Nuclear Translocation of Caspase-3 is Dependent on its Proteolytic Activation and Recognition of a Substrate-like Protein(s)*

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SUMMARY

Caspase-3 is thought to play an important role(s) in the nuclear morphological changes that occur in apoptotic cells and many nuclear substrates for caspase-3 have been identified despite the cytoplasmic localization of pro-caspase-3. Therefore, whether activated caspase-3 is localized in the nuclei and how active caspase-3 has access to its nuclear targets are important and unresolved questions. Here we confirmed nuclear localizations for both caspase-3-p17 and caspase-3-p12 subunits of active caspase in apoptotic cells using subcellular fractionation analysis. We also prepared polyclonal and monoclonal antibodies specific for active caspase-3 to define the subcellular localization of active caspase-3. Immunocytochemical observations using anti-active caspase-3 antibodies showed nuclear accumulation of active caspase-3 during apoptosis. In addition, caspase-3, but not caspase-7, translocated from the cytoplasm into the nucleus after induction of apoptosis. Mutations at the cleavage site between the p17 and p12 subunits and the substrate recognition site for the P3 amino acid of the DXXD substrate cleavage motif inhibited nuclear translocation of caspase-3, indicating that nuclear transport of active caspase-3 required proteolytic activation and substrate recognition. These results suggest that active caspase-3 is translocated in association with a substrate-like protein(s) from the cytoplasm into the nucleus during progression through apoptosis.
INTRODUCTION

Apoptosis is a fundamental cellular process involved in many biological phenomena, including morphogenesis and maintenance of tissue homeostasis. Apoptosis is morphologically characterized by a dramatic execution phase that includes loss of cell volume, plasma membrane blebbing and chromatin condensation, followed by packaging of the cellular contents into membrane-enclosed vesicles called apoptotic bodies. These changes reflect complex biochemical events carried out by a family of cysteine proteases called caspases (1). Caspases are divided into initiator caspases with long prodomains (caspase-8, -9, and -10), and effector caspases with short prodomains (caspase-3, -6, and -7). Initiator caspases activate effector caspases, which in turn cleave intracellular substrates, resulting in the dramatic morphological and biochemical changes characteristic of apoptosis (2-4).

Caspase-3 has been implicated as a key mediator of apoptosis in mammalian cells and is synthesized as a latent proenzyme composed of 277 amino acids (5-9). In response to various death signals, the caspase-3 proenzyme is cleaved by initiator caspases at Asp$^{28}$ and Asp$^{175}$ to generate the active large (p17) and small (p12) subunits, forming an active heterotetramer. Although the precursor form of caspase-3 is localized in the cytoplasm, caspase-3 plays essential roles in the nuclear changes in apoptotic cells (9, 10). These results suggested that some cytoplasmic substrates translocate into the nucleus after cleavage by caspase-3 leading to nuclear morphological changes. In this scenario, caspase-activated DNase (CAD)/DNA
fragmentation factor (DFF) 40 and apoptotic chromatin condensation inducer in the nucleus (Acinus) were identified in the cytoplasmic fraction of apoptotic cells (11-13). However, CAD/DFF40 and Acinus were suggested to be localized in the nucleus even before apoptosis induction (12-14). Furthermore, many substrates for caspase-3 have been identified in the nucleus (2-4, 15). Therefore, caspase-3 seems to translocate from the cytoplasm into the nucleus after apoptosis induction, and it was proposed that active caspase-3 is translocated into nuclei by simple diffusion after disruption of nuclear-cytoplasmic barrier (16). However, the precise molecular mechanism of nuclear translocation of active caspase-3 is still unknown.

In the present study, we have demonstrated the nuclear localization of active caspase-3 in apoptotic cells by using antibodies specific for the large and small subunits of active caspase-3. Furthermore, we have shown that the nuclear translocation of active caspase-3 required proteolytic activation and substrate recognition by using caspase-3 mutants, suggesting that the nuclear translocation of active caspase-3 is not mediated by simple diffusion but is operated by an active transport system.
EXPERIMENTAL PROCEDURES

Cell Culture and Apoptosis Induction - HepG2 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS). For induction of apoptosis, HepG2 cells were treated with 1 µg/ml of an agonistic anti-Fas antibody (CH-11; Kamiya Biomedical Company) in the presence of 0.2 µg/ml actinomycin D, or with 200 µg/ml etoposide. Transfection was performed using GenePORTER 2 (Gene Therapy Systems) according to the manufacturer’s instructions.

Subcellular Fractionation - For preparation of digitonin-lysed cell fractions, cells were washed with PBS and suspended in PBS followed by lysis with 0.3 mg/ml digitonin for 3 min at 37°C and centrifuged at 12,000 X g for 5 min at 4°C. The pellet and supernatant fractions were used for immunoblot analysis with the pellet fraction including nuclei and heavy membranes, and the supernatant fraction containing cytoplasm and light membranes.

For preparation of subcellular fractions, cells were washed with PBS and suspended in hypotonic solution [10 mM Hepes (pH 7.4), 10 mM MgCl\2, 42 mM KCl, 10 µM lactacystin] for 5 min on ice. Cells were lysed using a Dounce homogenizer and centrifuged at 600 X g for 10 min to collect crude nuclei that were further purified as described below. The supernatant was further centrifuged at 100,000 X g for 90 min. The pellet and supernatant were used as membrane and cytoplasmic fractions, respectively. The crude nuclear fraction was passed through a
27-gauge needle several times, extensively washed with hypotonic solution, and centrifuged through a 2 M sucrose cushion at 150,000 X g for 60 min. The pellet was used as a purified nuclear fraction. NP-40-treated nuclei were prepared by washing the purified nuclei with hypotonic solution containing 0.5% NP-40 to remove contaminating membranes.

Antibodies - Anti-active caspase-3 polyclonal (2622) and monoclonal (CS-1) antibodies, which recognize caspase-3-p12, were generated (see supplementary information). Anti-caspase-3 monoclonal antibody (C31720) was obtained from Transduction Laboratories; anti-caspase-3 polyclonal antibodies (sc-1224), anti-PKC polyclonal antibodies (sc-937) and anti-lamin B1 polyclonal antibodies (sc-6217) from Santa Cruz Biotechnology; anti-active caspase-3 pAb (G7481) from Promega; anti-green fluorescent protein (GFP) monoclonal antibody (8371) from Clontech; anti-caspase-3 polyclonal antibodies (9662) from Cell Signaling Technology.

Fluorescence Microscopy - For immunofluorescence analysis, cells were fixed with 3.7% formaldehyde in PBS for 10 min, washed with PBS twice, permeabilized in 0.5% Triton X-100 in PBS for 10 min, and washed with PBS twice. Cells were then incubated with primary antibodies in PBS containing 1% BSA overnight at 4°C. After washing with PBS twice, cells were incubated with Texas red (TXRD)-, FITC-, Alexa Fluor 488-, or Cy3-labeled secondary antibodies for 10 min at room temperature, and washed with PBS twice. After staining nuclei with 10 μM Hoechst
33342 (Calbiochem), cells were examined under a fluorescence microscope (Leitz Laborlux) or a confocal laser scanning microscope (Carl Zeiss).

**Plasmid Constructions** - The wild type pro-caspase-3 cDNA fragment was cloned into the *Eco*RI site of pUC-CAGGS (17) to generate pCAG-casp3. To construct expression plasmids for caspase fused to the N-terminus of GFP, PCR was carried out using caspase-3 and caspase-7 cDNAs as templates. The fragments encoding caspase-3 or caspase-7 were cloned into the *Eco*RI-*Bam*HI site of pEGFP-N1 (Clontech) to generate pcasp3-Wt-GFP or pcasp7-Wt-GFP. To generate C163S, D175A, R64E and R207E mutations, a PCR method employing mutagenic oligonucleotide primers was used. Caspase-3 cDNAs containing these mutations were cloned into the *Eco*RI-*Bam*HI site of pEGFP-N1 to generate pcasp3-C163S-GFP, pcasp3-D175A-GFP, pcasp3-R64E-GFP and pcasp3-R207E-GFP.
RESULTS AND DISCUSSION

Although it has been widely accepted that pro-caspase-3 is cleaved to generate the active form in the cytoplasm, the enzymatic activity of capase-3-like proteases can be found in the nuclear fraction of apoptotic cells (18-20). It is, however, necessary to determine whether activated caspase-3 itself is present in the nuclei of apoptotic cells, as opposed to other caspases such as caspase-7 and -8 that can also cleave caspase-3 substrates (21, 22). The p17 subunit of active caspase-3 is detected not only in the cytoplasmic and mitochondrial fractions but also in the nuclear fraction of apoptotic cells (23). However, the localization of the p12 subunit of active caspase-3 remained to be determined, which is important, because caspase-3 enzymatic activity requires both p17 and p12 subunits. Therefore, we initially investigated the localization of the caspase-3-p12 subunit. HepG2 cells treated with or without an agonistic anti-Fas antibody were separated into pellet and supernatant fractions after lysis with digitonin, followed by immunoblotting (Fig. 1A). Although pro-caspase-3 was present in the supernatant fraction irrespective of induction of apoptosis, the caspase-3-p12 subunit was present in both the pellet fraction (including nuclei) and the supernatant fraction after induction of apoptosis. Subcellular fractionation was used to confirm the nuclear localization of active caspase-3 in apoptotic cells. Since active caspase-3 may be degraded by the ubiquitin-proteasome pathway (24-26), pro-caspase-3 was transiently overexpressed to elevate the expression levels of caspase-3 and the preparation of subcellular fractions was carried out in the presence of a proteasome inhibitor,
lactacystin; PKC and lamin B1 were used as cytoplasmic and nuclear fraction markers, respectively. Normal non-transfected HepG2 cells (Fig. 1B, left panel) or transfected HepG2 cells treated with the anti-Fas antibody for 12 h (Fig. 1B, right panel) were fractionated into nuclear, membrane, and cytoplasmic fractions.

Although the expression level of pro-caspase-3 in transfected cells was more than five times higher than in non-transfected cells, the cytoplasmic localization of pro-caspase-3 was unaffected by overexpression. Both caspase-3-p17 and caspase-3-p12 were detected not only in the cytoplasmic fraction but also in the nuclear fraction even after treatment with 0.5% NP-40 (Fig. 1B, right panel). These results strongly suggested that active caspase-3 was present in the nuclei of apoptotic cells.

To directly assess the localization of active caspase-3, we prepared antibodies specific for active caspase-3-p12 subunit. Since pro-caspase-3 is processed at Asp²⁸ and Asp¹⁷⁵ sites to generate new N- and C- termini, we generated four affinity-purified polyclonal and three monoclonal antibodies that specifically recognize the newly exposed N-terminal region of caspase-3-p12 (see supplementary information). We also used the commercially available anti-active caspase-3 specific antibodies (G7481, Promega) that recognize the newly exposed C-terminus of caspase-3-p17.

Although pro-caspase-3 was detected in the cytoplasm of untreated normal cells (Fig. 2A), in most apoptotic cells stained with anti-active caspase-3 antibodies, which recognize the newly exposed N-terminus of caspase-3-p12 (Fig. 2B, a-l) or the newly exposed C-terminus of caspase-3-p17 (Fig. 2B, m-r), the signal was strongest around
the condensed nucleus. To determine whether active caspase-3 is actually present in
the nucleus, apoptotic cells were stained with the anti-active caspase-3 antibodies and
antibodies against lamin B1, a nuclear envelope marker, and analyzed by confocal
laser scanning microscopy (Fig. 2C). The signal for active caspase-3 was detected
both in and around the condensed nuclei. Taken together, these results indicate that
active caspase-3 is present not only in the cytoplasm but also in the nuclei of apoptotic
cells.

To examine the molecular mechanisms governing translocation of caspase-3
from the cytoplasm into the nucleus, we constructed various pro-caspase-3 mutants
fused with GFP. These constructs were transiently transfected into HepG2 cells.
After treatment with or without the agonistic anti-Fas antibody to induce apoptosis,
supernatant and pellet fractions were prepared after digitonin lysis, and
immunoblotted with the anti-GFP antibody (Fig. 3A). Although casp3-Wt-GFP was
predominantly present in supernatant, like the unprocessed form before Fas treatment,
proteolytically activated casp3-p12-GFP was recovered in both the supernatant and
pellet fractions after Fas treatment (Fig. 3A). In contrast, caspase-7, another effector
caspase, did not translocate to the nucleus, with both casp7-Wt-GFP and casp7-p12-
GFP being detected only in the supernatant fraction irrespective of Fas treatment.
Consistently, confocal laser scanning microscopy indicated that casp3-Wt-GFP was
present in both cytoplasm and nuclei, whereas casp7-Wt-GFP was found only in the
cytoplasm in apoptotic cells (Fig. 3B). These results suggested that the nuclear
translocation of effector caspases is specific for caspase-3, and that the nuclear translocation of caspase-3 is an active process and not simply entail diffusion after disruption of the nuclear-cytoplasmic barrier.

To determine whether caspase-3 activation is needed for nuclear translocation, we constructed pro-caspase-3 mutants that cannot be activated, recognize substrates or cleave substrates, respectively. Casp3-D175A-GFP, which is not activated due to mutation of the cleavage site between the p17 and p12 subunits, did not translocate after anti-Fas treatment, suggesting that the proteolytic activation of caspase-3 is necessary for its nuclear translocation (Fig. 3A). The three-dimensional structure of caspase-3 (27, 28), shows that Arg$^{64}$ and Arg$^{207}$ are essential for recognition of the P1 and P3 amino acids of the DXXD cleavage motif substrates, respectively. Cell fractionation analysis showed that casp3-R64E-GFP, but not casp3-R207E-GFP, translocated from cytoplasm to nuclei after Fas treatment, indicating that recognition of P3, but not P1, of a substrate(s) is necessary for nuclear translocation of active caspase-3 (Fig. 3A). Furthermore, casp3-C163S-GFP, in which the catalytic Cys is mutated, translocated from the cytoplasm into nuclei, indicating that substrate cleavage by caspase-3 is not essential for the translocation of active caspase-3. These results suggested that proteolytic activation and substrate recognition, but not substrate cleavage, are necessary for the nuclear translocation of active caspase-3.

Nuclear pore complexes (NPCs) mediate bidirectional transport between the cytoplasm and the nucleus (29). The NPC constitutes a passive diffusion channel,
which allows the diffusion of ions, metabolites and small proteins whose relative molecular mass is less than about 40 kDa. Proteins above the size limit can enter the nucleus by energy-dependent mechanisms. Caspase-3 lacks a typical consensus nuclear localization signal, and the active caspase-3 tetramer is too big to enter the nucleus passively. Recently, Faleiro and Lazebnik (16) reported that caspase-9 inactivates nuclear transport and increases the diffusion limit of the nuclear pore, leading to the entrance of caspase-3 into nuclei by diffusion. If caspase-3 enters the nucleus by simple diffusion, pro-caspase-3 as well as active caspase-3, and other caspases including caspase-7 would also be detected in the nuclear fraction of apoptotic cells. However, our data showed that neither pro-caspase-3 nor caspase-7 translocated into nuclei after apoptosis induction. Furthermore, active nuclear transport is required for the nuclear morphological changes induced by various apoptotic stimuli (30), and nuclear translocation of active caspase-3 required proteolytic activation and recognition of substrate-like protein(s). Therefore, we propose that the nuclear translocation of active caspase-3 is dependent on active nuclear transport. Identification of the substrate-like protein(s) which function as a carrier protein to transport active caspase-3 from the cytoplasm into nucleus in apoptotic cells is needed to clear the molecular mechanisms of nuclear translocation of active caspase-3.
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FOOTNOTES

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1The abbreviations used are: GFP, green fluorescent protein; TXRD, Texas red.
FIGURE LEGENDS

FIG. 1. **Nuclear localization of caspase-3-p17 and caspase-3-p12 subunits in apoptotic HepG2 cells.**

*A*, detection of caspase-3-p12 subunit in HepG2 cells. HepG2 cells were treated with or without the anti-Fas antibody in the presence of actinomycin D for 12 h, and pellet (P) and supernatant (S) fractions were prepared after lysis with digitonin. Each fraction was subjected to SDS-PAGE and immunoblotted with anti-caspase-3 polyclonal antibodies (sc-1224 from Santa Cruz Biotechnology), which detect both pro-caspase-3 and caspase-3-p12. 

*B*, subcellular fractionation of normal (left panel) and apoptotic (right panel) HepG2 cells. Subcellular fractions from apoptotic HepG2 cells were prepared after transfection with pCAG-casp3 followed by incubation for 24 h, and treatment with the anti-Fas antibody in the presence of actinomycin D for 12 h. Subcellular fractions, standardized to represent equal numbers of cells in each fraction, were subjected to SDS-PAGE and immunoblotted with anti-caspase-3 monoclonal antibody (C31720 from Transduction Laboratories), anti-caspase-3 antibody (9662 from Cell Signaling Technology), anti-caspase-3 polyclonal antibodies (sc-1224 from Santa Cruz Biotechnology), anti-PKC polyclonal antibodies or anti-lamin B1 polyclonal antibodies. 

T, total cell lysate; C, cytoplasmic fraction; M, membrane fraction; N, purified nuclear fraction; N⁺, purified nuclear fraction after treatment with 0.5% NP-40.
Fig. 2. **Nuclear accumulation of active caspase-3 in apoptotic cells.**

A, cytoplasmic localization of pro-caspase-3 in normal cells. After fixation and permeabilization, HepG2 cells were incubated with anti-caspase-3 polyclonal antibodies (sc-1224 from Santa Cruz Biotechnology) and TXRD-labeled secondary antibodies (a), followed by staining the nuclei with Hoechst 33324 (b). Photographs were taken of the same fields in a and b, and merged images of TXRD- and Hoechst-staining are shown in c. B, accumulation of active caspase-3 around the apoptotic nuclei. HepG2 cells were treated with the anti-Fas antibody in the presence of actinomycin D for 12 h (a-c, g-i, and m-o) or with etoposide for 40 h (d-f, j-l, and p-r). After fixation and permeabilization, the cells were incubated with anti-active caspase-3 polyclonal antibodies (2622) (a and d), anti-active caspase-3 monoclonal antibody (CS-1) (g and j), anti-active caspase-3 pAb (G7481 from Promega) (m and p), and TXRD-labeled secondary antibodies (a, d, g, j, m and p), followed by staining the nuclei with Hoechst 33324 (b, e, h, k, n and q). Photographs were taken of the same fields in a-c, d-f, g-i, j-l, m-o, and p-r, and merged images of TXRD- and Hoechst-staining are shown in c, f, i, l, o and r, respectively. C, detection of active caspase-3 in apoptotic nuclei. HepG2 cells were treated with the anti-Fas antibody in the presence of actinomycin D for 12 h or with etoposide for 40 h. After fixation and permeabilization, the cells were incubated with anti-active caspase-3 pAb (G7481 from Promega) and anti-lamin B1 polyclonal antibodies as a nuclear envelope marker,
and Cy3- and Alexa Fluor 488-labeled secondary antibodies, followed by staining the
nuclei with Hoechst 33324, and observed with confocal laser scanning microscope.
The z-series of images from five sections are shown. Bars, 10 μm.

**FIG. 3.** **Nuclear translocation of active caspase-3 requires proteolytic**
activation and substrate recognition, but not catalytic activity.  
**A,** localization of caspase-3- and caspase-7-GFP fusion proteins in HepG2 cells. One day before
transfection, HepG2 cells were seeded at a density of 2 x 10^5 cells per well in 6-well
dishes. In each well, 2 μg of plasmids expressing caspase-GFP fusion proteins were
transfected and incubated for 24 h. After treatment with or without the anti-Fas
antibody in the presence of actinomycin D for 12 h, cells were fractionated after lysis
with digitonin, and subjected to SDS-PAGE and immunoblotted with anti-GFP
monoclonal antibody.  
**B,** nuclear localization of caspase-3-, but not caspase-7-, GFP fusion protein in apoptotic cells
with confocal laser scanning microscopy. One day before transfection, HepG2 cells
were seeded at a density of 2 x 10^5 cells per 35-mm glass bottom dish. In each dish, 2
μg of pcasp3-Wt-GFP (a-i) or pcasp7-Wt-GFP (j-r) plasmids were transfected and
incubated for 24 h. After treatment without (a, b, c, j, k and l) or with the anti-Fas
antibody in the presence of actinomycin D for 12 h (d, e, f, m, n and o), or with
etoposide for 40 h (g, h, i, p, q and r), GFP expression (a, d, g, j, m and p) was
observed with confocal laser scanning microscope after staining with Hoechst 33342 (b, e, h, k, n and q). Photographs were taken of the same fields in a-c, d-f, g-i, j-l, m-o, and p-r, and merged images of GFP and Hoechst-staining are shown in c, f, i, l, o and r, respectively. Bar, 10 µm.
Fig. 1

A

Fas

|   | S | P |
|---|---|---|
| - |   |   |
| + |   |   |

kDa

37

25

15

10

pro-casp3

casp3-p12

B

normal cells

| T | C | M | N | N+ |
|---|---|---|---|----|

pro-casp3

PKC δ

lamin B1

apoptotic cells

| T | C | M | N | N+ |
|---|---|---|---|----|

pro-casp3

casp3-p17

casp3-p12

PKC δ

lamin B1
Fig. 2

A

\( \alpha \text{-casp-3 (poly) sc-1224} \)

TXRD  

Hoechst  

Merge


B

\( \alpha \text{-active casp-3 (poly) 2622} \)

TXRD  

Hoechst  

Merge

\( \alpha \text{-Fas + act D} \)

etoposide

\( \alpha \text{-active casp-3 (mono) CS-1} \)

TXRD  

Hoechst  

Merge

\( \alpha \text{-Fas + act D} \)

etoposide

\( \alpha \text{-active casp-3 pAb G7481} \)

TXRD  

Hoechst  

Merge

\( \alpha \text{-Fas + act D} \)

etoposide

C

\( \alpha \text{-Fas + act D} \)

etoposide

\begin{tabular}{|c|c|c|c|} 
\hline
active casp-3 & laminB1 & Hoechst & Merge \\
\hline
\end{tabular}

\begin{tabular}{|c|c|c|c|} 
\hline
active casp-3 & laminB1 & Hoechst & Merge \\
\hline
\end{tabular}
### Fig. 3

**A**

|        | casp3-Wt | casp3-C163S | casp3-D175A -GFP |
|--------|----------|-------------|------------------|
| Fas    | -        | +           | -                |
|        | S        | P           | S                |

**Gel Electrophoresis**

- **Pro-caspase-12**
- **Caspase-12**

**B**

|        | GFP | Hoechst | Merge |
|--------|-----|---------|-------|
| a      | b   | c       | normal|
| d      | e   | f       | α-Fas + act D|
| g      | h   | i       | etoposide|
| j      | k   | l       | normal|
| m      | n   | o       | α-Fas + act D|
| p      | q   | r       | etoposide|

**Caspase Analysis**

- **casp-3-Wt -GFP**
- **casp-7-Wt -GFP**
Supplementary Information

Supplemental EXPERIMENTAL PROCEDURES

Preparation of Active Caspase-3 Specific Antibodies - Anti-active caspase-3 polyclonal or monoclonal antibodies were generated by immunization of rabbits or mice with the synthetic peptide, Ser-Gly-Val-Asp-Asp-Asp-Cys, corresponding to the N-terminal sequence of caspase-3-p12 with a cysteine residue added to the C-terminus, conjugated to Diphtheria Toxoid-6-maleimido-caproic acid N-hydroxysuccinimide ester. Rabbits were immunized with the antigen conjugates 2 times at interval of 2 weeks; the first and the second injections included Freund’s Adjuvant, complete and incomplete, respectively. Antisera were obtained at 3 and 4 weeks after the final booster shot, and were affinity-purified by using immobilized antigen and designated 2622, 2623, 2624, and 2625, respectively. Anti-active caspase-3 monoclonal antibodies (isotype IgG1) were derived from the hybridomas (CS-1, CS-2 and CS-3) produced by the fusion of mouse myeloma cells (P3U1) and splenocytes from an immunized mouse (BALB/c).
Supplemental RESULTS

*Characterization of Active Caspase-3 Specific Antibodies* - To examine the specificities of the antibodies for detection of active caspase-3, HepG2 cells were treated with an agonistic anti-Fas antibody, and subjected to immunoblotting (Fig. S1A). The level of pro-caspase-3 gradually decreased (left and middle panels) and the level of processed p12 subunit increased (middle panel). Affinity-purified anti-active caspase-3 polyclonal antibodies detected only the p12 subunit of caspase-3 and not pro-caspase-3 (right panel), suggesting that the affinity-purified polyclonal antibodies specifically recognize active caspase-3.

To further characterize the anti-active caspase-3 antibodies, HepG2 cells treated with the anti-Fas antibody were fixed and permeabilized, followed by staining with the antibodies (Fig. S1B). We also used the commercially available anti-active caspase-3 specific antibodies (G7481, Promega) that recognize the newly exposed C-terminal region of caspase-3-p17. Apoptotic cells exhibiting fragmented and condensed nuclei detected with Hoechst 33324 were stained strongly with these antibodies (black arrowheads), whereas control cells with normal nuclei (white arrowheads) were not stained (Fig. S1B), indicating that these antibodies specifically recognize the corresponding antigens in apoptotic cells.
Supplemental FIGURE LEGENDS

FIG. S1. **Characterization of anti-active caspase-3 specific antibodies.** A, anti-active caspase-3 antibodies recognize caspase-3-p12 but not pro-caspase-3 in apoptotic cells. HepG2 cells were treated with the anti-Fas antibody in the presence of actinomycin D for the indicated periods. Cells were harvested and lysates were subjected to SDS-PAGE, followed by immunoblotting with anti-caspase-3 monoclonal antibody (C31720 from Transduction Laboratories) (left panel), anti-caspase-3 polyclonal antibodies (sc-1224 from Santa Cruz Biotechnology) (middle panel), or anti-active caspase-3 polyclonal antibodies (2622) (right panel). NS, non-specific band. B, immunocytochemical observation of active caspase-3. HepG2 cells were treated with the anti-Fas antibody in the presence of actinomycin D for 12 h. After fixation and permeabilization, the cells were incubated with anti-active caspase-3 polyclonal antibodies (2622) (a), anti-active caspase-3 monoclonal antibody (CS-1) (e) or anti-active caspase-3 pAb (G7481 from Promega) (i), and TXRD-labeled secondary antibodies (a, e and i), followed by staining the nuclei with Hoechst 33324 (b, f and j). Phase contrast images were shown in d, h and l, and the white or black arrowheads indicated normal or apoptotic cells. Photographs were taken of the same fields in a-d, e-h, and i-l, and merged images of TXRD- and Hoechst-staining are shown in c, g, and k, respectively. Bar, 10 µm.
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