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Proteolytic activation of PKN by caspase-3 or related protease during apoptosis

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ABSTRACT **PKN, a fatty acid- and Rho-activated serine/threonine kinase having a catalytic domain highly homologous to protein kinase C (PKC), was cleaved at specific sites in apoptotic Jurkat and U937 cells on Fas ligation and treatment with staurosporin or etoposide, respectively. The cleavage of PKN occurred with a time course similar to that of PKCδ, a known caspase substrate. This proteolysis was inhibited by a caspase inhibitor, acetyl-Asp-Glu-Val-Asp-aldehyde. The cleavage fragments were generated *in vitro* from PKN by treatment with recombinant caspase-3. Site-directed mutagenesis of specific aspartate residues prevented the appearance of these fragments. These results indicate that PKN is cleaved by caspase-3 or related protease during apoptosis. The major proteolysis took place between the amino-terminal regulatory domain and the carboxyl-terminal catalytic domain, and it generated a constitutively active kinase fragment. The cleavage of PKN may contribute to signal transduction, eventually leading to apoptosis.**

PKN is a serine/threonine kinase that has a catalytic domain highly homologous to protein kinase C (PKC) in the carboxyl-terminal region and a unique regulatory domain in the amino-terminal region (1–3). The amino-terminal region of PKN contains three repeats of a leucine zipper-like motif and the basic region adjacent to the first leucine zipper-like motif. This unique structure is conserved through evolution in vertebrates (4) and among the isoforms of PKN (5). The amino-terminal region plays a role in the regulation of PKN by suppressing the basal kinase activity as well as by providing the sites that interact with other components involved in the PKN-signaling pathway (2, 6). The suppressive effect of the amino-terminal region is suggested by the following observations: (*i*) truncation of the amino-terminal region by limited proteolysis results in the activation of PKN (2, 7), and (*ii*) the amino-terminal region contains the binding site for a small GTPase Rho that activates PKN in a GTP-dependent manner (6, 8, 9). This suppression is also supposed to be cancelled or reduced by unsaturated fatty acids such as arachidonic acid (AA) (2, 3), detergents (3), and phosphoinositides such as phosphatidylinositol 4,5-bisphosphate (10).

Apoptosis is characterized by structural changes that include actin cytoskeletal disruption, membrane blebbing, decreases in adhesion and intercellular contacts, chromatin condensation, nuclear fragmentation, and packing of the nuclear fragments into membrane-enclosed apoptotic bodies (11). Biochemical changes include limited proteolysis of cellular proteins by a caspase family of cysteine proteases with aspartate specificity (12–14). A growing number of cellular substrates for caspases have been reported (15, 16). The cleavage of certain protein kinases such as MEKK-1 (17), p21-activated kinase 2 (18), and PKCδ (19, 20) is accompanied by the induction of their kinase activities, suggesting that caspase-mediated cell death involves modulation of diverse signaling pathways, in addition to the destruction of structural components.

In the present study, we have examined whether PKN is cleaved during apoptosis by using well established apoptotic model systems of cultured cell lines. The results indicate that PKN is cleaved during apoptosis, presumably by caspase-3, which generates a constitutively active kinase fragment.

MATERIALS AND METHODS

Materials. Anti-Fas monoclonal antibody (α-Fas mAb) CH-11 was purchased from Medical and Biological Laboratories (Nagoya, Japan). Rabbit polyclonal antibodies against the carboxyl-terminal peptides of human PKN [PKN (C-19)] and PKCδ [nPKCδ (C-20)] were purchased from Santa Cruz Biotechnology. Antibody αC6 against the carboxyl-terminal fragment (amino acids 863–946) of rat PKN was described previously (21). Tetrapeptide caspase inhibitors acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO) and acetyl-Tyr-Val-Ala-Asp-aldehyde (YVAD-CHO) and the fluorogenic substrate acetyl-Asp-Glu-Val-Asp-4-methylcoumaryl 7-amide (DEVD-MCA) were purchased from Peptide Institute (Osaka). Staurosporin and etoposide phosphate were obtained from Research Biochemicals (Natick, MA) and Calbiochem–Nova Biochem, respectively.

Cell Culture, Induction of Apoptosis, and Inhibitor Treatment. Jurkat cells and U937 myeloid leukemia cells were grown in RPMI medium 1640 supplemented with 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine at 37°C in humidified air atmosphere with 5% CO_2. To induce apoptosis, the cells were treated with 150 ng/ml α-Fas mAb, 1 μM staurosporin, or 10 μg/ml etoposide for various time periods. The tetrapeptide inhibitor DEVD-CHO or YVAD-CHO was added to the cell suspension 1 h before the induction of apoptosis. Cell death was determined by the Annexin V Apoptosis Detection Kit (Genzyme), followed by analysis using a Cyto-ACE300 cytofluorometer (JASCO, Tokyo). Fluorescein isothiocyanate- and propidium iodide-double-negative cells were judged as viable cells.

Plasmid Constructions, *in Vitro* Mutagenesis, and Preparation of Recombinant Proteins. Plasmid pBlueBacHis/PKN to express His-tagged human PKN (His-PKN) in Sf9 cells was

Abbreviations: PKC, protein kinase C; DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; YVAD-CHO, acetyl-Tyr-Val-Ala-Asp-aldehyde; DEVD-MCA, acetyl-Asp-Glu-Val-Asp-4-methylcoumaryl 7-amide; GST, glutathione *S*-transferase; GST-caspase-6, GST-tagged caspase-6; AA, arachidonic acid; AF, apoptotic fragment; His-PKN, His-tagged human PKN; α-Fas mAb, anti-Fas monoclonal antibody; PKN-FLAG, a FLAG-tagged full length PKN; His-GST-PKN, His- and GST-tagged human PKN.

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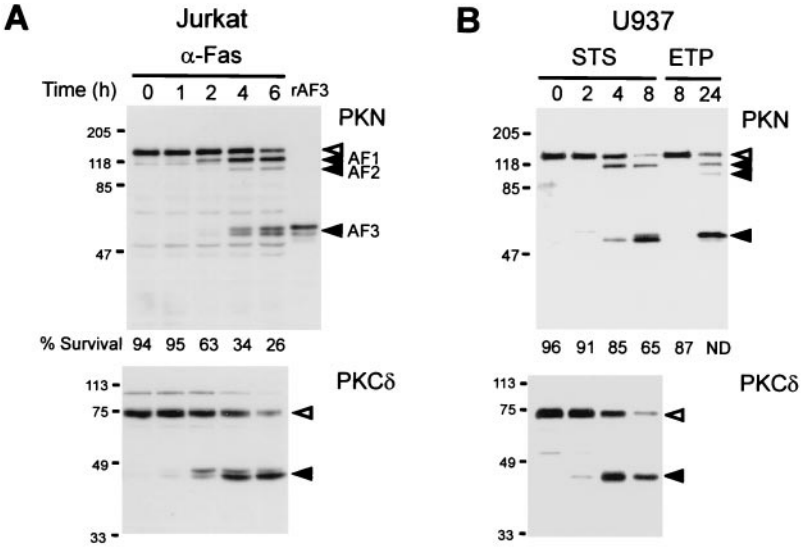

FIG. 1. Proteolytic cleavage of PKN in apoptotic cells. (*A*) Cleavage of PKN in Jurkat cells. Jurkat cells were treated with 150 ng/ml α-Fas mAb for the indicated time periods. (*B*) Cleavage of PKN in U937 cells. U937 cells were treated with 1 μM staurosporin (STS) or 10 μg/ml etoposide (ETP) for the indicated time periods. Cell lysates were subjected to immunoblot analysis with αC6 (*Upper*) or nPKCδ (C-20) (*Lower*). White and black arrowheads on the right indicate uncleaved and cleaved proteins, respectively. The cleavage products of PKN designated as AF1, AF2, and AF3 are shown with the black arrowheads in *A*. rAF3 indicates the recombinant AF3 expressed in COS-7 cells. Percentage of the viable cells determined by Annexin V-FITC apoptosis kit at each timepoint is given below each lane of PKN blots. ND, not determined. Molecular mass markers in kDa are indicated on the left of blots.

constructed by inserting the *Bgl*II–*Hin*dIII fragment of pBh-PKN4 (1) into pBlueBacHis (Invitrogen). The plasmids to express His-tagged caspase-3 (His-caspase-3) and glutathione *S*-transferase (GST)-tagged caspase-6 (GST-caspase-6) in *Escherichia coli* were kindly provided by Y. Tsujimoto (22) and E. S. Alnemri (23), respectively. Plasmid pBlueBacHis-GST/PKN for the expression of His- and GST-tagged human PKN (His-GST-PKN) was constructed by cloning of the *Sal*I fragment of phPKN-H4 (1) into pBlueBacHis together with the cDNA fragment encoding GST prepared by PCR from pGEX4T. Plasmid pBS/PRK2 containing full length cDNA coding for human PRK2 (5) was rescued from the clone obtained by screening of human lung λ ZAP II cDNA library. Mammalian expression plasmids pRc/CMV/PKN-FL and pRc/CMV/PKN/AF3-FL encoding a FLAG-tagged full length PKN (PKN-FLAG) and apoptotic fragment (AF)3 (AF3-FLAG, amino acids 561–942), respectively, were constructed as follows. The cDNA fragments coding for the corresponding regions of PKN were amplified by PCR from pMhPKN3 (1) by using primer sets to create FLAG-tag on the carboxyl terminus, then inserted into pRc/CMV (Invitrogen). Expression plasmid for AF3 without tag was constructed by inserting the cDNA fragment encoding amino acids 561–942 obtained by PCR into pTB701 (24). Mutant constructs of PKN replacing Asp with Ala were generated by QuickChange Site-Directed Mutagenesis Kit (Stratagene) from pRc/CMV/PKN-FL. His- and GST-tagged recombinant proteins were purified by using nickel nitrilotriacetic acid-agarose (Quiagen) and glutathione-Sepharose 4B (Pharmacia Biotech), respectively, according to the manufacturer's instructions.

***In Vitro* Cleavage of PKN by Recombinant Caspases.** Forty nanaograms of His-PKN was incubated with 30 ng of His-caspase-3 or 400 ng of GST-caspase-6 at 30°C for the indicated time periods and was subjected to immunoblot analysis with PKN (C-19). PKN mutants replacing Asp with Ala were expressed in COS-7 cells, then the cell lysates were incubated with 200 ng of His-caspase-3 at 30°C for 1 h followed by immunoblot analysis with PKN (C-19).

Separation of *in Vitro* Cleavage Products of PKN and Determination of Their Kinase Activity. Baculovirus-expressed His-GST-PKN was attached to glutathione-Sepharose beads and then incubated with His-caspase-3 at 30°C for 1 h. The proteins released from the beads were collected by centrifugation and applied onto a Mono Q HR5/5 (Pharmacia Biotech) column followed by elution with a linear gradient of 0–0.5 M NaCl in a buffer containing 50 mM Tris·Cl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10 μg/ml leupeptin, 10 mM NaF, and 1 mM $NaVO_3$ by using ÄKTA explorer 10S (Pharmacia Biotech). Each fraction was subjected to immunoblot analysis with αC6 and the kinase assay, as described previously (21).

Preparation of Recombinant PKN and Its Mutant AF3 Expressed in COS-7 Cells. PKN-FLAG and AF3-FLAG were expressed in COS-7 cells, then the cell lysates were applied to anti-FLAG M2 column (Eastman Kodak), followed by elution with 100 μg/ml FLAG peptide (Eastman Kodak). The eluates were subjected to SDS/PAGE and the kinase assay.

Preparation of Apoptotic Extracts. Jurkat cells were treated with α-Fas mAb for various time periods. Cell extracts were prepared by disrupting the cells in a buffer containing 25 mM Hepes (pH 7.4), 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 50 kallikrein inhibitor unit/ml aprotinin, and 1 mM EDTA, by repeated freeze-thawing. Caspase-3-like activity was monitored by using DEVD-MCA (25).

***In Vitro* Translation and Protease Cleavage Assays.** [^{35}S]Methionine-labeled full length PKN and PRK2 were prepared by coupled transcription/translation by using T3 polymerase in the TNT Reticulocyte Lysate System (Promega) from cDNA in phPKN-H4 (1) and pBS/PRK2, respectively. One microliter of the labeled protein was mixed with 8 μl of the apoptotic extracts prepared as above and incubated at 30°C for 1 h. The resultant products were separated on SDS/PAGE and visualized by autoradiography.

RESULTS

***In Vivo* Cleavage of PKN in Apoptotic Cells.** To examine whether PKN is cleaved during apoptosis *in vivo*, we used Jurkat cells treated with α-Fas mAb, a well established model system for apoptosis. Jurkat cells were incubated with α-Fas

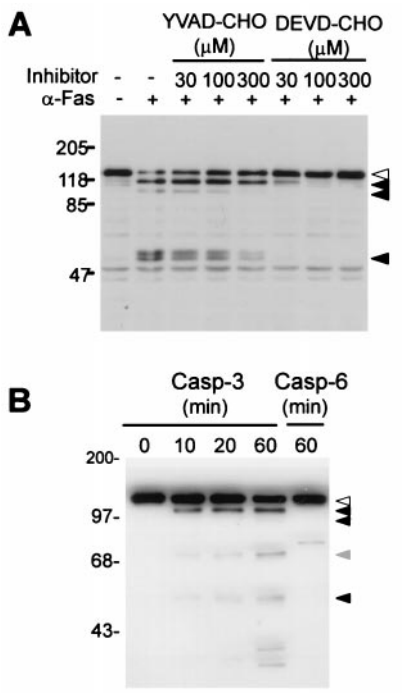

FIG. 2. Cleavage of PKN by caspase-3-like protease. (*A*) Inhibition of PKN cleavage during apoptosis by DEVD-CHO. Jurkat cells were pretreated with various concentrations of the tetrapeptide inhibitor of caspase-1 (YVAD-CHO) or caspase-3 (DEVD-CHO) for 1 h, as indicated, and then incubated with α-Fas mAb (150 ng/ml) for an additional 6 h. The cleavage of PKN was analyzed by immunoblotting with αC6. (*B*) *In vitro* cleavage of PKN by recombinant caspases. His-PKN (40 ng) was incubated with recombinant His-caspase-3 (Casp-3) or GST-caspase-6 (Casp-6) at 30°C for the indicated time periods, then analyzed by immunoblotting with PKN (C-19). The cleavage products corresponding to AF1, AF2, and AF3 were observed from 10-min incubation with caspase-3, while a cleavage product of a different size was observed from caspase-6 cleavage. White and black arrowheads on the right of the blot indicate the proteins uncleaved and cleaved by caspase-3, respectively. Gray arrowhead indicates a caspase-3 cleavage product that was not detected *in vivo*. Molecular mass markers in kDa are indicated on the left.

mAb for various time periods, and immunoblot analyses of the cell lysates were performed with αC6. As shown in Fig. 1*A* (*Upper*), the antiserum detected the full length PKN with a molecular mass of 120 kDa in the control Jurkat cells at 0 h, whereas several additional immunoreactive protein bands were observed during apoptosis. The cleavage product of approximately 105 kDa appeared 1 h after the addition of α-Fas mAb, and that of an approximately 55-kDa doublet appeared slightly later. A minor cleavage product of approximately 90 kDa was also observed in accordance with the 105-kDa product. The appearance of these PKN cleavage products was well correlated with the development of apoptosis, so we designated these fragments of 105 kDa, 90 kDa, and 55 kDa as AF1, AF2, and AF3, respectively. These fragments were also detected by the antibody PKN (C-19) raised against the carboxyl-terminal peptide of human PKN (data not shown), suggesting that these fragments did not result from crossreactivity of αC6 with some proteins induced by apoptosis, but were derived from PKN with an intact carboxyl terminus. AF1 and AF2 seem to be the intermediates of AF3. The time course of PKN cleavage was compared with that of PKCδ, previously reported to be proteolytically activated by caspase-3-like protease during apoptosis (19, 20), by immunoblot analysis of the same cell lysates as above with nPKCδ (C-20) (Fig. 1*A*, *Lower*). The cleavage product was faintly detected at 1 h of treatment and became intense at 2 to 4 h, which coincided with the appearance of AF1 of PKN. Similar results were obtained in another model system of apoptosis, U937 myeloid leukemia cells treated with the kinase inhibitor staurosporin or the topoisomerase II inhibitor etoposide (Fig. 1*B*).

DEVD-CHO Inhibited the Cleavage of PKN in Apoptotic Cells. To examine the effect of caspase inhibitors on the cleavage of PKN, Jurkat cells were preincubated with the tetrapeptide inhibitor YVAD-CHO for caspase-1/ICE-1 or DEVD-CHO for caspase-3/CPP32. Then the cells were treated with α-Fas mAb for an additional 6 h. As shown in Fig. 2*A*, DEVD-CHO completely inhibited the appearance of the apoptotic fragments at 100 μM, while YVAD-CHO did so only partially at 300 μM. These results suggest that PKN is cleaved by caspase-3 or a related protease during apoptosis.

PKN Was Specifically Cleaved by Recombinant Caspase-3 *in Vitro*. We next examined whether PKN could be cleaved *in vitro* by recombinant caspase-3. Baculovirus-expressed His-PKN was incubated with *E. coli*-expressed His-caspase-3 or GST-caspase-6, then analyzed by immunoblotting with PKN (C-19). As shown in Fig. 2*B*, *in vitro* cleavage of PKN with caspase-3 gave the cleavage products corresponding to AF1 (105 kDa), AF2 (90 kDa), and AF3 (55 kDa), observed in apoptotic cells (Fig. 1). An additional proteolytic product of approximately 70 kDa was also observed. Caspase-6 gave a cleavage product of a different size that was not detected in apoptotic cells, indicating that caspase-6 is not involved in the fragmentation of PKN *in vivo*. All of the caspase-3 cleavage products disappeared by addition of DEVD-CHO in the reaction (data not shown), indicating that the specific cleavage of PKN is mediated by caspase-3 or related protease.

Determination of the Cleavage Sites in PKN by Site-Directed Mutagenesis. To determine the cleavage sites in human PKN, we constructed mutant PKNs replacing Asp with Ala at various sites by site-directed mutagenesis from PKN-FLAG and expressed them in COS-7 cells. Then the cell lysates were incubated with His-caspase-3 followed by immunoblotting with PKN (C-19). AF1 disappeared upon mutation at D108 (Fig. 3*A*, lane 2). Mutation preventing the appearance of AF2 has not been found thus far. The 70-kDa fragment disappeared after mutation at either D451 (Fig. 3*A*, lane 6) or D454 (Fig. 3*A*, lane 8). Generation of AF3 was prevented when both D558 and D560 were mutated (compare Fig. 3*A*, lane 16 with Fig. 3*A*, lanes 12 and 14). Amino acid sequences around these mutated residues are presented in Fig. 3*B* with schematic representation of the structure of PKN. Among the above amino acid sequences, that of the 70-kDa fragment contains the consensus sequence for caspase-3 cleavage site DXXD (25), suggesting that the 70-kDa fragment is generated by the cleavage at D454. This site may be protected from protease attack *in vivo*, presumably by binding with some cellular protein, because this site was not cleaved in the apoptotic cells (Fig. 1). AF1 may be cleaved at D108, and AF3 at D558 and/or D560, although the putative cleavage site for either of them contains Asp in the substrate P1 position but not in the P4 position. The predicted positions of the apoptotic fragments are presented in Fig. 3*B* (*Lower*).

To confirm the cleavage site(s) for AF3, we constructed a mutant AF3-FLAG encoding amino acids 561–942 and expressed it in COS-7 cells for immunoblot analysis. The recombinant AF3-FLAG (Fig. 3*A*, lane 17) co-migrated with the band corresponding to AF3-FLAG generated by cleavage of PKN-FLAG (for instance, Fig, 3*A*, lane 14), indicating that the cleavage site for AF3 was at D560 or very close to this site. The recombinant AF3 without tag co-migrated with AF3 observed in apoptotic cells as doublet or triplet bands (Fig. 1*A, Upper,* rAF3 vs. α-Fas 6h). Moreover, phosphatase treatment of the recombinant AF3 resulted in the mobility shift to the fastest migrating band, suggesting that the doublet or triplet bands of

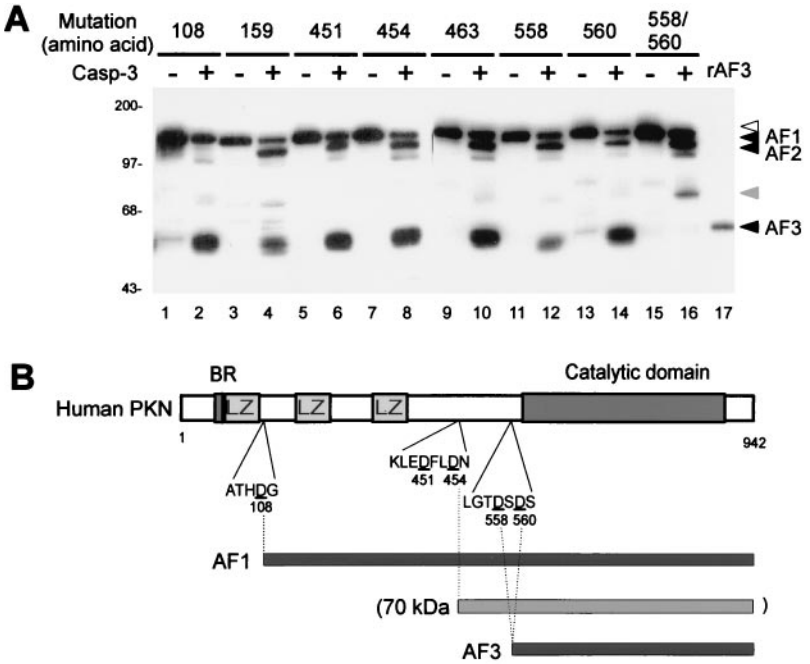

FIG. 3. Determination of the caspase-3 cleavage sites in PKN. (*A*) Cleavage of PKN point mutants replacing Asp with Ala by caspase-3. Extracts of COS-7 cells expressing the FLAG-tagged PKN mutants were incubated with or without recombinant His-caspase-3 (Casp-3), and were analyzed for the cleavage of PKN by immunoblotting with PKN (C-19). Mutated amino acid number is shown on the top. White and black arrowheads on the right of the blot indicate the uncleaved and cleaved bands, respectively. Gray arrowhead indicates the cleavage product detected only in the *in vitro* reaction. rAF3 indicates the recombinant AF3-FLAG expressed in COS-7 cells. Molecular mass markers in kDa are indicated on the left. (*B*) Schematic representation of the structure of human PKN with the predicted cleavage sites and locations of AF1, AF3, and the *in vitro* 70-kDa product. The mutated Asp residues giving rise to the resistance to the cleavage are presented with neighboring amino acid sequences below the structure of PKN. The predicted positions of AF1, AF3, and the 70-kDa fragment are shown on the bottom. LZ, leucine zipper-like motif; BR, basic region.

AF3 are attributed to the difference in the phosphorylation state (unpublished work).

Generation of Constitutively Active Fragment of PKN by Apoptotic Cleavage. As the cleavage products of PKN identi-

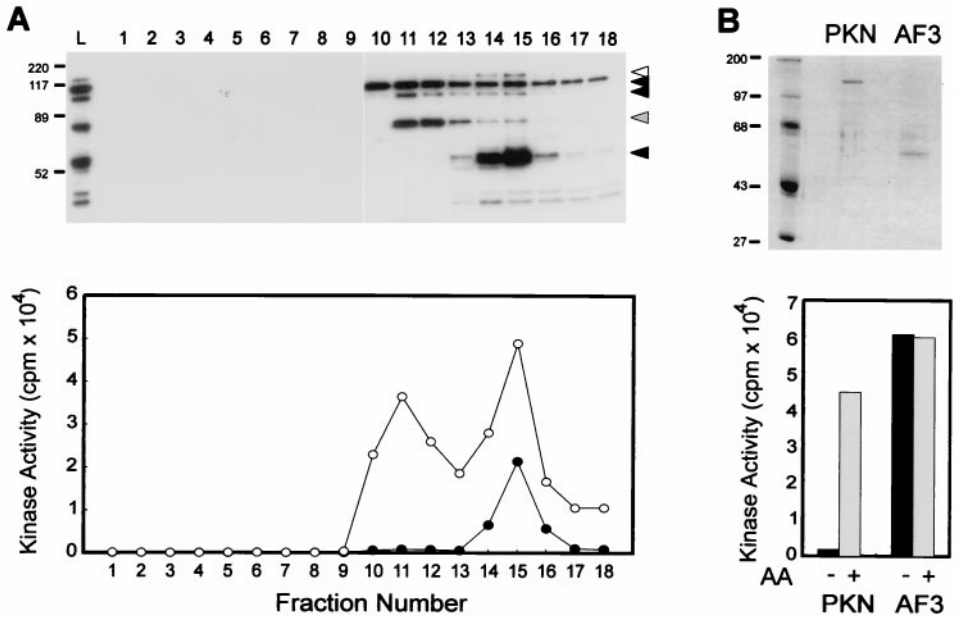

FIG. 4. Generation of constitutively active kinase after caspase-3-mediated proteolysis of PKN. (*A*) Mono Q column chromatography of the *in vitro* cleavage products of PKN. Baculovirus-expressed His-GST-PKN was digested with His-caspase-3, and the cleavage products were separated by Mono Q column chromatography. Each fraction was subjected to immunoblotting with αC6 (*Upper*) and the kinase assay in the presence (○) or absence (●) of AA (*Lower*). L indicates the sample loaded to the column. Positions of uncleaved and cleaved proteins are indicated with black and white arrowheads, respectively. Gray arrowhead indicates a cleavage product detected only in the *in vitro* reaction. (*B*) comparison of kinase activity between the recombinant full length PKN and its mutant AF3. PKN-FLAG (PKN) and AF3-FLAG (AF3) expressed in COS-7 cells were purified by anti-FLAG column, then equal amount of the proteins were subjected to SDS/PAGE followed by Coomassie brilliant blue staining (*Upper*) and the kinase assay in the presence or absence of AA (*Lower*). The results are representative of three independent experiments. Molecular mass markers in kDa are indicated on the left of the gel.

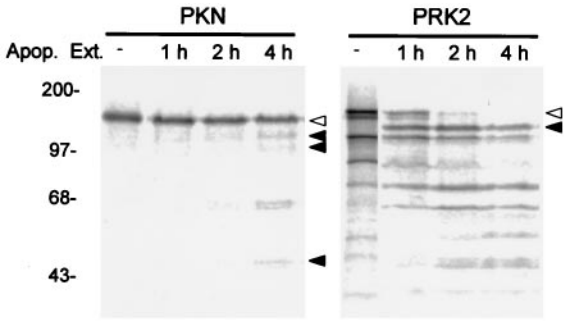

FIG. 5. Cleavage of PKN and PRK2 by apoptotic extracts from Jurkat cells treated with α-Fas mAb. *In vitro* translated PKN and PRK2 were incubated with apoptotic extracts (Apop. Ext.) prepared from Jurkat cells treated with 150 ng/ml α-Fas mAb for the indicated time periods. White and black arrowheads indicate uncleaved and cleaved proteins, respectively. Molecular mass markers in kDa are indicated on the left.

fied above contained the intact kinase domain and lacked the amino-terminal regulatory domain to various extents, the kinase activity of these products was measured. The cleavage products of PKN were separated by Mono Q column chromatography, and each fraction was subjected to immunoblotting and the kinase assay in the presence or absence of AA, an activator of PKN. As shown in Fig. 4*A*, fractions rich in AF1, AF2, and the *in vitro* 70-kDa fragment possessed AA-dependent kinase activity, while AF3 in fractions 14–16 possessed the constitutively active kinase activity. To confirm the activity of AF3, the recombinant AF3-FLAG and PKN-FLAG expressed in COS-7 cells were subjected to the kinase assay (Fig. 4*B*). The kinase activity of AF3 was not affected by the presence of AA and was comparable to that of PKN in the presence of AA, indicating that AF3 is constitutively active.

Cleavage of PKN and a Closely Related Kinase PRK2 by Apoptotic Extracts. In the course of this study, PRK2, a kinase closely related to PKN, was reported to be rapidly cleaved by caspase-3 or related protease during apoptosis, whereas PKN (or PRK1) was not (26). This was demonstrated *in vitro* by incubating the radiolabeled proteins with the extracts prepared from apoptotic Jurkat cells induced by Fas ligation for 1 h. We compared the time course of the cleavage between PKN and PRK2. Apoptotic extracts were prepared from Jurkat cells treated with α-Fas mAb for various time periods and examined for the cleavage of *in vitro*-translated PKN and PRK2 (Fig. 5). It was confirmed that PKN was not cleaved by the 1-h extract, whereas PRK2 was readily cleaved. However, PKN was cleaved by the 2-h and 4-h treated extracts. Caspase-3-like activity measured with the synthetic substrate DEVD-MCA was rather low in the 1-h extract, compared with those in the 2-h and 4-h extracts (about 2- and 4-fold higher, respectively; data not shown). PKCδ was also cleaved by the 2-h and 4-h extracts, but not by the 1-h extract (data not shown). Thus, PRK2 may be a highly sensitive substrate for caspase-3 or related protease.

DISCUSSION

The present study demonstrated that PKN is proteolytically cleaved to generate fragments designated as AF1, AF2, and AF3 during the execution phase of apoptosis. Inhibitor sensitivity and the *in vitro* cleavage study suggest the involvement of caspase-3 in this reaction, although the cleavage sites of PKN *in vivo* are not DXXD/(caspase-3 consensus sequence) but XXXD/ (Fig. 3*B*). Some proteins such as β-catenin (27) are also cleaved during apoptosis at the sequence containing Asp in the P1 position but not in the P4 position. The enzyme responsible for cleavage of such proteins will be clarified by further investigation of the substrate specificity of caspases. The predicted cleavage site of AF1 (D108) corresponds to that of PRK2 (D117) (26) based on the sequence alignment, suggesting that this site is exposed on the surface of the molecule and is susceptible to protease attack. The region corresponding to the cleavage site of AF3 is divergent in PRK2 (5), whereas it is highly conserved in rat PKN (1), suggesting that AF3 is a PKN-specific product.

AF3, which seems to be the final product of PKN during apoptosis, is generated in different cell lines (Jurkat and U937) induced by different apoptotic stimuli such as Fas ligation, staurosporin, and etoposide. Further, similar fragmentation of PKN has been demonstrated in animal models of apoptosis. The delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is proposed to be mediated by apoptosis (28). We have found that an αC6-reactive band of approximately 55 kDa appeared in the hippocampus extract after ischemic insult (T. Hashimoto, Y.O., & C. Tanaka, unpublished work). An immunoreactive band co-migrating with AF3 has also been observed in the ischemia/reperfusion model of rat retina (K. Sumioka, Y.O., & N. Saito, unpublished work). Therefore, generation of AF3 representing constitutively active kinase activity may be a general phenomenon in apoptosis. In this context, it is of interest that PKN is expressed ubiquitously in human tissue (1), and relatively higher in testis, spleen, and thymus (3), where the apoptotic process is suggested to be active (29–31), although the physiological role of the cleavage products of PKN in apoptosis remains unknown.

It is possible that AF3 constitutively phosphorylates substrates of PKN by escaping from the control of physiological regulators such as Rho. Previously, we demonstrated that PKN binds to and phosphorylates intermediate filaments, such as subunits of neurofilament (32), vimentin, and glial fibrillary acidic protein (33), and that the phosphorylation results in the inhibition of the filament assembly *in vitro* (32, 33). PKN also efficiently phosphorylates actin and caldesmon *in vitro* (21). Phosphorylation of such proteins by AF3 might be involved in the morphological changes of the cells undergoing apoptosis, together with the caspase-mediated cleavage of cytoskeletal components (34–36). However, the possibility may not be ruled out that the proteolysis of PKN described above reflects a process of degradation of this enzyme during apoptosis. Additional investigation will be required to clarify the role of the cleavage of PKN in apoptosis.

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