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Cleavage at the Carboxyl-Terminus of Ku80 during Apoptosis in Human Jurkat T Cells

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We have previously reported that the amount of Apg-2, an Hsp110 family protein, decreases during apoptosis in Jurkat T cells. Since we hypothesized that Apg-2 would be cleaved by caspase-3 during apoptosis, a cleavage-site-directed antibody was raised against the carboxyl-terminus of the Apg-2 fragment that appears after the cleavage by caspase-3. Although this antibody could not detect the Apg-2 fragment in apoptotic cells, three additional fragments were unexpectedly detected. Based on the results of microsequencing, one of these fragments was identified as Ku80. Ku80 is a nuclear protein and a component of DNA-dependent protein kinase (DNA-PK). In this study, we observed that Ku80 is cleaved at Asp-730 residue during apoptosis, and this cleavage occurs in the nucleus in the early apoptotic phase. Furthermore, Ku80 is distributed in the cytoplasm of nuclear fragmented apoptotic cells, although the cleaved fragment contains the nuclear-localization signal (NLS). Our study clearly shows that Ku80 is cleaved in the nucleus, and distributes in the cytoplasm during apoptosis.

Key words: Apg-2, apoptosis, caspase, cleavage-site–directed antibody, Ku80.

Abbreviations: Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-YVKD-CHO, acetyl-Tyr-Val-Lys-Asp-aldehyde; Z-Asp-CH_2-DCB, carbobenzoxy-Asp-[(2,6-dichlorobenzoyl)oxy] methane; Z-VAD-fmk, carbobenzoxy-Val-Ala-Asp-fluoromethylketone.

Apoptosis is a major form of physiological cell death that is important in development and organ homeostasis. In apoptosis, caspases, a family of cysteine proteases, play important roles in apoptotic signal transduction and executing processes (*1*, *2*). All the known caspases are synthesized as inactive precursors and require proteolytic processing for activation (*3*). Among the caspases, caspase-8 and caspase-9, a subset of initiator caspases, are activated by aggregation associated with caspase-adapter molecules, such as Fas-associated protein with death domain (FADD) or Apaf-1 (*4–6*). The activated initiator caspases activate the effector caspases, caspase-3, caspase-6, and caspase-7, by direct cleavage (*7–9*). Caspase-3 in particular has been identified as an important protease in the apoptosis process because a specific inhibitor can suppress apoptosis (*10*). Previous studies have shown that caspase-3 recognizes the DXXD sequences found in poly (ADP-ribose) polymerase (PARP) ($DEVD_{213}$) (*11*), calpastatin ($DAID_{233}$) (*12*), inhibitor of caspase-activated DNase (ICAD) ($DETD_{116}$ and $DAVD_{224}$) (*13*), and DNA topoisomerase I ($DDVD_{146}$) (*14*). Caspase-7 resembles caspase-3 in its consensus motif, but the subcellular distribution of caspase-7 differs from that of caspase-3 during apoptosis (*15*, *16*).

A cleavage-site–directed antibody is a very useful tool with which to analyze cleaved fragments because this antibody is designed to react either with the amino-terminus or the carboxyl-terminus that appears after cleavage by proteolysis (*17–19*). By using a cleavage-site–directed antibody, we observed that the nonmuscle myosin heavy chain-A is cleaved during apoptosis (*20*). In our previous report, we also observed that the level of an ATP- and peptide-binding protein in germ cells-2 (Apg-2), an Hsp110 family protein, decreases during apoptosis. In this study, we attempted to detect the cleaved Apg-2 fragment using a cleavage-site–directed antibody for a caspase-3 consensus motif. This antibody detected Apg-2 in non-apoptotic cells, whereas a cleaved Apg-2 fragment could not be detected in apoptotic cells. Although the Apg-2 fragment was not found, three additional fragments (125 kDa, 93 kDa, and 85 kDa) were detected unexpectedly in apoptotic cells. Among these apoptosis specific fragments, the 85-kDa fragment was identified as Ku80. Ku80 is a nuclear protein that is well known as a component of DNA-dependent protein kinase (DNA-PK), which is involved in the repair of DNA double-strand breaks and V(D)J recombination (*21*, *22*). Ku80 combines with Ku70, and this heterodimeric protein complex is called as Ku (*23*, *24*). Ku shows double-strand DNA end–binding activity and acts as a DNA-binding component of DNA-PK (*25–28*).

Previous studies have shown that the DNA-PK catalytic subunit (DNA-PKcs) is cleaved by caspase-3 during apoptosis (*29–33*). These studies showed that Ku80 is not degraded and cleaved during apoptosis since the molecular size of Ku80, as determined by SDS-PAGE, did not differ between non-apoptotic and apoptotic cells (*29–31*). However, in our study, we detected the cleaved Ku80 fragment by using a cleavage-site–directed antibody. In this study, we determined the cleavage site of Ku80 by

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immunoabsorption and EGFP-tagged Ku80 mutant analysis. Furthermore, we observed that the cleavage of Ku80 occurs in the cell nucleus in the early apoptotic phase, and becomes distributed in the cytoplasm of nuclear fragmented apoptotic cells.

MATERIALS AND METHODS

Reagents—The reagents for analysis were obtained from the following sources: carbobenzoxy-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk), Bachem (Bubendorf, Switzerland); carbobenzoxy-Asp-[(2,6-dichlorobenzoyl) oxy] methane (Z-Asp-CH_2-DCB), acetyl-Tyr-Val-Lys-Asp-aldehyde (Ac-YVKD-CHO), and acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO), Peptide Institute Inc. (Osaka, Japan); staurosporine, Sigma Chemicals (St. Louis, MO, USA).

Cell Culture and Apoptosis Induction—Human T-lymphoblastoid Jurkat cells (Japan Research Resources Bank) were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM Gln, 100 U/ml penicillin G, and 200 μg/ml streptomycin. HeLa cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% (v/v) fetal calf serum and 2 mM Gln. All cells were maintained at 37°C under a humidified atmosphere of 5% (v/v) CO_2. The cells were challenged with 25 ng/ml anti-Fas mAb (for Jurkat, MBL., Nagoya, Japan) and 0.5 μM staurosporine (for Jurkat and HeLa) for the induction of apoptosis.

Immunoblotting—The following antibodies were raised in rabbits by synthetic peptides as haptens conjugated with a carrier protein, and purified from antisera using affinity chromatography on the immobilized antigen peptides: cleavage-site–directed antibody for Apg-2 (#1339) against residues 712–727 (IISSFKNKEDQYDHLD), anti-Ku80 antibody against residues 702–717 (KKFLAPKDK-PSGDTAA), and anti–cleaved Ku80 antibody against residues 715–730 (TAAVFEEGGDVDDLLD).

The procedure used for immunoblotting has been described in the literature cited (*12*). Immunoabsorption using synthetic peptides was performed as follows. The synthetic peptide (2 nmole) was incubated with an antibody for 4 h at 4°C. The peptide–antibody mixture was then used as the primary antibody for immunoblotting.

Purification and Characterization of Apoptotic Specific 85-kDa Fragment—The cytosol fraction of apoptotic Jurkat cells was prepared by sonication in lysis buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA] and applied to a DEAE-Sepharose CL-6B (Amersham Biosciences Corp., Piscataway, NJ, USA) column. Proteins were eluted from the column with a linear gradient of 0–0.5 M NaCl in lysis buffer, and fractions containing the 85-kDa fragment were detected by immunoblotting. These fractions were diluted in 1 M $(NH_4)_2SO_4$ in lysis buffer and applied to a Butyl-Toyopearl (Tosoh., Tokyo, Japan) column. Proteins were eluted from the column with a linear gradient of 1–0 M $(NH_4)_2SO_4$, and fractions containing the 85-kDa fragment were detected by immunoblotting. These fractions were concentrated by 10% (w/v) trichloroacetic acid and blotted onto a nitrocellulose filter after SDS-PAGE. The 85-kDa fragment band was cut from the filter paper and digested by lysyl endopeptidase (Wako, Osaka, Japan). The resulting peptides were separated by reverse-phase high performance liquid chromatography (RP-HPLC) (C_{18} column). The peptide fractions were eluted from the column with a linear gradient of acetonitrile, and the amino terminal sequences of the separated peptides were analyzed by Edman degradation using a protein sequencer LF3000 (Beckman Coulter., Fullerton, CA, USA).

Expression of the EGFP-Ku80 Fusion Protein in HeLa Cell—cDNAs of Ku80 [full length (Ku80), a site-directed mutant of Ku80 (Ku80 (D730E)), and carboxyl-terminus truncated Ku80 (Ku80 ΔMI)] were generated from Jurkat mRNA using RNA LA PCR Kit (AMV) ver. 1.1 (Takara., Kyoto, Japan). The oligonucleotides used for the PCR sense primer was 5′-GGAATTCCATGGTGCGGTCGG-GGAATAA-3′ and for the antisense primers were 5′-CGGGATCCCGCTATATCATGTCCAATAAAT-3′ (Ku80), 5′-CGGGATCCCGCTATATCATCTCCAATAAAT-3′ [Ku80 (D730E)], and CGGGATCCCGCTAGTCCAATAAATCG-TCCA-3′ (Ku80ΔMI). Each cDNA was digested with *Eco*RI and *Bam*HI and cloned into pEGFP-C1 mammalian expression vector (BD Biosciences Clontech., Palo Alto, CA, USA). HeLa cells were grown to 70% confluence in 12-well culture dishes for the transient expression of the EGFP-Ku80 proteins and then transfected with LIPOFECTAMINE PLUS reagent (Invitrogen Corp., Carlsberg, CA, USA) according to the manufacturer's instructions.

Confocal Immunofluorescence Microscopy—Non-apoptotic and apoptotic Jurkat cells were harvested and washed in phosphate-buffered saline (PBS). The cells were suspended in PBS and placed on a poly-L-lysine coated cover glass for 15 min, fixed in 4% (w/v) paraformaldehyde in PBS for 30 min at room temperature, washed twice for 5 min each in PBS, then permeabilized and blocked with 3% (w/v) BSA in PBS containing 0.1% (w/v) Triton X-100 for 30 min. The cells were incubated with primary antibodies for 1 h at 37°C, washed three times for 5 min each in Tris-buffered saline (TBS) containing 0.05% (w/v) Tween 20, and incubated with FITC-conjugated anti-rabbit secondary antibody for 1 h at room temperature. After incubation with the secondary antibody, the cells were further incubated with propidium iodide (50 ng/ml) for 20 min at room temperature in order to stain the nuclear DNA. After the final wash in PBS, the cells were mounted in fluorescence mounting medium (Vector Lab. Inc., Burlingame, CA, USA) and viewed using an MRC-1024 confocal laser microscope (Bio Rad Laboratories., Hercules, CA, USA).

Preparation of EGFP-Ku80 transfected HeLa cells was carried out as followes. HeLa cells were grown on cover glasses and transfected with plasmid DNA as described above. After 48 h, the cells were washed with PBS, and fixed in 4% (w/v) paraformaldehyde in PBS for 30 min. The fixed cells were permeabilized with 0.2% (w/v) Triton X-100 in PBS for 5 min, then incubated with TO-PRO-3 (Molecular Probes Inc., Eugene, OR, USA) in order to stain the nuclear DNA.

RESULTS

Detection of Apoptosis Specific Fragments by a Cleavage-Site–Directed Antibody—We have previously reported that the level of Apg-2, a heat shock protein 110 (Hsp110) family protein, decreases during apoptosis in human

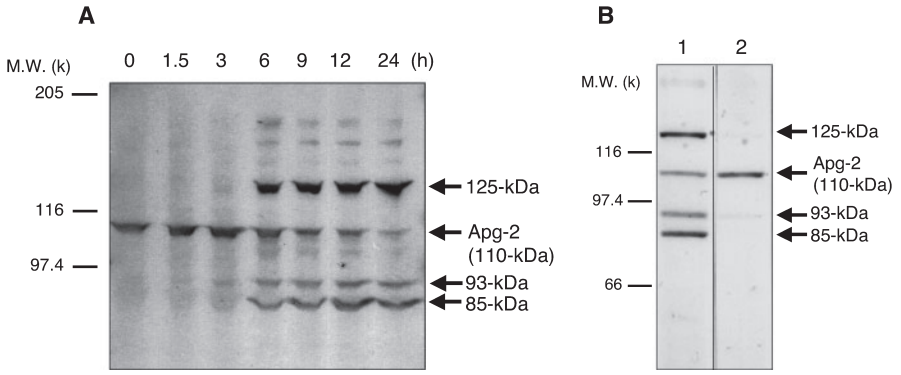

Fig. 1. **Immunoblotting using the cleavage-site-directed antibody #1339.** A: Jurkat cells were treated with anti-Fas mAb and sampled for immunoblotting using #1339 at the specified times (0 to 24 h). B: Immunoblotting of an apoptotic cell lysate using #1339 incubated with the synthesized peptide, AAAAAADHLD. Lane 1, no-peptide; and 2, incubation with peptide.

Jurkat T cells (*20*). We hypothesized that the decrease in Apg-2 is caused by proteolysis because many proteases are involved in apoptosis. Since caspase-3 is known to be an important protease involved in the apoptotic process and cleaves many substrates during apoptosis, we considered that the decrease would be mediated by caspase-3. Then, we tried raising cleavage-site–directed antibodies for Apg-2 to test whether it is cleaved by caspase-3 during apoptosis. Apg-2 contains four caspase-3 consensus motifs (DXXD): $DGED_{665}$/Q, $DQYD_{724}$/H, $DHLD_{727}$/A, and $DAAD_{730}$/M. Firstly, we produced a cleavage-site–directed antibody (#1339) against a caspase-3 consensus motif DHLD. #1339 detected Apg-2 in non-apoptotic Jurkat cells, and the decrease in Apg-2 during anti-Fas mAb–induced apoptosis was also detected by this antibody (Fig. 1A). Although #1339 detected Apg-2, it also detected three additional fragments with molecular sizes of 125-kDa, 93-kDa, and 85-kDa in apoptotic cells (Fig. 1A). These fragments were detected at 6 h after the addition of anti-Fas mAb, and were also detected in staurosporine, a PKC inhibitor, induced apoptosis (data not shown). Next, we tried to determine the cleaved Apg-2 fragment. Because #1339 is a polyclonal antibody and it also detects unprocessed Apg-2 in non-apoptotic cells, this antibody should comprise at least two different types of antibodies: one that reacts with the carboxyl-terminus of antigen peptide, and another that reacts with antigen peptide other than the carboxyl-terminus. Then, we attempted to use the antibody that does not react with the carboxyl-terminus of the antigen peptide to detect the cleaved Apg-2 fragment. To immunoabsorb the carboxyl-terminus reacting antibody, #1339 was incubated with a synthetic peptide, AAAAAADHLD, and the peptide incubated #1339 was used as the primary antibody for immunoblotting. After incubating with the peptide, #1339 detected unprocessed Apg-2, but other fragments were hardly detected in apoptotic cells (Fig. 1B).

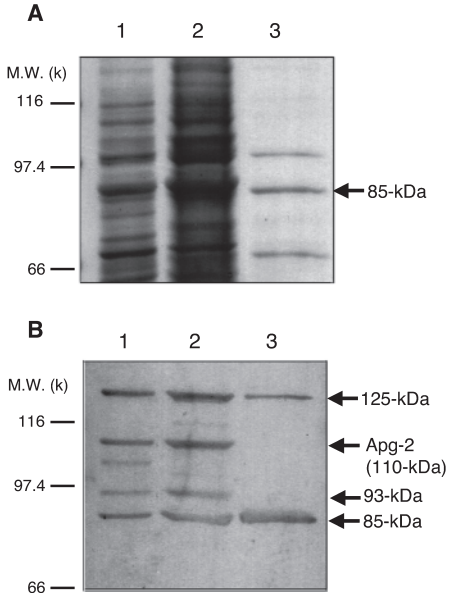

Fig. 2. **Purification of the apoptosis-specific 85-kDa fragment.** Purification of the apoptosis specific #1339 positive 85-kDa fragment was performed as described in "MATERIALS AND METHODS." Aliquots from each purification step were subjected to SDS-PAGE and proteins were detected by Coomassie Brilliant Blue staining (A) and immunoblotting using antibody #1339 (B). Lane 1, apoptotic cytosol fraction; 2, DEAE-Sepharose fraction; and 3, Butyl-Toyopearl fraction.

Although the cleaved Apg-2 fragment was not determined definitely, the three apoptosis specific fragments should be produced by proteolysis, because they could not be detected by the peptide incubated #1339, indicating that the carboxyl-terminus reacting antibody detected these fragments in apoptotic cells. At this time, we changed the subject of the study and tried to characterize these apoptosis specific fragments.

Identification of Apoptosis Specific 85-kDa Fragment—To characterize the apoptosis specific fragments detected by #1339, we attempted to purify these fragments. Among the fragments, the 85-kDa fragment was purified to an extent to which it could be isolated as a single band on SDS-PAGE (Fig. 2). Following this, the three peptides were chosen for amino-terminal sequencing after RP-HPLC separation. As shown in Table 1, the sequences of these peptides were identified in the sequence of human Ku80, a component of DNA-dependent protein kinase (DNA-PK).

Determination of Cleavage Site in Ku80—The apoptosis specific 85-kDa fragment was identified as Ku80 based on the results of microsequencing. DNA-PK

Table 1. **Partial amino acid sequences of the 85-kDa fragment.**

Sequences	Identification in Ku80
EDGSGDRGD	$_{172}$EDGSGDRGD$_{180}$
EDIIQGFRYG	$_{308}$EDIIQGFRYG$_{317}$
DQVTAQEIF	$_{546}$DQVTAQEIF$_{534}$

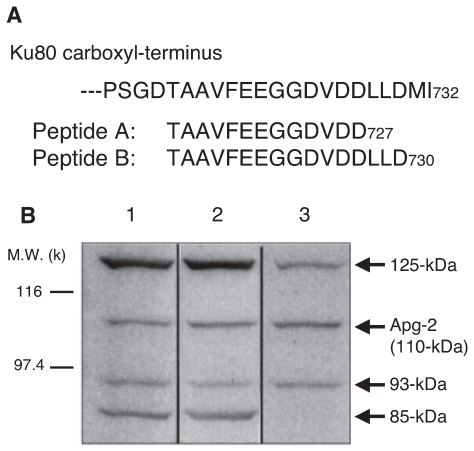

Fig. 3. **Immunoabsorption of #1339 using synthesized peptides derived from the Ku80 carboxyl-terminus.** Two peptides derived from the Ku80 carboxyl-terminus were synthesized and immunoblotting was performed by incubating these peptides with #1339. A: A diagram representing the amino acid sequence of the carboxyl-terminus of human Ku80 (amino acid residues 721–732) and the two peptides (peptide A and B). B: Immunoblotting using a #1339 incubated with synthesized peptide. Lane 1, no-peptide; 2, incubation with peptide A; and 3, incubation with peptide B.

consists of a catalytic subunit termed DNA-PKcs and a DNA-binding component Ku antigen (Ku70 and Ku80). Previous studies have shown that although DNA-PKcs is cleaved by caspase-3 during apoptosis, the molecular size of Ku70 and Ku80 do not differ. However, we found that Ku80 is cleaved during apoptosis because Ku80 was identified in this study by using the cleavage-site–directed antibody (#1339) designed to react with a cleaved fragment. Furthermore, since #1339 was designed to react with the carboxyl-terminus region that contains the aspartic acid residue, it can be inferred that cleaved Ku80 contains the aspartic acid residue at the carboxyl-terminus. Therefore, we hypothesized that Ku80 would be cleaved at the aspartic acid residue in the carboxyl-terminus region, since the molecular size of Ku80 has been reported as 80–86 kDa on SDS-PAGE, which is not significantly different from the #1339 detected Ku80 in this experiment. We then investigated the epitope of #1339 in Ku80 because its epitope coincides with the cleavage site. We examined the immunoabsorption by synthetic peptides from the carboxyl-terminus region of Ku80 to determine the epitope (Fig. 3). Ku80 contains three aspartic acid residues in the carboxyl-terminus, and two caspase-3 consensus motifs are formed by these aspartic acid residues ($DVDD_{727}$/L and $DLLD_{730}$/M). Between the two synthetic peptides (Fig. 3A, peptides A and B), peptide B, which contains DLLD at the carboxyl-terminus, inhibited Ku80 detection by #1339 (Fig. 3B, lane 3).

Moreover, to determine the epitope of #1339, we transfected the EGFP-tagged Ku80 mutant expression constructs into HeLa cells. All constructed EGFP-Ku80 mutants [EGFP-Ku80, EGFP-Ku80 (D730E), and EGFP-Ku80ΔMI described in "MATERIALS AND METHODS"] were localized in the nuclei in transfected HeLa cells (Fig. 4A). EGFP-Ku80 was detected as a 112-kDa polypeptide on SDS-PAGE by an anti-Ku80 antibody (Fig. 4B, lane 3 and 4). EGFP-Ku80 (D730E), in which Asp-730 is replaced

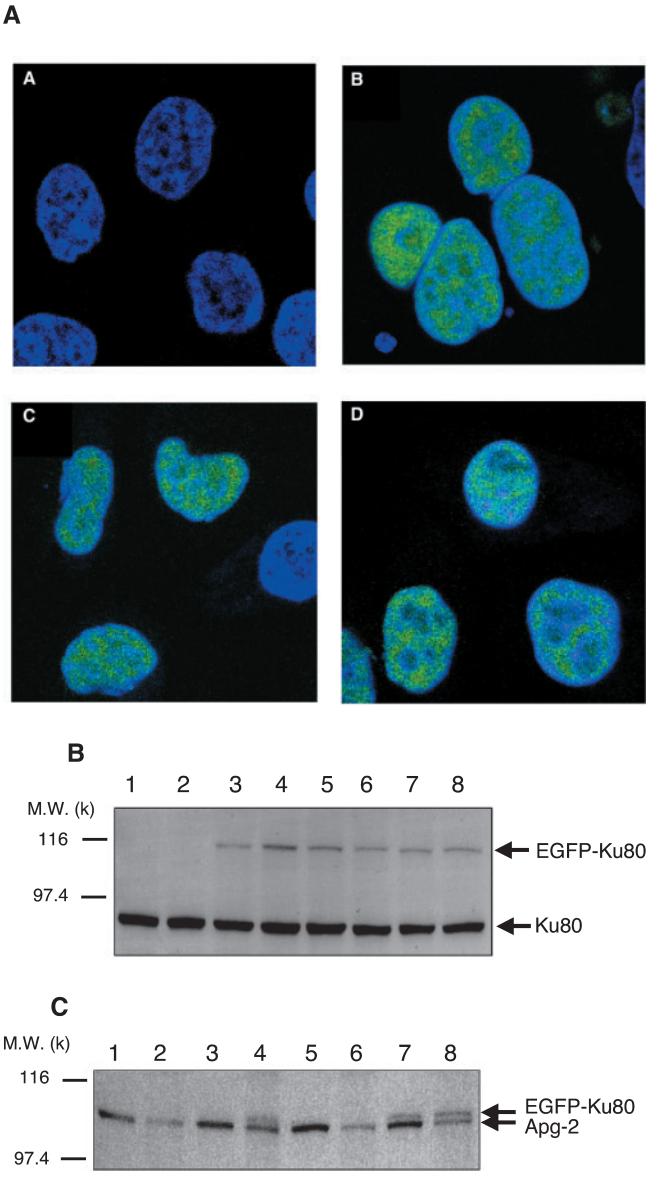

Fig. 4. **Detection of EGFP-Ku80 mutants by #1339.** EGFP-tagged Ku80 mutants were expressed in HeLa cells, treated with staurosporine for 6 h, and sampled for immunoblotting. A: EGFP image of EGFP-Ku80 mutants expressed in HeLa cells without staurosporine treatment. Nuclear DNA is stained by TO-PRO-3. EGFP-Ku80 mutants are shown in green and nuclear DNA was shown in blue. A: untransfected cells. The following EGFP-Ku80 mutants were expressed: B, EGFP-Ku80; C, EGFP-Ku80 (D730E); and D, EGFP-Ku80ΔMI. B and C: Immunoblotting was performed using an anti-Ku80 antibody (panel A) and #1339 (panel B). Lanes 1 and 2, untransfected HeLa cells; Lanes 3 and 4, EGFP-Ku80; Lanes 5 and 6, EGFP-Ku80 (D730E); and Lanes 7 and 8, EGFP-Ku80ΔMI. 1, 3, 5, and 7, not treated with staurosporine; 2, 4, 6, and 8, treated with staurosporine.

with Glu, and EGFP-Ku80ΔMI, which lacks the two amino acid residues at the carboxyl-terminus of Ku80, were also detected as 112-kDa fragments (Fig. 4B, lanes 5–8). The molecular sizes of these EGFP-tagged proteins did not change in staurosporine-induced apoptosis (Fig. 4B). Next, we examined whether EGFP-Ku80 mutants could be detected by #1339. EGFP-Ku80 was detected by

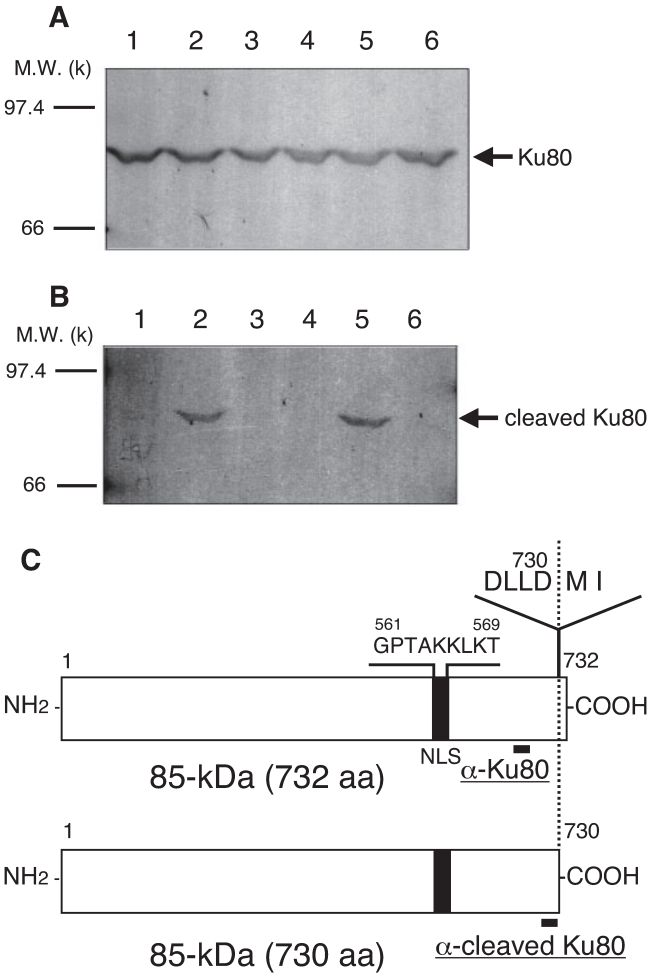

Fig. 5. **Effect of caspase inhibitors on Ku80 cleavage.** After pretreatment with caspase inhibitors for 2 h, cells were treated with anti-Fas mAb for 24 h. Immunoblotting was performed using an anti-Ku80 antibody (panel A) and anti–cleaved Ku80 antibody (panel B). Lane 1, control cells without mAb. The following inhibitors (100 μM) were added: 2, none; 3, Z-Asp-CH_2-DCB; 4, Z-VAD-fmk; 5, Ac-YVKD-CHO; and 6, Ac-DEVD-CHO. C: Schematic structure of Ku80. Ku80 contains the nuclear localization signal (NLS, shown as a black box) reported by Koike *et al.* (*39*). The cleavage site is indicated, and the lengths of unprocessed and the cleaved fragment shown in panels A and B are expressed as molecular size on SDS-PAGE as well as residue number. The epitope regions of the anti-Ku80 antibody and anti–cleaved Ku80 antibody are indicated by bold bars.

#1339 in apoptotic transfected HeLa cells but not in non-apoptotic cells (Fig. 4C, lane 3 and 4). Furthermore, as expected, EGFP-Ku80 (D730E) was not detected in either apoptotic or non-apoptotic transfected cells, whereas EGFP-Ku80ΔMI was detected in both apoptotic and non-apoptotic cells (Fig. 4C, lane 5–8). These results show that #1339 reacts with carboxyl-terminus truncated Ku80 in apoptotic cells, and also that Ku80 is cleaved at Asp-730 during apoptosis.

Detection of Cleaved Ku80 in Apoptotic Cells—The results of immunoabsorption and EGFP-tagged mutant proteins indicate that Ku80 is cleaved at Asp-730 during apoptosis. We then raised a cleavage-site–directed antibody (anti-cleaved Ku80 antibody) that reacts with the carboxyl-terminus sequence of cleaved Ku80. The molecular size of Ku80 on SDS-PAGE was similar in both non-apoptotic and apoptotic Jurkat cells (Fig. 5A, lane 1 and 2), whereas the anti-cleaved Ku80 antibody detected cleaved Ku80 in apoptotic cells but not in non-apoptotic cells (Fig. 5B, lane 1 and 2). Moreover, the molecular size of the cleaved Ku80 was same as that of unprocessed Ku80 (Fig. 5, A and B, lane 2). We then investigated the effect of caspase inhibitors on the detection of cleaved Ku80. Z-VAD-fmk and Z-Asp-CH_2-DCB, which are caspase inhibitors with broad specificities, inhibited the detection of Ku80 by anti-cleaved Ku80 antibody (Fig. 5B, lane 3 and 4). Ac-DEVD-CHO, a caspase-3/caspase-7 specific inhibitor, also inhibited the detection (Fig. 5B, lane 6). However, Ac-YVAD-CHO, which inhibits caspase-1, did not inhibit the detection of Ku80 (Fig. 5B, lane 5). Schematic representations of Ku80, including the cleavage site and antibody recognition regions, are presented in Fig. 5C.

We further examined the subcellular localization of cleaved Ku80 by confocal immunofluorescence microscopy (Fig. 6). In this study, we classified the three apoptotic phases depending on the staining patterns of nuclear DNA by propidium iodide: (i) non-apoptotic phase, (ii) early apoptotic phase (a little nuclear DNA condensed), and (iii) late apoptotic phase (nuclear fragmented). As reported, Ku80 localizes in the nucleus during the interphase and is distributed in the cytoplasm in the mitotic phase in non-apoptotic cells (Fig. 6, panel A). Although Ku80 was localized in the nucleus in the early apoptotic phase (panel B), it was distributed in the cytoplasm in the late apoptotic phase (panel C). Cleaved Ku80 was not detected in non-apoptotic cells (panel D). However, the cleaved fragment was detected in the nucleus in the early apoptotic phase (panel E), and this fragment was distributed in the cytoplasm in the late apoptotic phase (panel F, arrowed). Moreover, cleaved Ku80 was also detected in the nucleus of the cells with completely condensed chromatin (panel F, arrowhead). These results show that the cleavage of Ku80 occurs in the nucleus in the early apoptotic phase, and that the localization of Ku80 changes during apoptosis.

DISCUSSION

Many proteases are involved in the process of apoptosis. Among these proteases, caspases play an important role in apoptotic-signal transduction and executing processes (*1*, *2*). Caspase-3, in particular, is known to be an important protease in the process of apoptosis (*10*). Several substrates cleaved by caspase-3 during apoptosis have been reported. Although caspase-3 is localized in the cytoplasm in non-apoptotic cells, this protease translocates into the nuclei during apoptosis and cleaves nuclear proteins such as PARP (*34*). Ku80, a nuclear protein, is a component of DNA-dependent protein kinase (DNA-PK) , which is involved in the repair of DNA double-strand breaks and V(D)J recombination (*21*, *22*). Previous studies have reported that the DNA-PK catalytic subunit (DNA-PKcs) is cleaved by caspase-3 during apoptosis, but Ku80 is not cleaved (*29–31*). However, we observed that Ku80 is cleaved during apoptosis as described in this study.

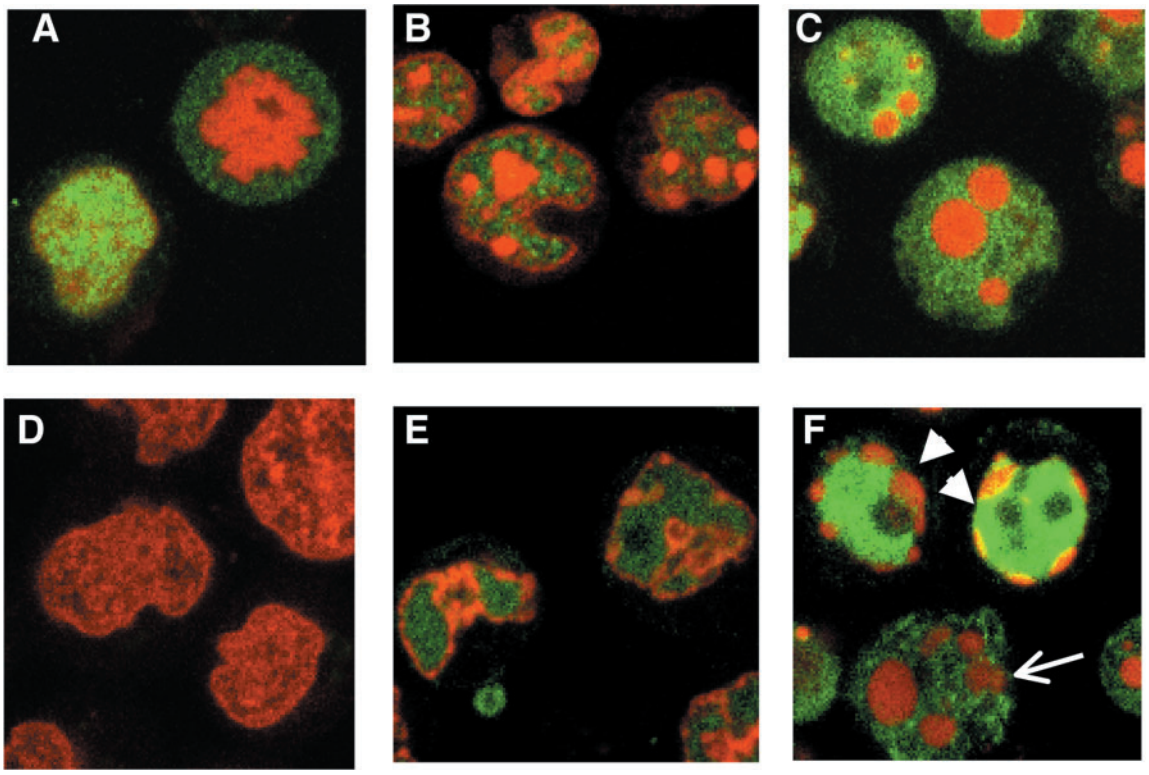

Fig. 6. **Cellular localization of Ku80 in apoptotic Jurkat cells.** Non-apoptotic and apoptotic Jurkat cells were fixed as described in "MATERIALS AND METHODS" and prepared for confocal immunofluorescence microscopy using anti-Ku80 antibody (panels A, B, and C) and anti–cleaved Ku80 antibody (D, E, and F). Cells were double labeled with propidium iodide, which binds to nuclear DNA. Antibody staining is shown in green and nuclear DNA staining is shown in red. The apoptotic phase was classified into three stages depending on the staining pattern of nuclear DNA: non-apoptotic phase (panels A and D), early apoptotic phase (panels B and E), and late apoptotic phase (panelsC and F). In panel F, the cells with completely condensed chromatin are shown by an arrowhead and a nuclear fragmented cell is shown by an arrow.

Firstly, we attempted to determine the cleaved Apg-2 fragment in apoptotic cells. Apg-2 is a member of the Hsp110 family and is expressed in all tissues (*35*). It has previously been reported that the level of Apg-2 decreases during apoptosis in human Jurkat cells, as shown in Fig. 1A; therefore, we hypothesized that Apg-2 would be cleaved by caspase-3 during apoptosis. Then, we raised a cleavage-site–directed antibody (#1339) designed to react with the carboxyl-terminus of the Apg-2 fragment cleaved by caspase-3. Since apoptosis specific fragments were detected by #1339, we tried to identify the cleaved Apg-2 fragment by using peptide incubated #1339, which was immunoabsorbed with an antibody reacting with the carboxyl-terminus of the antigen peptide. Although peptide incubated #1339 detected unprocessed Apg-2, other fragments were hardly detected in apoptotic cells, as shown in Fig. 1B. However, a 93-kDa fragment, one of the apoptosis specific fragments, was detected faintly. Based on the principle of immunoabsorption, this fragment could be identified as the cleaved Apg-2 fragment, but the intensity of the 93-kDa fragment detected by peptide incubated #1339 is much lower than that detected by peptide unincubated #1339 (Fig. 1B, lane 1 and 2). For this reason, we could not confirm that the 93-kDa fragment is the cleaved Apg-2 in apoptotic cells.

We could not detect the cleaved Apg-2 fragment definitely. Apg-2 contains four caspase-3 consensus motifs (DXXD): DGED$_{665}$/Q, DQYD$_{724}$/H, DHLD$_{727}$/A, and DAAD$_{730}$/M, and a peptide derived from Apg-2 residues 717–727 was synthesized and used as an antigen to produce a cleavage-site–directed antibody (#1339). Because this antigen peptide contains two caspase-3 consensus motifs, DQYD$_{724}$/H and DHLD$_{727}$/A, and #1339 did not react with the cleavage site only, this antibody would detect an Apg-2 fragment cleaved at Asp-724 or Asp-727. Furthermore, #1339 would detect the fragment cleaved at Asp-730 because a consensus motif, DAAD$_{730}$/M, is present downstream of the antigen peptide. Based on these facts, one possible explanation is that Apg-2 is cleaved at Asp-665 during apoptosis. In the next study, we will produce another antibody to determine whether Apg-2 is cleaved during apoptosis, and also check whether purified Apg-2 is cleaved by recombinant caspase-3 *in vitro*.

In this study, we detected the cleaved Ku80 fragment by using a cleavage-site–directed antibody. Furthermore, we also detected the cleavage site of Ku80 by immunoabsorption and EGFP-tagged Ku80 mutant protein analysis. Ku80 is cleaved during apoptosis at the Asp-730 residue, and the sequence of the cleavage site is DLLD$_{730}$/M, which fits the caspase-3/caspase-7 consensus motif DXXD. This cleavage is inhibited by treatment with a caspase-3/caspase-7 specific inhibitor. Since it was observed that caspase-3 translocates to the nucleus dur-

ing apoptosis (*34*), we conclude that Ku80 is cleaved by caspase-3 during apoptosis. In previous studies, it was considered that Ku80 was not cleaved during apoptosis because the molecular size of Ku80 is similar in non-apoptotic and apoptotic cells. However, in our study, we detected the cleaved Ku80 fragment by using a cleavage-site–directed antibody. This type of antibody is useful to analyze cleaved fragments. It can detect a cleaved fragment such as Ku80, the molecular size of which does not differ significantly from the unprocessed polypeptide.

As shown in Fig. 6, we determined that the cleavage of Ku80 occurs in the nucleus in the early apoptotic phase. As previously described, Ku80 would be cleaved by caspase-3 because this protease translocates to the nucleus during apoptosis. Furthermore, we found that Ku80 is distributed in the cytoplasm of nuclear fragmented apoptotic cells. Song *et al.* also observed this change in the localization of Ku80 during apoptosis in pancreatic acinar AR42J cells (*36*). During the cell cycle, Ku80 localizes in the nucleus in interphase and is distributed in the cytoplasm during mitosis (*37*, *38*). Since Ku80 contains the nuclear localization signal (NLS), the distributed Ku80 is re-localized into the nucleus during late telophase/early G1 phase by the importin α and importin β mediated transport pathway (*39*, *40*). Previous studies have shown that La autoantigen, a nuclear caspase-3 target protein, is distributed from the nucleus to the cytoplasm during apoptosis because it loses the NLS by cleavage (*41*). However, it was observed that Ku80 does not lose the NLS by cleavage and that EGFP-Ku80ΔMI localizes in the nucleus as shown in Fig. 4A. Consequently, the localization change of Ku80 during apoptosis is not caused by NLS loss or cleavage. Since cleaved Ku80 is localized in nuclei with condensed chromatin, the localization change could be caused by nuclear fragmentation in the late apoptotic phase.

A cleavage-site–directed antibody, #1339, was designed to react with the Apg-2 fragment cleaved by caspase-3. However, this antibody unexpectedly reacted with cleaved Ku80. The carboxyl-terminal sequence of the antigen peptide for #1339 is DHLD, whereas the sequence for cleaved Ku80 is DLLD. By comparing these sequences, it was observed that the P_1, P_2, and P_4 residues are identical, but the P_3 residues (histidine in the antigen peptide and leucine in the cleaved Ku80) are classified into different amino acid groups. #1339 cross reacts with the carboxyl-terminal sequence of cleaved Ku80, since the P_1, P_2, and P_4 residues between the antigen peptide and cleaved Ku80 are the same.

We found three apoptosis specific fragments by #1339, and one of these fragments was characterized as Ku80. However, two fragments (125 kDa and 93 kDa) have not been characterized, and their identification will be the target of future studies.

Finally, what is the biological significance of Ku80 cleavage? DNA-PK is composed of DNA-PKcs and Ku (a complex of Ku80 and Ku70). Several studies have indicated that DNA-PK is involved in the repair of DNA double-strand breaks. Previous studies have reported that DNA-PKcs is cleaved by caspase-3 during apoptosis, and this cleavage induces a decrease in catalytic activity (*30*, *31*). During apoptosis, chromatin DNA is degraded into nucleosome-sized fragments by CAD (*13*); the cleavage of DNA-PKcs is then important to prevent the repair of DNA double-strand breaks. In this study, we observed that Ku80 was cleaved at the carboxyl-terminus during apoptosis. Since the carboxyl-terminus of Ku80 is identified as the DNA-PKcs binding site (*42*), the cleaved Ku80 can not associate with DNA-PKcs. Based on these results, the repair of DNA double-strand breaks during apoptosis might be prevented by the cleavage of the two components of DNA-PK, DNA-PKcs and Ku80.

Ku has a non–sequence-specific double-strand DNA end-binding activity, and acts as a DNA-binding component of DNA-PK (*25*, *26*, *28*). As shown in Fig. 6, Ku80 is distributed in the cytoplasm in nuclear fragmented cells, and Song *et al.* have reported that Ku70 is also distributed in the cytoplasm during apoptosis (*36*). Since the carboxyl-terminus truncated Ku80 associates with Ku70, it will be present as the Ku complex in apoptotic cells, and the DNA-binding activity of Ku is retained (*43*, *44*). As described previously, many double-strand DNA ends are present in the nucleus of apoptotic cells because CAD cleaves chromatin DNA. Ku would be observed in fragmented nuclei if Ku binds to DNA-ends mediated by CAD, but Ku80 and Ku70 are distributed in the cytoplasm during apoptosis. One possibility for the localization change of Ku80 is that Ku may not bind to the CAD cleaved double-strand DNA ends, and this possibility will be examined in our next study.

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