La Autoantigen Is Cleaved in the COOH Terminus and Loses the Nuclear Localization Signal during Apoptosis*

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La autoantigen is a 47-kDa nuclear protein that binds to nascent polymerase III transcripts and a number of viral RNAs. We show that La protein was cleaved to generate a 43-kDa fragment during apoptosis of human leukemic HL-60 cells treated with camptothecin or etoposide. Immunofluorescence microscopy showed that the La protein level was increased in the cytoplasm during apoptosis of HL-60 cells. In addition, UV irradiation of HeLa cells led to the cleavage and redistribution of La protein upon apoptosis. Several lines of evidence show that La protein is cleaved by caspase-3 or closely related proteases at Asp-374 in the COOH terminus. When the full-length (La) and COOH-terminally truncated (LaΔC374) forms of La protein were expressed as fusion proteins with green fluorescence protein (GFP), GFP-LaΔC374 was predominantly cytoplasmic, whereas GFP-La was localized in the nucleus. These results suggest that La protein loses the nuclear localization signal residing in the COOH terminus upon cleavage and is thus redistributed to the cytoplasm during apoptosis.

Apoptosis is accompanied by disorganization of the nuclear architecture, cytoskeleton, and cell membrane. Proteolytic cleavage of key substrates is an important biochemical mechanism underlying the apoptotic process, and interleukin-1β-converting enzyme-like proteases have been reported to play crucial roles as mediators of apoptosis (1–3). Identifying substrates of interleukin-1β-converting enzyme or interleukin-1β-converting enzyme-like proteases and determining cleavage sites are important to understand the apoptotic process. A variety of antitumor drugs have been shown to induce apoptosis and to cause changes in nuclear morphology in rapidly proliferating cells, lymphoid tissues, and tumors (4, 5). Camptothecin and etoposide, which are antitumor drugs, inhibit proliferating cells, lymphoid tissues, and tumors (4, 5). Camptothecin and etoposide, which are antitumor drugs, inhibit proliferating cells, lymphoid tissues, and tumors (4, 5). Camptothecin and etoposide, which are antitumor drugs, inhibit proliferating cells, lymphoid tissues, and tumors (4, 5).

EXPERIMENTAL PROCEDURES

Cell Culture Conditions, Reagents, and Irradiation—Mouse myeloma P3-X63-Ag8.653 cells that were used for production of hybridoma were obtained from the RIKEN Gene Bank. HL-60, Jurkat, U937, and HeLa cells were obtained from American Type Culture Collection. Leukemia cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum in an atmosphere of 95% air and 5% CO2. Camptothecin (TopoGEN) and etoposide (Nippon-Kayaku) were dissolved in a 1:1 (v/v) mixture of dimethyl sulfoxide and ethanol and stored at −20 °C until used. HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum. Subconfluent monolayer cultures of HeLa cells were washed twice with PBS (2.7 mM KCl, 1.5 mM KH2PO4, 137 mM NaCl, and 8 mM Na2HPO4) and irradiated in PBS with 100 J of 254 nm UV light/m2 (Spectrolinker XL-1500, Spectronics Corp., Westbury, NY). The anti-poly(ADP-ribose) polymerase (PARP) C-2-10 antibody was obtained from Oncogene Research Products (Cambridge).

Preparation of a Triton X-100-insoluble Fraction of HL-60 Cells and Production of mAb—After washing with PBS, HL-60 cells were extracted on ice for 5 min with 0.5% Triton X-100 in cytoskeleton buffer (10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM EDTA, 4 mM vanadyl riboside complex, and 1 mM phenylmethlysulfonyl fluoride). The insoluble fraction was collected by centrifugation at 600 × g for 5 min. For immunization, chromatin was removed from the Triton X-100-insoluble material by DNAse I (Sigma) digestion, followed by 0.25 M ammonium sulfate extraction as described by Fey and Penman (23). The chromatin-depleted insoluble materials were mixed with Freund’s complete adjuvant for the first immunization or with Freund’s incomplete adjuvant for booster immunizations. Spleen cells were collected from immunized BALB/c mice and were fused with mouse myeloma cells by the polyethylene glycol method (24).

Identification of Antigens by Immunodetection of the cDNA Library—Total RNA was isolated from HL-60 cells, and mRNA was purified on an oligo(dT)-cellulose column (25). cDNAs were synthesized using random hexadeoxynucleotides as the primer and cloned into the EcoRI site of Agt11 arms according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).
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The agt11 cDNA libraries were immunoscreened with this mAb (24) as described (25). The cloned cDNAs were amplified by polymerase chain reaction using agt11 forward and reverse primers and introduced into the vector pGEX-1T-1 (Amersham Pharmacia Biotech) for expression as a glutathione S-transferase fusion protein. The glutathione S-transferase fusion proteins were subjected to Western blot analysis using the anti-La mAb to exclude false-positive cDNA clones. The polymerase chain reaction product of the cDNA clone was directly sequenced by the dye-oxey chain termination method (26). To determine the region of La protein recognized by mAb 24 more precisely (see “Results”), the coding sequence was amplified by polymerase chain reaction, inserted into pGEX-1T-1, and expressed as a glutathione S-transferase fusion protein.

Immunofluorescence Microscopy—HL-60 cells were collected by centrifugation at 600 × g for 5 min, placed on 14-well Teflon-coated slide glasses (Cell-Line/Erie Scientific, Portsmouth, NH), and fixed with 70% ethanol or acetone/methanol (1:1) for 15 min. After soaking in PBS containing 1% bovine serum albumin for 15 min, samples were incubated with the first antibody at room temperature for 60 min and then stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Dako Japan, Kyoto, Japan) in 20 μg/ml propidium iodide. Samples were examined using a fluorescence microscope (Axioskop, Carl Zeiss, Oberkochen, Germany).

Apoptotic cells were determined by the terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL) assay (27). DNA strand breaks were demonstrated by labeling 3'-OH termini with fluorescein isothiocyanate-labeled deoxyuridine using an in situ apoptosis detection kit (Takara, Kyoto). After TUNEL staining, cells were immunostained with anti-La mAb. In this case, tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG (Dako Japan) was used as the second antibody.

In Vitro Cleavage with Recombinant Caspase-3—HL-60 cells (1 × 10⁶) were washed with Krebs-Ringer buffer and lysed on ice with 1 ml of cell lysate were incubated at 37 °C in the presence or absence of 2 μl of 0.37 mg/ml (3300 units/ml) recombinant caspase-3 (Medical and Biological Laboratories Co., LTD, Nagoya, Japan) according to the manufacturer’s instructions.

Production of Antibodies against the NH₂ and COOH Termini of La—An NH₂-terminal peptide (‘MAENGDNEKMAALEA’25) and a COOH-terminal peptide (‘DYTEDEEPSKQKQKRT’) of La were synthesized by an automated solid-phase peptide synthesis (Takara). A cysteine residue was added to the NH₂ terminus of each of the synthetic peptides for coupling to bovine serum albumin. The peptides were coupled to bovine serum albumin using maleimidobenzoic acid hydroxysuccinimide ester as a linker (29). The peptide-coupled bovine serum albumin was used to immunize New Zealand White rabbits, and antisera were obtained.

Expression of the Full-length and COOH-terminally Truncated Forms of La Proteins as Fusion Proteins with GFP—cDNAs of full-length (La) and COOH-terminally truncated (LaC374) forms of La proteins were synthesized by reverse transcription and polymerase chain reaction. An antisense primer of La mRNA (‘5'-TAAAACTACTG-GTCTCCAGCA-3’) was used for reverse transcription. cDNAs of La and LaC374 were amplified by polymerase chain reaction using the sense primer ‘5'-CGGATATCCGCTGAAAAATGTGATATTG-3’ and the antisense primers ‘5'-ACGGCTGCACCTAGCTGCTGCGACCAT-3’ and ‘5'-ACGGCTGACGCTATATGCTGATCTG-3’, respectively. Each cDNA was digested with EcoRI and SalI and cloned into the pEGFP-c2 mammalian expression vector (CLONTECH). Exponentially growing COS-7 cells were plated in 6-well tissue culture dishes for Western blot analysis or on coverslips for immunofluorescence staining and incubated for 24 h at 37 °C. Each construct was transfected into COS-7 cells by lipofection using Transome™ (Wako, Osaka, Japan) according to the manufacturer’s instructions.

Determination of Molecular Mass by Delayed Extraction Matrix-assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (DE-MALDI-TOF-MS)—Hybridoma was cultured in protein-free hybridoma media (Life Technologies Inc.), and anti-La mAb was purified from culture fluid using protein A-Sepharose CL-4B (Amersham Pharmacia Biotech). The purified anti-La mAb was conjugated to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech). HL-60 cells that were treated with 10 μg/ml etoposide for 12 h were lysed in 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), and the protease inhibitors phenylmethylsulfonyl fluoride, aprotinin, antipain, papain, and leupeptin. The cell lysate was clarified by centrifugation at 10,000 × g for 20 min and incubated with anti-La mAb-conjugated Sepharose 4B for 1 h at 4 °C. After elution with 0.1 M glycine (pH 2.5), the affinity-purified proteins were concentrated and desalted using a centrifugal filter device (Microcon YM-30, Millipore Corp.). DE-MALDI-TOF-MS was carried out on a Voyager Elite XL Biochemical Workstation (6.5-m flight length linear mode; Perseptive Biosystems, Framingham, MA). Sinapinic acid was used as a matrix. A nitrogen laser (337 nm) was used for ionization. Acceleration voltage was set 20,000 V; grid voltage was set at 74% of the acceleration voltage; and guide wire voltage was 0.05% of the acceleration voltage. Delay time was 250 ns.

RESULTS

Production of Anti-La mAb—To examine changes in nuclear architecture during apoptosis, we developed mAbs against nuclear matrix proteins (23) in apoptotic HL-60 cells. Labeling with one mAb, designated mAb 24, was examined using immunofluorescence microscopy, and marked changes in HL-60 cells during apoptosis were observed. We immunoscreened agt11 HL-60 cDNA libraries with mAb 24 and isolated one cDNA clone encoding a polypeptide that was identical to La protein from amino acids 320 to 393. La is a nuclear protein composed of 408 amino acids with a predicted molecular mass of 46,837 Da (30, 31). mAb 24 recognized a polypeptide with a molecular mass of ~47 kDa upon Western blotting (Fig. 1) and stained the nuclei of various human cells upon immunofluorescence microscopy.

Cleavage of La Protein upon Apoptosis—Western blot analysis of cell lysates with anti-La mAb revealed extra polyepitopes in addition to the native 47-kDa polypeptide of La in HL-60 cells treated with camptothecin (Fig. 1a) or etoposide (data not shown). A 43-kDa fragment was prominent in lysates. PARP is a well documented caspase-3 substrate and is cleaved in the early stages of apoptosis (32–34). We compared La protein with PARP in HL-60 cells and found that after incubation with camptothecin for 12 h, >80% of PARP was cleaved (data not shown), but only ~35–40% of La protein was cleaved (Fig. 1a). This result indicates that La protein is not as good a substrate for caspases as PARP. Similarly, camptothecin induced apoptosis of human monocytic leukemia U937 cells and T-cell lymphoma Jurkat cells, where the 43-kDa fragment was generated (data not shown).

Cellular Localization of La Protein in Non-apoptotic and Apoptotic HL-60 Cells—Immunofluorescence analysis with anti-La mAb revealed marked changes in HL-60 cells treated with camptothecin (Fig. 1a) or etoposide (data not shown). A 43-kDa fragment was prominent in lysates. PARP is a well documented caspase-3 substrate and is cleaved in the early stages of apoptosis (32–34). We compared La protein with PARP in HL-60 cells and found that after incubation with camptothecin for 12 h, >80% of PARP was cleaved (data not shown), but only ~35–40% of La protein was cleaved (Fig. 1a). This result indicates that La protein is not as good a substrate for caspases as PARP. Similarly, camptothecin induced apoptosis of human monocytic leukemia U937 cells and T-cell lymphoma Jurkat cells, where the 43-kDa fragment was generated (data not shown).

TUNEL staining and various morphological features such as reduction in cell volume and cytoplasmic blebbing were used to identify apoptotic cells. After treatment with camptothecin or etoposide, apoptotic cells were found to increase as shown in Fig. 1b. TUNEL staining clearly demonstrated that the redistribution of La protein was associated with DNA fragmentation (Fig. 1c). These findings were observed in Jurkat and U937 cells, where La protein levels were increased in the cytoplasm during apoptosis induced by the antitumor drugs (data not shown).

Cytoplasmic La staining was seen in most of the TUNEL-
positive cells (Fig. 2). Here, we must emphasize that cytoplasmic La protein levels were variable among TUNEL-positive cells, and La staining was still seen in the nuclei of most of these cells (Fig. 1c). La protein exhibited two changes upon apoptosis, i.e., proteolytic cleavage and nuclear-to-cytoplasmic redistribution. We next investigated the relationship between the generation of the 43-kDa polypeptide and the nuclear-to-cytoplasmic redistribution of La antigen.

Cleavage and Redistribution of La Protein in Apoptotic HeLa Cells—To investigate whether these changes in La protein seen in apoptotic leukemia cells occurred in adherent cells upon apoptosis, we analyzed the apoptotic process in HeLa cells. UV irradiation of HeLa cells led to nuclear condensation and morphological changes characteristic of apoptosis after ~6–24 h. La protein was predominantly nuclear in non-apoptotic HeLa cells, but it translocated to the cytoplasm upon apoptosis (Fig. 3b). The cleavage product of the 43-kDa polypeptide was increased during apoptosis induced by UV irradiation, and a good correlation was observed between cleavage of La and the number of cells exhibiting cytoplasmic La staining (Fig. 3c). We compared La protein with PARP in HeLa cells and found that La protein was not as good a substrate for caspases as PARP (Fig. 3a), as found in HL-60 cells. These results in HeLa cells were consistent with those in leukemia cells, indicating that the cleavage and redistribution of La protein are common in various types of cells during apoptosis.

Cleavage of La Protein in the COOH Terminus—The native 47-kDa La polypeptide is cleaved to generate a 43-kDa fragment during apoptosis. This result indicated that La protein is cleaved at ~30–40 amino acids downstream from the NH₂ terminus or upstream from the COOH terminus. We developed antisera against the NH₂ and COOH-terminal peptides of La protein in rabbits (Fig. 4a). As shown in Fig. 4b, the anti-
COOH-terminal peptide antibody recognized the native 47-kDa polypeptide, but not the cleaved 43-kDa polypeptide, whereas the anti-NH₂-terminal peptide antiserum recognized both the 47- and 43-kDa polypeptides. These results demonstrate that La protein is cleaved at ~30–40 amino acids upstream from the COOH terminus (Fig. 4a).

The DEXD motif of the caspase-3 cleavage site (35, 36) is present in the COOH terminus of human La protein, 360.DEH-DEHD374 (Fig. 4c). As mentioned above, mAb 24 used in this study was identified as an anti-La mAb recognizing a polypeptide of La protein from amino acids 320 to 393. The putative caspase-3 cleavage site(s) was present from amino acids 320 to 393. We then expressed a polypeptide of La from amino acids 320 to 371 as a fusion protein with glutathione S-transferase using the pGEX-4T-1 expression vector. Western blotting showed that anti-La mAb 24 recognized the fusion protein (data not shown), indicating that the epitope of anti-La mAb 24 is present from amino acids 320 to 371 of La protein.

We tested whether exogenous caspase-3 would cleave La protein as seen during apoptosis. Incubation of untreated cell lysate with recombinant caspase-3 resulted in cleavage of the 47-kDa La polypeptide to a 43-kDa fragment, identical in size to the fragment observed during apoptosis (Fig. 5a). We next examined the effects of caspase inhibitors on cleavage of La protein in intact cells. A specific inhibitor of caspase-3, acetyl-DEVD-aldehyde, decreased generation of the 43-kDa fragment in HL-60 cells treated with antitumor drugs (Fig. 5b). In contrast, the interleukin-1β-converting enzyme inhibitor acetyl-YVAD-aldehyde did not inhibit cleavage of La protein.

There are two possible caspase-3 cleavage sites (Asp-371 and Asp-374) in the COOH terminus of La protein. To map the cleavage site, we isolated La protein and its fragment from etoposide-treated HL-60 cells by anti-La mAb affinity chromatography and determined the molecular mass by DE-MALDI-TOF-MS (Fig. 6). The molecular masses of the native La polypeptide and the 43-kDa polypeptide are 46,831 ± 31 and 43,182 ± 44 Da (n = 5), respectively. Based on the amino acid sequence, the molecular mass of the native La protein is 46,837 Da, and those of two possible fragments are 42,816 Da for Asp-371 and 43,197 Da for Asp-374. This result indicates that...
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FIG. 7. Effects of COOH-terminal truncation of La protein on its localization. The full-length (GFP-La) or COOH-terminally truncated (GFP-LaΔC374) forms of La protein were expressed in COS-7 cells as GFP fusion proteins using the pEGFP-c2 expression vector. a, cell lysates expressing GFP fusion proteins or GFP only were subjected to Western blotting with anti-GFP antibody. The anti-GFP antibody used in this study recognized an unknown polypeptide of HL-60 cells to Western blotting with anti-GFP antibody. The anti-GFP antibody used in this study recognized an unknown polypeptide of HL-60 cells. b, COS-7 cells expressing GFP fusion proteins or GFP only were fixed and stained with propidium iodide (PI, red). Green fluorescence (GF) shows the distribution of GFP fusion proteins (GFP-LaΔC374 and GFP-La) or GFP alone.

suggest that loss of NLS may be responsible for the redistribution of La protein to the cytoplasm during apoptosis.

**DISCUSSION**

Our results demonstrate that La protein loses its COOH-terminal NLS by proteolytic cleavage during apoptosis and that the loss of NLS leads to the redistribution of La protein from the nucleus to the cytoplasm. Apoptosis is accompanied by disorganization of the nuclear architecture, cytoskeleton, and cell membrane. Members of the caspase family of cysteine proteases play essential roles in apoptosis (1–3). The caspase family of cysteine proteases have overlapping substrate specificities. For example, caspase-2, caspase-3, and caspase-7 each display similar specificities, which suggests that their roles in cells are at least overlapping, if not completely redundant (33, 34). The DEXD motif is present in the cleavage sites of several cell maintenance and/or repair proteins that are proteolytically cleaved during apoptosis, including poly(ADP-ribose) polymerase (32–34), the catalytic subunit of DNA-dependent protein kinase (28), the 70-kDa protein component of the U1 small ribonucleoprotein (28), D4-GB1 (38), and protein kinase C (28), the 70-kDa protein component of the U1 small ribonucleoprotein (28), D4-GB1 (38), and protein kinase C (28). The DEXXD motif, 368DEHDEHD374, thus, there are two possible cleavage sites for caspase-3, Asp-371 and Asp-374; but La protein is cleaved only at Asp-374 in apoptotic HL-60 cells upon apoptosis. This result demonstrates that the cleavage site in apoptotic cells is not solely determined by the DXXD motif, but that other factors such as the amino acid sequence surrounding the DXXD motif, interaction with other molecules, or conformation may influence the acceptability of each DXXD sequence.

La protein is cleaved less efficiently than the well documented caspase-3 substrate of PARP, indicating that the cleavage site of La protein is less accessible to caspases than that of PARP. After submission of this manuscript, Rutjes et al. (40) reported that La protein was proteolytically cleaved in vivo, generating a 45-kDa fragment. Based on the molecular size of the fragment upon SDS-polyacrylamide gel electrophoresis and the observation that caspase-specific inhibitors prevented cleavage of La protein, they proposed that La protein might be cleaved at Asp-371 and/or Asp-374 upon apoptosis. Their results are consistent with ours.

Sera from patients with rheumatoid diseases such as systemic lupus erythematous and Sjögren’s syndrome frequently contain antibodies directed against La (18, 19). The mechanisms responsible for production of autoantibodies against La protein are not fully understood. As shown in this study, La protein shows distinctive behavior during apoptosis, and this may be a general phenomenon associated with apoptosis of various types of cells. Apoptotic cell antigens have been identified as the targets of autoantibodies in autoimmune diseases, and it was proposed that apoptotic cells were the primary source of autoantigens (20, 22).

Cascoli-Rosen et al. (20) showed that La antigen was found to rim the core of nucleic acid in early apoptosis and then was found surrounding apoptotic bodies (large blebs) at the cell surface in later stages of apoptosis in UV-irradiated keratinocytes. Miranda et al. (21) reported that nuclear La staining became weaker and that La-stained blebs emerged from the cell membrane in later stages of apoptosis in cardiac myocytes. It would be interesting to determine whether loss of NLS by caspases is responsible for the redistribution of La protein in all of these cases.

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