Specific Proteolysis of the Kinase Protein Kinase C-related Kinase 2 by Caspase-3 during Apoptosis

IDENTIFICATION BY A NOVEL, SMALL POOL EXPRESSION CLONING STRATEGY*

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The caspase family of proteases plays a critical role in the execution of apoptosis. However, efforts to decipher the molecular mechanisms by which caspases induce cell death have been greatly hindered by the lack of systematic and broadly applicable strategies to identify their substrates. Here we describe a novel expression cloning strategy to rapidly isolate cDNAs encoding caspase substrates that are cleaved during apoptosis. Small cDNA pools (approximately 100 clones each) are transcribed/translated in vitro in the presence of 35S-methionine; these labeled protein pools are then incubated with cytosolic extracts from control and apoptotic cells. cDNA pools encoding proteins that are specifically cleaved by the apoptotic extract and whose cleavage is prevented by the caspase inhibitor acetyl-Tyr-Val-Ala-Asp chloromethylketone are subdivided and retested until a single cDNA is isolated. Using this approach, we isolated a partial cDNA encoding protein kinase C-related kinase 2 (PRK2), a serine-threonine kinase, and demonstrate that full-length human PRK2 is proteolized by caspase-3 at Asp117 and Asp700 in vitro. In addition, PRK2 is cleaved rapidly during Fas- and staurosporine-induced apoptosis in vivo by caspase-3 or a closely related caspase. Both of the major apoptotic cleavage sites of PRK2 in vivo lie within its regulatory domain, suggesting that its activity may be deregulated by proteolysis.

Caspases are a novel family of cysteine proteases with aspartate specificity that are related to the Caenorhabditis elegans cell death gene product CED-3. Evidence from many laboratories indicates that caspases play a critical role in the execution of apoptosis. Ectopic expression of these proteases induces programmed cell death. Caspases are normally present in cells as catalytically inactive proenzymes and are proteolytically processed and activated during the induction of apoptosis. Moreover, viral, peptide, and dominant negative inhibitors of caspases delay or prevent programmed cell death (reviewed in Ref. 1). Finally, homozymous inactivation of caspase-1 (2) and caspase-3 (3) in mice results in selective defects in apoptosis.

Because caspase activation is a crucial event in apoptosis, it is essential to identify the downstream molecular targets of these proteases whose selective proteolysis is likely to underlie the characteristic morphological features of apoptotic cell death. Although a number of structural and signaling proteins have been shown to be cleaved by caspases during programmed cell death (reviewed in Ref. 4), our understanding of the molecular mechanisms by which caspases induce cell death has been greatly hindered by the lack of systematic and broadly applicable strategies to identify these substrates. Given the absence of such methods and the growing number of caspase family members, it seems likely that the majority of apoptotic caspase targets have yet to be identified.

In this report, we describe a method to identify caspase substrates directly and rapidly using labeled protein pools derived from small cDNA library pools (5) that have been transcribed/translated in vitro (6). These protein pools are incubated with cell-free extracts prepared from nonapoptotic and apoptotic cells. cDNA pools encoding proteins specifically cleaved by an apoptotic extract (and prevented by caspase inhibitors) are subdivided and re-examined until a single cDNA is isolated. Using this approach, we demonstrate that the serine-threonine kinase PRK2 (7) is rapidly and specifically cleaved by caspase-3 during the induction of apoptotic cell death.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Human Jurkat cells were grown in RPMI 1640 medium (Life Technologies, Inc.) with 10% heat-inactivated fetal calf serum (FCS). Acetyl-Tyr-Val-Ala-Asp chloromethyl ketone (YVAD-CMK), acetyl-Tyr-Val-Ala-Asp aldehyde (YVAD-CHO), and acetyl-Asp-Glu-Val-Ala aldehyde (DEVD-CHO) were obtained from BACHEM Bioscience, Inc. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyIxetrazolium bromide (MTT) was purchased from Sigma. Stock solutions were prepared according to the manufacturer’s instructions.

Preparation of 35S-Labeled Protein Pools—Pre-existing cDNA pools of approximately 100 clones each from a Xenopus gastrula-stage library (5) were transcribed and translated in vitro using the TnT coupled

The abbreviations used are: PRK2, protein kinase C-related kinase 2; FCS, fetal calf serum; YVAD-CMK, acetyl-Tyr-Val-Ala-Asp chloromethyl ketone; YVAD-CHO, acetyl-Tyr-Val-Ala-Asp aldehyde, DEVD-CHO, acetyl-Asp-Glu-Val-Ala aldehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyIxetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody.

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Reticulocyte Lysate System (Promega) in the presence of [35S]methionine and SP6 RNA polymerase. Reactions were scaled down from the manufacturer’s instructions to a 5-μl volume using 100–500 ng of total DNA/pool; all other reagents were proportionately reduced.

Preparation of Control and Apoptotic Cytosolic Extracts—Jurkat cells were grown to near confluency in RPMI 1640 with 10% FCS and concentrated to 2 × 10⁷ cells/ml in fresh medium immediately prior to making extracts. An aliquot of concentrated cells was treated with 250 ng/ml of anti-Fas monoclonal antibody (mAb) CH-11 (Kamiya Biomedical Company, Thousand Oaks, CA) for 1 h. Cytosolic extracts from untreated cells (control extract) and anti-Fas-treated cells (apoptotic extract) were prepared as described previously (6) and stored at −80 °C until used. Each μl of extract (control or apoptotic) was derived from 4 × 10⁶ cells and contained approximately 8–10 μg of total protein.

Proteolytic Cleavage Reactions—Protein pools were analyzed in triplicate. 1.5-μl aliquots of each [35S]-labeled protein pool were incubated for 1 h at 37 °C with 5 μl of (i) control extract, (ii) apoptotic extract, or (iii) apoptotic extract preincubated for 15 min with 10 μM YVAD-CMK, a specific caspase inhibitor that inhibits many caspases under these conditions (8). Cleavage reactions were stopped by the addition of an equal volume of 2 × protein lysis buffer (125 mM Tris-Cl (pH 6.8), 2% SDS, 20% glycerol and 10% β-mercaptoethanol) and boiled for 5 min. The protein products were separated by SDS-PAGE and visualized by autoradiography as detailed previously (6). The cleavage reactions were run, 35S-labeled poly(ADP-ribose) polymerase (PARP) (prepared from pBSK-PARP using the Tnt C Coupled Reticulocyte Lysate System), a well characterized caspase-3 substrate (9, 10), was incubated with the above-noted three cleavage conditions to verify the specificity of the cleavage activity (i.e., present in the apoptotic extract only) and its sensitivity to inhibition by the caspase inhibitor YVAD-CMK.

Identification of Putative Apoptotic Caspase Substrates—Labeled protein pools containing putative caspase substrates cleaved during apoptosis were identified by (i) the disappearance of a protein in the apoptotic extract that was present in both the control extract and in the apoptotic extract preincubated with caspase inhibitor and/or (ii) the appearance of a novel protein fragment in the apoptotic extract only. Once a positive protein pool was confirmed, the corresponding cDNA pool was progressively subdivided and re-examined in the same manner until a single positive cDNA clone was isolated (6). Positive clones were sequenced and examined for homology to known sequences by internet BLAST search (National Center for Biotechnology Information).

Proteolytic Cleavage of Human PRK2 in Vitro—pcDNA3 plasmids containing full-length human PRK2 and PRK1 cDNAs were transcribed and translated in vitro in the presence of [35S]methionine using T7-C Coupled Reticulocyte Lysate System (Promega) according to the manufacturer’s instructions. 35S-Labeled PRK2 and PRK1 were incubated with 10 μl of control and apoptotic extracts and analyzed as above. 35S-Labeled PRK2 was also incubated for 1 h at 37 °C with apoptotic extracts that had been preincubated for 15 min with either DEVD-CHO (1–100 μM) or YMAD-CMK (1–100 μM) YVAD-CMK (1:200 dilution) (7). A pool of 75 labeled protein pools was screened in this manner until a single positive cDNA clone was isolated (6). Positive clones were sequenced and examined for homology to known sequences by internet BLAST search (National Center for Biotechnology Information).

Determination of Caspase-3 Cleavage Sites in PRK2 in Vitro—Based on the size of the observed cleavage fragments and examination of the amino acid sequence of human PRK2 for potential caspase-3 cleavage sites (DXXD motifs) (12, 13), two mutant human PRK2 constructs, D117A and D700E, were made using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions with the following oligonucleotide primers: 5′-GAAGATATCAGCAGTGCAGGCGTCCG-3′ and 5′-GGAGCCCTGTGAGGAACCCTTGTGATCCGCTG-3′ and 5′-CAGCATACACCCTGTGATCCGCTG-3′ (D117A) 5′-GAAGATATCAGCAGTGCAGGCGTCCG-3′ and 5′-GGAGCCCTGTGAGGAACCCTTGTGATCCGCTG-3′ (D700E). All constructs were verified by automated DNA sequencing of both strands. Mutant PRK2 cDNA constructs were transcribed and translated in vitro, and the corresponding 35S-labeled proteins were incubated with extracts and bacterially expressed caspases as above.

Result and Discussion

75 cDNA pools containing approximately 100 cDNAs each were transcribed/translated in vitro in the presence of [35S]methionine, usually resulting in the production of only 15–28 labeled proteins/pool. This discrepancy likely reflects the presence of empty or noncoding inserts in the cDNA library and variable transcription/translation efficiencies among cDNAs, some of which are not full-length and lack transcription initiation sequences (6). The labeled protein pools were then examined for protein bands specifically modified by a cell-free apoptotic extract derived from cells treated with anti-Fas monoclonal antibody. These cysteolic extracts have been shown to faithfully reproduce many of the characteristic features of apoptosis observed in vivo, including selective proteolysis of caspase substrates and fragmentation of added nuclei (14–16). Of the 75 labeled protein pools screened, fifteen contained a single protein that was cleaved by the apoptotic extract but not by this same extract preincubated with the caspase inhibitor YVAD-CMK; one pool contained two such proteins (data not shown). In this report, we describe the isolation and characterization of one of these proteins.

As shown in Fig. 1A, incubation of 35S-labeled protein pool 25 with an apoptotic extract (lane A) resulted in the production of...
a novel protein fragment of approximately 36 kDa (indicated by the arrow) that was not observed when this pool was incubated with either control extract (lane C) or apoptotic extract preincubated with the caspase activator OVA3 (lane A+O). The specificity of the proteolytic activity of the apoptotic extract (lane A) and its sensitivity to inhibition by OVA3 (lane A+O) was verified by using 35S-labeled PARP, a well-characterized caspase-3 substrate, as a control (Fig. 1B). The observed pattern for protein pool 25 suggests that the novel protein band seen in the apoptotic extract represents a cleavage fragment of a caspase substrate. In this case, disappearance of the intact caspase substrate in the apoptotic extract was not observed because it was only partially cleaved (Fig. 1C). DNA pool 25 was progressively subdivided into smaller pools, and the smaller pools were retested to find a single cDNA encoding a protein with the above-noted cleavage pattern was isolated (Fig. 1C). DNA sequencing of clone 25 revealed that it is a partial Xenopus PRK2 cDNA containing the entire C-terminal kinase domain. Comparison of clone 25 with human PRK2 (Fig. 2A) revealed a striking degree of homology in the kinase domains: >90% identity at the amino acid level.

Because clone 25 is a partial Xenopus PRK2 cDNA, we next examined whether full-length human PRK2 was cleaved by the apoptotic extract. As shown in Fig. 2A (left-hand panel), 35S-labeled human PRK2 was completely cleaved by the apoptotic extract (lane A) into three fragments of approximately 110, 70, and 36 kDa (indicated by the arrows). However, the closely related kinase PRK1 was not cleaved by the apoptotic extract (Fig. 2A, right-hand panel). Moreover, the apoptotic cleavage of PRK2 in vitro was completely inhibited by 10 μM DEVD-CHO (Fig. 2B), a peptide caspase inhibitor that preferentially inhibits caspase-3 and related subfamily members (10). In contrast, the apoptotic proteolysis of PRK2 was only partially prevented by 1000-fold higher (10 μM) concentrations of DEVD-CHO (Fig. 2B), a selective inhibitor of caspase-1 and related subfamily members (17). Taken together, these results indicate that human PRK2 is cleaved at two distinct sites by a DEVD-inhibitable caspase (caspase-3 or a caspase-3-like family member) during apoptosis in vitro.

In an effort to better characterize the caspase(s) responsible for PRK2 proteolysis in the apoptotic extract, we incubated 35S-labeled human PRK2 with caspase-1, caspase-2, and caspase-3 (the prototypical members of the three caspase subfamilies). Bacterially expressed caspase-3 cleaved PRK2 into three fragments of identical size seen in the apoptotic extracts
Apoptotic Cleavage of PRK2 by Caspase-3

(Fig. 3A, left-hand panel). However, incubation of PRK2 with amounts of caspase-1 or caspase-2 that were 5-fold greater than that needed to cleave pro-interleukin-1β and pro-caspase-2, respectively, did not result in any detectable proteolysis of PRK2 (Fig. 3A, both panels). These findings are consistent with the inhibitor profile of the PRK2 protease and strongly implicate caspase-3 as the protease responsible for PRK2 cleavage during apoptosis in vitro.

To identify the caspase-3 cleavage sites in human PRK2, we examined its amino acid sequence and identified two DXXD motifs (the consensus caspase-3 cleavage sequence (12, 13)) that would result in cleavage fragments of the expected size: DIID\textsuperscript{117}C (in the N-terminal regulatory domain) and DEVD\textsuperscript{700}S (in the C-terminal kinase domain). By site-directed mutagenesis of the critical aspartic acid residues, we created two mutant PRK2 constructs (D117A and D700E). As shown in Fig. 3B, substitution of aspartate 700 with glutamic acid (D700E) prevented cleavage at this site (the 70- and 36-kDa cleavage fragments were not formed) but not at the second site (the 110-kDa cleavage fragment was produced). In contrast, substitution of aspartate 117 with alanine (D117A) prevented cleavage at this site (the 110-kDa fragment was not formed) but did not prevent cleavage at Asp\textsuperscript{700}, thereby resulting in cleavage fragments of 36 kDa and approximately 90 kDa (appropriately larger than the corresponding 70-kDa fragment seen in wild-type PRK2 because the 20-kDa N-terminal cleavage site is missing). These cleavage patterns are illustrated schematically in Fig. 3C. Interestingly, cleavage of full-length PRK2 in the D117A mutant was reproducibly diminished compared with either wild-type PRK2 or the D700E mutant (Fig. 3B), suggesting that proteolysis at this N-terminal site may facilitate subsequent cleavage at Asp\textsuperscript{700}. These results unambiguously establish Asp\textsuperscript{117} and Asp\textsuperscript{700} as the two sites in human PRK2 cleaved by caspase-3 during apoptosis in vitro.

Having demonstrated that human PRK2 is cleaved by caspase-3 in vitro, we next wanted to determine whether PRK2 was cleaved during apoptosis in vivo. To this end, we performed Western analysis of PRK2 in lysates from Jurkat cells treated with anti-Fas monoclonal antibody or staurosporine for various time periods. Using a polyclonal peptide antibody directed against the C terminus of human PRK2 (7), a 110-kDa cleavage fragment (indicated by an arrow) was readily detected within 2 h of treatment with either anti-Fas mAb or staurosporine (Fig. 4A) and was observed as early as 1 h after these treatments on prolonged exposure of the blot (data not shown). This fragment is identical in size to the N-terminal cleavage fragment produced in vitro by cleavage at Asp\textsuperscript{117} (data not shown). A smaller fragment of approximately 100 kDa (indicated by an arrow) was first observed 4 h after treatment with anti-Fas mAb and 2 h after staurosporine treatment. In addition, several faint, smaller fragments (approximately 50–70 kDa in size) were observed at later time points (after 4 h), especially in staurosporine-treated cells; these minor fragments are likely produced by proteases activated after the initiation of apoptosis. Of note, no cleavage fragment in the 30–40-kDa range was detected during Fas- or staurosporine-induced cell death in vivo, indicating that proteolysis at Asp\textsuperscript{700} does not occur during apoptosis in vivo. Within 6 h of anti-Fas treatment or 4 h of staurosporine treatment, virtually all of the full-length PRK2 had been proteolysed into the above-noted cleavage fragments.

Importantly, PRK2 proteolysis occurs early during the induction of Fas- and staurosporine-mediated apoptosis. As noted, the 110-kDa cleavage fragment can be detected within 1–2 h of exposure to either of these apoptotic stimuli. Within this time period, 88–96% of the anti-Fas mAb-treated cells and 52–75% of the staurosporine-treated cells are still viable. Furthermore, at all time points examined, the extent of PRK2 proteolysis was correlated with the degree of cell death. Fas- and staurosporine-induced PRK2 cleavage also coincided temporally with caspase-3 proteolytic activation (as manifested by reduction in intensity of its proenzyme) and proteolysis of the well characterized caspase-3 substrate PARP (Fig. 4A), suggesting that caspase-3 (or a related subfamily member) is the protease responsible for PRK2 cleavage during apoptosis in vivo. Further evidence implicating caspase-3 in this role comes from the in vivo inhibitor studies. Preincubation of cells with 100 μM DEVD-CHO, a peptide inhibitor that preferentially inhibits caspase-3 (10), prevented Fas-induced PRK2 cleavage and cell death (Fig. 4B). The same concentration of YVAD-CHO, a caspase-1 selective inhibitor (17), had little effect on Fas-mediated PRK2 cleavage and cell death. Interestingly, the proteolytic formation of the smaller, 100-kDa PRK2 cleavage fragment was particularly sensitive to inhibition by DEVD-CHO. Concentrations of DEVD-CHO as little as 1–10 μM inhibited formation of this fragment and resulted in the accumulation of excess amounts of the larger, 110-kDa cleavage fragment (Fig. 4B, right-hand panel) suggesting that proteolysis at Asp\textsuperscript{700} contributes significantly to formation of the 100-kDa fragment.

FIG. 4. Specific and rapid cleavage of PRK2 during Fas- and staurosporine-induced apoptosis in vivo by a caspase(s) sensitive to inhibition by DEVD-CHO. A, similar time course for proteolytic cleavage of PRK2, caspase-3, and PARP during Fas- and staurosporine-induced apoptosis in vivo. Jurkat cells were treated with 250 ng/ml anti-Fas mAb or 2 μM staurosporine for 0–8 h or 0–6 h, respectively. Cell lysates were prepared and subsequently analyzed by Western blotting using the appropriate antibodies as detailed under “Experimental Procedures.” α-Tubulin was used as a control to verify that equivalent amounts of protein were loaded in each lane. B, Fas-induced proteolysis of PRK2 is potently inhibited by DEVD-CHO. Jurkat cells were preincubated for 2 h with 0–100 μM DEVD-CHO or YVAD-CHO and then treated for an additional 5 h with 250 ng/ml anti-Fas mAb. For each condition, the corresponding cell viability was determined by MTT conversion assay (8) and is indicated at the bottom. The molecular mass of markers in kDa is indicated at the left of each panel.
Apoptotic Cleavage of PRK2 by Caspase-3

4B), suggesting that apoptotic PRK2 proteolysis in vivo may be executed by two distinct caspase-3-like proteases with different sensitivities to DEVD-CHO.

In short, the serine-threonine kinase PRK2 is rapidly and specifically cleaved by caspase-3 (and/or a caspase-3-like subfamily member) during apoptosis in vitro and in vivo. Although one of the cleavage sites of PRK2 in vitro lies within its kinase domain (D700), both of its major apoptotic cleavage sites in vivo lie within its N-terminal regulatory domain. Given the evidence that both PRK2 and the closely related kinase PRK1 are activated by limited tryptic proteolysis (18, 19), presumably by removal of an inhibitory N-terminal domain (20), it is tempting to speculate that the activity of PRK2 might be deregulated by its cleavage during apoptosis. Indeed, several other kinases, including protein kinase C δ (21, 22) and θ (23), the p21-activated kinase PAK2 (24), and MEKK-1 (25), are cleaved and activated by caspase-3 during apoptotic cell death; proteolytic activation of these kinases may contribute to the morphological manifestations of apoptosis.

In addition, the successful isolation of PRK2 as a caspase substrate cleaved during apoptosis demonstrates the utility of the small pool expression cloning strategy presented here. By providing a rapid and direct method to identify the downstream targets of these critical apoptotic proteases, this approach should enable investigators to systematically dissect the molecular events/signaling pathways that play an important role in the execution of apoptosis. Although we used an apoptotic extract to screen for caspase substrates, individual recombinant caspases could be used to identify substrates of a particular caspase. Furthermore, the approach has much broader potential applications and can be used to identify substrates of a variety of enzymatic activities, including kinases (6, 26). We are currently exploring several of these applications in our studies of apoptotic signal transduction.

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