated the p38/SAP2K pathway in transfected HEK293 cells, resulting in phosphorylation of both p38 and the downstream substrate MAPKAP kinase-2. As expected, phosphorylation of MAPKAP kinase-2 was completely inhibited by SB203580, an inhibitor of p38\(\alpha\) and p38\(\beta\). SB203580 also partially inhibited phosphorylation of p38 itself, as previously described for the \(\beta\)-isoform (28, 29). However, no significant phosphorylation of caspase-9 at Thr\(^{125}\) occurred when p38 was stimulated (Fig. 1B). TPA-stimulated phosphorylation of caspase-9 at Thr\(^{125}\) was completely inhibited by PD184352 but not by SB203580. Together these results show that caspase-9 is selectively phosphorylated in cells by ERK1/2 but not by JNK or p38\(\alpha/\beta\) MAPKs.

**A Putative ERK Docking Site in the N-terminal Domain of Caspase-9**—We wished to elucidate the mechanism by which ERK1/2 specifically recognize and phosphorylate caspase-9. A clue was uncovered with the observation that phosphorylation of caspase-9 by recombinant ERK2 was substantially reduced upon removal of the first 79 amino acids of the prodomain region of caspase-9 (Fig. 2A). We therefore examined the sequence of caspase-9 in this region for potential ERK1/2 docking motifs that might promote recognition of the substrate. We identified amino acids 6–16 of caspase-9 as a putative MAPK docking motif known as a D domain or kinase interaction motif (KIM), which is characterized as a cluster of basic residues often situated N-terminal to a hydrophobic motif containing Leu, Ile, or Val separated by one amino acid (30, 31). Related sequences are found in a number of classical MAPK-interacting proteins, including substrates, regulators, and scaffolding proteins (Fig. 2B). This motif lies within the prodomain of caspase-9, which also contains a CARD region that is involved in interactions with Apaf-1 (Fig. 2C).

**Requirement of the D Domain of Caspase-9 for Binding and Phosphorylation by Endogenous ERK1/2**—To determine if this motif forms part of an ERK1/2 docking site (D domain), we mutated to alanine three residues in the basic cluster (arginines 7, 10, and 11) or two hydrophobic residues (leucines 14 and 16) that might form the \(\phi_1-X-\phi_3\) motif and tested the effect on phosphorylation by purified ERK2 of recombinant caspase-9 expressed as fusions with GST (Fig. 3A). In an in vitro kinase assay under conditions where phosphorylation was linear with respect to time, wild type caspase-9 was phosphorylated by ERK2, and this phosphorylation was dependent on Thr\(^{125}\), the site of phosphorylation. Phosphorylation of the triple arginine mutant (R7A/R10A/R11A) at Thr\(^{125}\) was strongly reduced...
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compared with the wild type protein, whereas the double leucine mutation (L14A/L16A) only slightly reduced phosphorylation. This indicates that the one or more basic amino acids within this region of caspase-9 are important for recognition by ERK2.

To examine whether the D domain of caspase-9 is required for the binding and phosphorylation by endogenous ERK1/2 under nearly physiological conditions, we tested the phosphorylation of recombinant caspase-9 in cell extracts that reproduce the regulation of caspase-9 in vitro (12). Previous studies have shown that incubation of such extracts with okadaic acid (OA), an inhibitor of PP1 and PP2A phosphatases, induces activation of endogenous ERK1/2 and phosphorylation of caspase-9 at Thr125 (12). As expected, we found that GST-caspase-9 (WT) recovered from cell extracts was phosphorylated on Thr125 in response to treatment with OA (Fig. 3B). Coprecipitation of ERK1/2 with caspase-9 was also stimulated by OA. Interestingly, mutation of the Thr125 phosphorylation site to the nonphosphorylatable amino acid alanine resulted in an increase in ERK1/2 binding. These results suggest that the interaction of ERK1/2 with caspase-9 is likely to be promoted by the phosphorylation and activation of ERK1/2, whereas subsequent phosphorylation of the substrate destabilizes the interaction. Phosphorylation of caspase-9 at Thr125 was strongly reduced by mutation of basic residues of the D domain to Ala (R7A/R10A/R11A), and this decrease in phosphorylation correlated with loss of ERK1/2 binding to caspase-9 containing Thr125 and a substantial decrease in binding to the T125A mutant, demonstrating that the basic residues in the D domain play an important role in the binding and phosphorylation of caspase-9 by ERK1/2 in cell extracts.

To investigate the sequence requirements of the D domain in more detail, residues were individually mutated to Ala, and recombinant proteins were purified for use as substrates (Fig. 4). Mutation of Arg-10 had a strong inhibitory effect, with Leu9 and Arg11 substantially reducing phosphorylation, indicating that these residues are particularly important for the recognition of caspase-9 by ERK2. Mutation of the hydrophobic residues Leu14 and Leu16 also partially inhibited phosphorylation, whereas one or both of these sites were mutated to Ala in peptides R10A and R10A/R11A, respectively. An unrelated peptide (R-DIS) was used as an additional control (Fig. 5A). Increasing concentrations of WT peptide were found to inhibit phosphorylation of caspase-9 by ERK2, whereas peptide R10A inhibited to a lesser extent (Fig. 5B). Peptide R10A/R11A produced very poor inhibition of phosphorylation, confirming the importance of these residues in the interaction of caspase-9 with ERK2. Together with mutational analysis of caspase-9, these results confirm the role of the D domain in caspase-9 for its recognition and phosphorylation by ERK2 in vitro.

The D Domain of Caspase-9 Is Essential for Mediating Phosphorylation by ERK1/2 in Cells—We extended this work on the role of docking interactions for regulating caspase-9 phosphorylation by ERK1/2 to studies on intact cells. In initial experiments, we failed to observe any necessity for the docking site for the phosphorylation of caspase-9 at Thr125 in cell lines transiently expressing caspase-9 (data not shown). However, since docking domains are thought to work by increasing the efficiency and specificity of phosphorylation by kinases, we were concerned that strong overexpression of caspase-9 and therefore a greatly increased substrate concentration may have swamped this level of regulation. We therefore opted for an inducible system that permitted expression of wild type caspase-9 and docking site mutants in cells at levels resembling that of the endogenous protein. Selective antibiotics were used to isolate human U2OS cell lines stably expressing the construct for either WT caspase-9 or docking site mutant caspase-9 (R10A). Expression of exogenous proteins was stimulated with increasing concentrations of the inducing agent ponasterone A (Pon A) (Fig. 6A). The exogenous caspase-9 proteins were HA-tagged to discriminate from endogenous caspase-9 and to allow their specific retrieval by immunoprecipitation. A concentration of 2.5 μM ponasterone A for 24 h was chosen, since this induced caspase-9 to levels comparable with those of the endogenous protein in both wild type and docking site mutant cell lines (Fig. 6B).

To test if the docking site was required for caspase-9 phosphorylation by ERK1/2 in cells, expression of wild type and mutant caspase-9 was induced for 24 h followed by overnight serum starvation. Cells were then stimulated for 5 min with EGF before lysates were harvested and proteins were recovered by HA-immunoprecipitation. Fig. 5B shows equal induction of wild type and docking site mutant caspase-9 prior to immunoprecipitation and shows that immunoprecipitation of HA-caspase-9 was efficient in both lysates. We found that
immunoprecipitated WT caspase-9 was phosphorylated on Thr\textsuperscript{125} in response to treatment with EGF, correlating with activation of ERK1/2. In contrast, there was minimal phosphorylation of caspase-9 mutated at the docking site, despite ERK1/2 being similarly activated. Furthermore, the MEK1/2 inhibitor UO126 inhibited phosphorylation of wild type caspase-9 in response to EGF, showing again that phosphorylation was dependent on the ERK1/2 pathway (Fig. 6C). Thus, the D domain of caspase-9 is required for its phosphorylation by ERK1/2 in cells in response to a physiological stimulus.

ERK2 Recognition of Caspase-9 Does Not Require the CD Domain or Docking Groove Residues but Involves a TCCD Motif in ERK2—Having identified a docking domain in caspase-9 as necessary for phosphorylation by ERK1/2, we wished to uncover the complementary regions of the kinase that participate in the interaction. This might reveal the basis for the selectivity of caspase-9 phosphorylation by ERK1/2. Several regions have been identified in MAPKs that play roles in docking interactions with other proteins (see Fig. 10 for a structural model of ERK2 indicating potential docking regions). Mutational studies with ERK2 have defined an acidic patch on the surface-exposed L16 loop of the kinase opposite to its catalytic cleft which acts as a MAPK conserved docking motif (CD site) common to substrates, activators, and inactivators (32).

To assess the relative importance of the CD domain in ERK1/2 docking to caspase-9, we made mutations in ERK2 at Asp\textsuperscript{316} and Asp\textsuperscript{319}, previously identified as key residues mediating CD interactions (Fig. 7A). Cells were transfected with the wild type and mutant ERK2 constructs for 24 h and then serum-starved before subsequent stimulation of the ERK1/2 pathway by EGF. Immunoprecipitation was then performed, followed by \textit{in vitro} kinase assays using recombinant caspase-9 or p90 ribosomal S6 kinase (RSK) as substrates, with immunoprecipitated ERK as the source of active kinase. As shown in Fig. 6B, both single and double mutations in the CD domain of ERK2 did not inhibit its ability to phosphorylate caspase-9. In contrast, these mutations almost entirely abolished RSK phosphorylation by ERK2, confirming previously published results (33). This shows that, unlike RSK, these residues in the CD domain are not important for ERK2 docking to caspase-9.

Chang \textit{et al.} (34) have identified a separate hydrophobic docking groove in p38\textit{\alpha} in which residues Ile\textsuperscript{116} and Gln\textsuperscript{120} are essential for recognition of a peptide containing a docking domain (Fig. 7A). Conservation of a docking groove similar to that of p38 has been proposed to exist in other MAPK family members (34). To examine the possibility that this type of interaction plays a role in the recognition of caspase-9 by ERK2, we

\begin{figure}[h]
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\caption{Inhibition of ERK-mediated caspase-9 phosphorylation by peptides mimicking the D domain. A, sequences of soluble synthetic peptides used; substitutions in peptides derived from caspase-9 are underlined. B, inhibition by docking site peptides of ERK2-catalyzed phosphorylation of caspase-9. Recombinant GST-caspase-9 (0.5 \mu g) was incubated with purified active His-ERK2 and \textsuperscript{32}P]ATP for 15 min at 30 °C with the indicated concentrations of WT, R10A, R10A/R11A, or R-DIS peptides. Caspase-9 phosphorylation was analyzed by SDS-PAGE and autoradiography (top). Gels were analyzed by staining with Coomassie Blue for visualization of caspase-9 to verify equal amounts in the reaction (bottom). Results are plotted as percentage phosphorylation relative to that observed in the absence of any added peptide. Experiments were conducted at least three times, and representative data from one experiment are shown.}
\end{figure}
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FIGURE 6. The D domain of caspase-9 is essential for mediating phosphorylation of caspase-9 at Thr$^{125}$ in cells by endogenous ERK1/2. A, inducible expression of exogenous wild type HA-caspase-9 (WT) or HA-caspase-9 mutated at Arg10 to Ala (10A) in stably transfected U2OS cell lines by stimulation with the indicated concentrations of the inducing agent ponasterone A (Pon A). B, Western blot analysis of endogenous levels of wild type HA-caspase-9 (WT) or mutated HA-caspase-9 (10A) induced in stably transfected U2OS cell lines by 2.5 μM ponasterone A. Samples are shown prior to (pre) or after (post) immunoprecipitation of HA-caspase-9. C, phosphorylation of caspase-9 by ERK1/2 stimulated by EGF is inhibited by mutation of Arg$^{10}$. WT or mutant R10A (10A) HA-caspase-9 expression was induced in U2OS stable cell lines by 2.5 μM ponasterone A for 24 h before serum starvation overnight. Cells were then stimulated for 5 min with 50 ng ml$^{-1}$ EGF before HA-caspase-9 was precipitated from cell lysates with an HA-antibody/agarose conjugate. Immunoprecipitates (IP) were immunoblotted with antibodies to phospho-Thr$^{125}$ caspase-9 or caspase-9 (top). Cell lysates were also immunoblotted with antibodies to phospho-ERK1/2 and ERK1/2 (bottom). Experiments were conducted at least three times and representative data from one experiment is shown.

mutated residues within the proposed docking groove of ERK2. Mutation of Leu$^{113}$ and Gln$^{117}$ failed to significantly inhibit caspase-9 phosphorylation by ERK. Indeed, we found that mutation of Gln$^{117}$ greatly increased phosphorylation. These mutations had little effect on ERK2 phosphorylation of RSK (Fig. 7B). Thus, key residues within the proposed hydrophobic docking groove are not required for ERK2 docking to either caspase-9 or RSK, which argues against this mechanism of interaction being important for the recognition of these substrates.

Last, we tested whether residues within an adjacent acidic patch of ERK2 might play a role in interaction with caspase-9. The sequence Thr$^{157}$.Thr$^{158}$.Cys$^{159}$.Asp$^{160}$ (157$^{	ext{TTCD}}$160$^{	ext{A}}$) located within this acidic patch is different from that found in p38α (Glu$^{160}$-Asp$^{161}$-Cys$^{162}$-Glu$^{163}$), which has been referred to as the ED site (35), whereas other important residues are largely conserved. In JNK1, the corresponding residues are Ser$^{161}$-Asp$^{162}$-Cys$^{163}$-Thr$^{164}$. This region may therefore play a role in the ability of different MAPKs to distinguish between substrates containing D domains (31, 35). We found that mutation of both Thr$^{157}$ and Thr$^{158}$ to Ala (T157A/T158A) greatly reduced phosphorylation of caspase-9 by ERK2 and had a lesser effect on the phosphorylation of RSK in vitro. Similarly, mutation of Thr$^{157}$ and Thr$^{158}$ to a more “p38-like” sequence (T157E/T158D) also dramatically decreased phosphorylation of caspase-9 by ERK2 (Fig. 8). This was in contrast to mutation to a more “JNK-like” sequence (T157S/T158D), which had no inhibitory effect on either the phosphorylation of caspase-9 or RSK.

Mutation of Asp$^{160}$ in ERK2 to Ala or Asn also substantially reduced phosphorylation of caspase-9 (Fig. 9). Similarly, mutation to Glu, as found in p38α at this position, or Thr, as in JNK2, greatly inhibited phosphorylation of caspase-9, showing the critical importance of Asp$^{160}$ in ERK2 for its ability to recognize and phosphorylate caspase-9. By contrast, although mutation of Asp$^{160}$ to Asn or Thr greatly decreased ERK2 phosphorylation of RSK, mutation to alanine or glutamate had little effect, so there are likely to be differences between the interaction of this region of ERK2 with caspase-9 and RSK. Together, these data show that the TTCD motif within an acidic patch on ERK2 can account for its ability, in contrast to p38α and JNK, to recognize and phosphorylate caspase-9 at Thr$^{125}$.

Molecular Modeling Indicates a Direct Interaction between the D Domain of Caspase-9 and the TTCD Motif in ERK2—We used the ClusPro program developed by Comeau et al. (24) as a nonbiased predictor of complex formation between phosphorylated and active ERK2 (23) and the prodomain of caspase-9 (21, 22). The best fit model, derived from shape complementarity and then desolvation and electrostatic energies, showed an interaction involving the D domain of caspase-9 with ERK2, consistent with our identification of this domain in caspase-9 by mutational and deletion analysis (Fig. 10). The total solvent-accessible surface area buried between the two domains is 1489 Å, a value that is comparable with those observed for the formation of stable protein-protein interfaces (e.g. an antibody-antigen complex). Hydrogen bonds involving residues Arg$^{16}$ with Gln$^{117}$, Gln$^{117}$ with Ser$^{157}$, Arg$^{16}$ with Glu$^{113}$, Asp$^{16}$ with Glu$^{113}$, Asp$^{16}$ with Tyr$^{131}$, and Glu$^{131}$ with Tyr$^{131}$ are established between caspase-9 and ERK2, respectively. Most notably, a hydrogen bond between Arg$^{13}$ with Thr$^{157}$ and a salt bridge between Arg$^{16}$ and Asp$^{160}$ of ERK2 and caspase-9, respectively, are formed, in strong agreement with our identification of these residues as being critical for the interaction between ERK2 and caspase-9. Together with the mutational analysis, this strongly suggests that the TTCD motif of ERK2 interacts directly with the D domain of caspase-9, and this interaction is critical for the recognition of caspase-9 as a substrate by ERK2. Additional runs of ClusPro performed using structures of ERK2 where Asp$^{160}$ was “in silico” mutated to either Ala, Thr, or Asn resulted in the abolishment of the binding mode previously observed, suggesting that Asp$^{160}$ is crucial for this modeled interaction.

DISCUSSION

We have previously established caspase-9 as a target of ERK1/2, with phosphorylation at a single major site, Thr$^{125}$, inhibiting caspase-9 activation (12). Here, we have revealed a docking mech-
anism for the interaction of ERK1/2 with caspase-9. This interaction is related to previously identified interactions between MAPKs and their substrates/regulators but shows distinctive features. We propose that our findings uncover the molecular basis for selective phosphorylation of caspase-9 by ERK1/2 but not other MAPKs, such as p38 and JNK.

The sequence of the docking site that we have identified in caspase-9 that enables interaction with ERK1/2 resembles previously characterized D domains in other MAPK substrates, defined as a cluster of basic amino acids followed by a hydrophobic motif (ϕ_A-X-ϕ_B, where ϕ is a small hydrophobic amino acid) (30). Several residues in the N-terminal prodomain region of caspase-9 are important for ERK1/2 docking and phosphorylation of caspase-9, with Arg_10 being particularly significant. Arg_10, Arg_11, and other basic residues are exposed on the surface of this domain in caspase-9 and form a highly positively charged patch that is available for interaction with other proteins (21, 22). However, Leu_14 and Leu_16, which would form the hydrophobic ϕ_A-X-ϕ_B motif for the D domain in caspase-9, appear to be less important for phosphorylation by ERK1/2. Although it has been suggested that hydrophobic residues of the ϕ_A-X-ϕ_B motif in MAPK substrates are more critical for docking than other residues in the docking domain (30, 36, 37), in some MAPK substrates, such as RSK, this feature is not conserved (38). Therefore, although many MAPK substrates share related D domain docking motifs, in any given substrate, the specificity determinants for docking may differ, for instance in the number or spacing of basic residues or the importance of a hydrophobic ϕ_A-X-ϕ_B motif for docking interactions.

In some MAPK substrates, additional motifs have been recognized that play a role in interaction with the kinase. Originally described as a specific docking domain for ERK1/2, recent studies have shown that the DEF (docking site for ERK) motif (39) may also allow docking of caspase-9. A, sequence alignment of potential docking domains (CD domain and docking groove) in the MAPK family. Amino acids already characterized to be important in docking are highlighted in boldface type. These correspond to Asp_116 and Asp_119 in the CD domain of ERK2 and to Ile_113 and Gin_120 in the docking groove of p38α. B, CD domain (D319N, D316N/D319N) and docking groove (L113A, Q117A) HA-ERK2 mutants were expressed in HEK 293 cells, immunoprecipitated (IPP), and then used in kinase reactions using either GST-caspase-9 or GST-RSK (each 0.5 μg) as a substrate and [γ-32P]ATP. The 32P-labeled bands detected by autoradiography that correspond to GST-caspase-9 and GST-RSK, identified by protein staining, are shown. Levels of HA-ERK2 (ERK) and phosphorylated HA-ERK2 (pERK) in the immunoprecipitates are shown by immunoblotting. Experiments were conducted at least three times, and representative data from one experiment are shown.
p38α (40). With regard to caspase-9, we found that mutation of potential DEF sites consisting of Phe residues in the C-terminal half of caspase-9 did not reduce the capacity of ERK2 to phosphorylate caspase-9 (data not shown). Given the observation that mutation of the D domain strongly reduces phosphorylation by ERK1/2 both in vitro and in cells, our studies indicate that the D domain is the major docking site in caspase-9 that directs phosphorylation at Thr125 by ERK. Nevertheless, weak binding of ERK1/2 to the caspase-9 R7A/R10A/R11A mutant, particularly when combined with the T125A mutation, does suggest an additional mode of interaction that is independent of the D domain. Residual phosphorylation of D domain mutants in extracts and cells could be due to a weak interaction with ERK1/2 or might represent a relatively minor additional kinase activity that targets Thr125 independently of the D domain.

We have also investigated the regions of ERK2 that are involved in the interaction with caspase-9. We found no evidence that key acidic residues within the CD domain (32) of ERK2 are required; although mutation of Asp316 and Asp319 did
not affect phosphorylation of caspase-9, these mutations inhibited phosphorylation of RSK, showing a clear difference in the recognition of these two substrates by ERK2. We also found no evidence that the hydrophobic docking groove identified in p38 and proposed to be present in other MAPKs by Chang et al. (34) is involved in the interaction of ERK1/2 with caspase-9 or indeed with RSK.

Our study did, however, reveal that the sequence 157TTCD160 in ERK2 governs docking to caspase-9. We refer to this site in ERK2 as the TTCD motif. Interestingly, conversion of Thr157 and Thr158 to Glu and Asp, respectively, as found at the corresponding positions in p38 ("ED site") (35), significantly reduced the ability of ERK2 to phosphorylate caspase-9, whereas mutation to Ser and Asp, found in JNK1, did not. This suggests that Thr157 in the 157TTCD160 motif of ERK2 is particularly important for the interaction with caspase-9. Although a conservative change in this position to Ser is tolerated, an acidic residue (Glu) is not. Notably, a dramatic reduction in phosphorylation of caspase-9 by ERK2 was observed when Asp160 was mutated. By contrast, recognition of RSK by ERKs tolerated mutation of Asp160 to alanine or glutamate, although not asparagine or threonine. This residue has also been shown to be important for the interaction of ERK2 with the tyrosine phosphatases PTP-SL and STEP (41) and its regulator MAPK phosphatase 3 (42), whereas in Drosophila ERK2 D160N is a gain-of-function mutation (43). Thus, it appears that Asp160 is a critical residue for the docking interactions of ERK2 with certain upstream regulators and some but not all substrates.

Recently, Liu et al. (31) determined the crystal structure of ERK2 bound to a D domain peptide derived from MKP3 (KIMMKP3) and showed that an acidic patch and a hydrophobic groove in ERK2 engaged the basic and $\phi_\lambda$-$X$-$\phi_\eta$ residues, respectively, in the peptide. The acidic patch included CD domain residues Asp316 and Asp319, together with Asp160, whereas the hydrophobic groove included Leu113. Our molecular modeling indicates the likely basis for the similarities and differences between the interaction of KIMMKP3 and the prodomain of caspase-9 with ERK2. Similar to KIMMKP3, the interaction between the prodomain of caspase-9 and ERK2 is likely to involve a salt bridge formed between Asp160 of ERK2 and Arg10 within the D domain of caspase-9, which would account for the essential role of these residues in the interaction of the proteins. However, the CD domain residues Asp316 and Asp319 of ERK2 do not form close contacts with the prodomain of caspase-9, being closest to the neutral residues of the N-terminal of caspase-9. This may account for the lack of requirement of these residues in ERK2 for the recognition of caspase-9 as a substrate. Furthermore, the docking groove in ERK2 also does not have close contacts with the proposed hydrophobic $\phi_\lambda$-$X$-$\phi_\eta$ motif (Leu14-Arg15-Leu16) or indeed other hydrophobic residues in the caspase-9 prodomain. This may explain the lack of requirement for Leu113 and Gln117 of ERK2 and the only minor role of Leu14 and Leu16 of caspase-9 in the recognition of caspase-9 by ERK2. Overall, the prodomain of caspase-9 is predicted to form a smaller area of interaction with ERK2 than MKP3 that is highly dependent on the interaction of Asp160 with Arg10 (Fig. 10). Firm conformation of this interaction will, however, require solution of the crystal structure of the ERK2-caspase-9 complex.

Importantly, mutation of Asp160 in ERK2 to residues found in either p38 or JNK strongly inhibited its ability to phosphorylate caspase-9.
Docking Interaction between Caspase-9 and ERK2

FIGURE 11. Model for the dynamic interaction between ERK2 and caspase-9. Once phosphorylated and activated by MEK, ERK2 docks with the D domain within the prodomain of caspase-9. Further interactions between the catalytic site of ERK2 and the target region of caspase-9 result in the phosphorylation of Thr\(^{125}\), which destabilizes the complex, allowing ERK2 to catalyze the phosphorylation of further substrate molecules.

rlylate caspase-9. Thus, the TTCD motif can account for the ability of ERK1/2, but not p38 and JNK, to phosphorylate and inhibit caspase-9 activation. This specificity and recognition of a critical component of the intrinsic apoptotic pathway may be significant for the ability of the classical MAPKs to suppress the apoptosis, whereas the stress-activated p38 and JNK pathways often promote apoptosis (20).

Consistent with our previous studies (12), our results also show that the interaction of ERK1/2 with caspase-9 in cell extracts is enhanced when ERK1/2 is phosphorylated and activated by OA treatment (Fig. 4). This suggests a specific interaction of caspase-9 with the activated form of ERK1/2, although a role for another phosphorylation event stimulated by OA cannot be excluded. Conversely, the interaction of caspase-9 with ERK1/2 is greatly increased by mutation of the phosphorylated residue in caspase-9, Thr\(^{125}\), to nonphosphorylatable alanine. This indicates that once caspase-9 is phosphorylated by ERK1/2, the affinity of the interaction is decreased. These results suggest a model (Fig. 11) for the dynamic interaction of ERK1/2 with caspase-9, in which active ERK1/2 interacts through its TTCD motif with the D domain on caspase-9. In addition to this tethering interaction, there are likely to be other interactions that bring the phosphorylation site in caspase-9 in close proximity to the active site of ERK2. Once ERK1/2 catalyzes the phosphorylation of Thr\(^{125}\), it appears to be released, presumptively through a conformational change in caspase-9 that disrupts the interactions between the proteins. Thus, the catalytic efficiency of ERK2 toward caspase-9 would be enhanced.

Interference with the docking interaction by small molecules could potentially prevent the inhibition of caspase-9 activation by phosphorylation at Thr\(^{125}\) and therefore promote apoptosis, for instance in cancer cells in which classical MAPKs are constitutively activated. The differences in the interaction of ERK2 with caspase-9 and other substrates suggest that specificity in the modulation of this anti-apoptotic function might be achieved while maintaining other essential functions of the kinase. However, the basic region near to the N terminus of caspase-9, including Arg\(^{111}\) and Arg\(^{112}\), is also involved in the interaction with acidic residues in the CARD of Apaf-1 (21, 22). Thus, promotion of apoptosis by preventing the interaction of ERK1/2 with caspase-9 would need to be selective for interaction of the kinase and not simultaneously prevent the binding of Apaf-1, which is required for caspase-9 activation. Indeed, it is possible that ERK1/2 and Apaf-1 can each antagonize the other’s antiapoptotic and proapoptotic functions, respectively, through competition for binding to caspase-9.

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