Inositol 1,4,5-Trisphosphate Receptor Type 1 Is a Substrate for Caspase-3 and Is Cleaved during Apoptosis in a Caspase-3-dependent Manner*

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The inositol 1,4,5-trisphosphate (IP₃) receptor (IP₃R), an IP₃-gated Ca²⁺ channel located on intracellular Ca²⁺ stores, modulates intracellular Ca²⁺ signaling. During apoptosis of the human T-cell line, Jurkat cells, as induced by staurosporine or Fas ligation, IP₃R type 1 (IP₃R1) was found to be cleaved. IP₃R degradation during apoptosis was inhibited by pretreatment of Jurkat cells with the caspase-3 (-like protease) inhibitor, Ac-DEVD-CHO, and the caspases inhibitor, z-VAD-CH₂DCB but not by the caspase-1 (-like protease) inhibitor, Ac-YVAD-CHO, suggesting that IP₃R1 was cleaved by a caspase-3 (-like) protease. The recombinant caspase-3 cleaved IP₃R1 in vitro to produce a fragmentation pattern consistent with that seen in Jurkat cells undergoing apoptosis. N-terminal amino acid sequencing revealed that the major cleavage site is 1889DEVD₁₈₉₂R (mouse IP₃R₁), which involves consensus sequence for caspase-3 cleavage (DEVD). To determine whether IP₃R1 is cleaved by caspase-3 or is proteolyzed in its absence by other caspases, we examined the cleavage of IP₃R1 during apoptosis in the MCF-7 breast carcinoma cell line, which has genetically lost caspase-3. Tumor necrosis factor-α or staurosporine-induced apoptosis in caspase-3-deficient MCF-7 cells failed to demonstrate cleavage of IP₃R1. In contrast, MCF-7/Casp-3 cells stably expressing caspase-3 showed IP₃R1 degradation upon apoptotic stimuli. Therefore IP₃R1 is a newly identified caspase-3 substrate, and caspase-3 is essential for the cleavage of IP₃R1 during apoptosis. This cleavage resulted in a decrease in the channel activity as IP₃R1 was digested, indicating that caspase-3 inactivates IP₃R1 channel functions.

Apoptosis is an evolutionary conserved form of cell death by which normal cellular development and homeostasis are maintained. This programmed cell death is regulated by a series of biochemical events, namely activation of a family of cysteine proteases, caspases, which in turn cleave specific intracellular proteins resulting in an irreversible commitment to cell death.

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Among the caspase family, caspase-3 plays a crucial role in execution of apoptosis. Known substrates for caspase-3 (1) involve poly(ADP-ribose) polymerase, p21-activated kinase 2 (1, 2), gelsolin (3), DNA-dependent protein kinase catalytic subunit, DNA fragmentation factor 45 kDa subunit (4), and α-fodrin (5, 6). By analogy of the cleavage site of these substrates, amino acid sequence of DEVD is considered to be a recognition motif of caspase-3.

Inositol 1,4,5-trisphosphate (IP₃)³ receptor (IP₃R), an IP₃-gated Ca²⁺ channel located on intracellular Ca²⁺ stores, plays a crucial role in a variety of cell functions, including fertilization, cell proliferation, metabolism, secretion, contraction of smooth muscle, and neural signals (7, 8). Molecular cloning studies revealed that there are three types of IP₃Rs: IP₃R type 1 (IP₃R₁), IP₃R type 2 (IP₃R₂), and IP₃R type 3 (IP₃R₃) (9–12). The involvement of IP₃Rs during apoptosis has been proposed (13–15). Khan et al. (15) reported that mRNA and protein of IP₃R3 increase in B and T lymphocytes in response to anti-IgM antibodies and dexamethasone, respectively. Reduction of IP₃R3 expression by antisense construct of IP₃R3 cDNA blocked the dexamethasone-induced apoptosis. Jayaraman and Marks (14) reported that a stable transfectant of the human T-cell line, Jurkat, expressing an antisense cDNA construct of IP₃R1 is resistant to apoptotic stimuli, including Fas, dexamethasone, and γ-irradiation, despite the finding that T-cells in IP₃R1-deficient mice normally develop and respond to proliferative and death signals (16). Sugawara et al. (15) reported that IP₃/ Ca²⁺ signaling is involved in B-cell antigen receptor-induced apoptosis in a chick B-cell line, DT40 cells. In their experiments, IP₃R-deficient cells showed a reduction in apoptosis in which the degree of resistance depend on the number of IP₃Rs depleted, i.e. triple IP₃R-deficient cells were more resistant than single IP₃R-deficient cells.

Although it has been demonstrated that IP₃Rs are involved in the process of apoptosis, less attention has been directed to the relationship between IP₃Rs and caspases. Among the IP₃R family, IP₃R1 is the most widely expressed in tissues and is recognized as an ubiquitous type of IP₃R. In the primary amino acid sequence of IP₃R1, there is the DEVD consensus sequence for caspase-3 cleavage at 1889–1892 amino acids of mouse IP₃R₁ (DEVD) and IP₃R₁ (DEVD) human IP₃R₁. In the present studies, we asked whether IP₃R1 could serve as a substrate of caspase-3 during apoptosis, and we obtained evidence that IP₃R1 is a newly identified substrate for caspase-3. Using caspase-3-deficient

* The abbreviations used are: IP₃, D-myoinositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; IP₃R₁, IP₃R type 1; IP₃R₂, IP₃R type 2; IP₃R₃, IP₃R type 3; mAb, monoclonal antibody; IICR, IP₃-induced Ca²⁺ release; TNF-α, tumor necrosis factor-α.
cells, MCF-7 (17), we found that caspase-3 is essential for the cleavage of IP$_3$R1. In addition, effects of the cleavage by caspase-3 on the IP$_3$R1 channel function were also given attention.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies—**Monoclonal antibodies (mAbs) KM1112, KM1083, and KM1082 against the C terminus of IP$_3$R1, IP$_3$R2, and IP$_3$R3, respectively, were prepared as described elsewhere (18, 19). A monoclonal antibody against pro-caspase-3 was obtained from Transduction Laboratories (Lexington, KY). Anti-Fas IgM CH-11 was purchased from Medical and Biological Laboratories Co., Ltd. (Nagoya, Japan). Recombinant human tumor necrosis factor-α (TNF-α) was obtained from Pepro Tech EC, Ltd. (London, UK). Staurosporine and cycloheximide were purchased from Sigma and Wako Chemical (Tokyo, Japan), respectively. Ac-YVAD-CHO (inhibitor for caspase-1-like protease) and Ac-DEVD-CHO (inhibitor for caspase-3-like protease) were purchased from Peptide Institute (Osaka, Japan). z-VAD-CH$_2$DCB (inhibitor for caspases with broad specificity) was obtained from Phoenix Pharmaceutical, Inc. (Mountain View, CA).

**Cell Lines and Culture Conditions—**The human lymphoblastoid T-cell line, Jurkat, was obtained from the ATCC (Manassas, VA), and was maintained in RPMI 1640 medium with 2 mM l-glutamine and 10% of fetal calf serum. The human breast carcinoma cell line, MCF-7, was obtained from Dainippon-Seiyaku, Co., Ltd. (Osaka, Japan) and was maintained in Dulbecco’s modified Eagle’s medium containing 2 mM l-glutamine and 10% fetal calf serum.

**Induction of Apoptosis—**To induce apoptosis, Jurkat cells were treated with staurosporine (1 or 2 μM) or with anti-Fas IgM CH-11 (500 μg/ml). MCF-7 cells or MCF-7/Casp-3 cells were treated with staurosporine (1 μM) or a combination of TNF-α (30 ng/ml) and cycloheximide (10 μg/ml).

**Western Blot Analysis—**Cells (1 × 106 cells) treated with apoptotic stimuli were harvested at the indicated time and washed with phosphate-buffered saline and then were solubilized in 100 μl of the lysis buffer [150 mM NaCl, 5 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol, 1% Triton X-100, 0.1 mM phenylmethylsulfonfyl fluoride, 10 μM leupeptin, 10 μM pepstatin A, 10 μM E-64, and 20 mM tri-HCl, pH 7.5] on ice for 15 min. The insoluble fraction was removed by centrifugation at 15,000 rpm for 15 min at 4 °C. The resultant supernatant was subjected to SDS-5% polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunodetected using mAbs specific for each type of IP$_3$R.

**Cleavage of IP$_3$R1 by the Recombinant Caspase-3 in Vitro—**The recombinant histidine-tagged human caspase-3 (CPP32) was kindly provided by Dr. M. Miura (Osaka University, Osaka, Japan). The recombinant caspase-3 was purified using a nickel column according to the protocol of the manufacturer (Amersham Pharmacia Biotech). The cerebellar microsome fraction (200 μg/ml), in which IP$_3$R1 is dominantly expressed was incubated with the purified recombinant caspase-3 (10 or 50 μg/ml) at 37 °C for 10 min in the presence or absence of 10 μM caspase-3 inhibitor, Ac-DEVD-CHO. The reaction mixture were then subjected to Western blot analysis as described above.

**Determination of Cleavage Sites of IP$_3$R1 by Caspase-3—**Cleavage sites of IP$_3$R1 by caspase-3 were determined by N-terminal amino acid sequencing, as described, but with some modification (20). Briefly, IP$_3$R1 was purified, as described previously (21) then was treated with the recombinant caspase-3. The reaction mixture was subjected to Western blot analysis as described above.

**Stable Transfection of Caspase-3 cDNA in MCF-7 Cells—**The expression vector for FLAG-tagged human caspase-3, pM136, was a kindly provided by Dr. M. Miura (Osaka University). pM136 was used to transfect MCF-7 cells using LipofectAMINE (Life Technologies, Inc.). After 2 weeks of selection in growth medium containing 700 μg/ml of G418, 1.58 (National Institutes of Health, Bethesda, MD).

**RESULTS**

**Specific Degradation of IP$_3$R$_1$ during Apoptosis—**IP$_3$R1 has the DEVD consensus sequence for caspase-3 cleavage within its modulatory domain at 1835DEVD of mouse IP$_3$R1, which is conserved among species (rat: 1835DEVD and human: 1835DEVD) (Fig. 1A). To determine whether IP$_3$R1 is cleaved during apoptosis, Jurkat cells were treated with either 2 μM staurosporine or 500 ng/ml anti-Fas IgM CH-11 and then were subjected to Western blot analysis with specific mAbs against the C terminus of IP$_3$R$_1$, KM1112 (anti-IP$_3$R$_1$), KM1083 (anti-IP$_3$R$_2$), and KM1082 (anti-IP$_3$R$_3$).
**Specific Degradation of IP₃R1 by Caspase-3**

**Fig. 2. Inhibition of specific degradation of IP₃R1 during apoptosis by caspase-3 (like protease) inhibitors.** Specific degradation of IP₃R1 was inhibited by pretreatment of Jurkat cells with caspase-3 (like protease) inhibitors, DEVD-CHO (100 μM), and z-VAD (100 μM) but not caspase-1 (like) protease inhibitor, YMAD-CHO (100 μM). Jurkat cells were treated with caspase inhibitors for 1 h prior to addition of 1 μM staurosporine. IP₃R1 was detected using KM1112.

**Fig. 3. In vitro digestion of IP₃R1 by the recombinant caspase-3.** Mouse cerebellar microsome fractions (200 μg/ml), in which IP₃R1 is dominantly expressed, were treated with recombinant caspase-3 for 10 min at 37°C in the presence or absence of caspase-3 inhibitor, DEVD-CHO (10 μM). IP₃R1 was detected using KM1112.

Partially cleaved, whereas IP₃R2 and IP₃R3 were resistant to specific degradation. All three types of IP₃R, however, disappeared 10 h after stimulation, a time when almost all the Jurkat cells had died, as determined by trypan blue exclusion. In the case of anti-Fas antigen, specific degradation of IP₃R1 was also observed during apoptosis. In both cases, one major fragment with an estimated molecular weight of 95,000 and two minor fragments of 215,000 and 160,000 were detected, using a mAb against the C terminus of IP₃R1, KM1112.

To determine whether degradation of IP₃R1 during apoptosis is mediated by caspase-3 or caspase-3-like proteases, we examined effects of caspase inhibitors on IP₃R1 degradation. Jurkat cells were pretreated with caspase inhibitors 1 h prior to apoptotic stimuli and then were incubated during the stimuli. Fig. 2 shows that pretreatment of Jurkat cells with either a caspase-3-like protease inhibitor (Ac-DEVD-CHO) or a caspase inhibitor of broad specificity (z-VAD-CH₂DCB) inhibited the degradation of IP₃R1 during the apoptosis induced by staurosporine, whereas caspase-1 inhibitor (Ac-YVAD-CHO) showed no such inhibition (Fig. 2). The same results were obtained in the case of anti-Fas stimulation (data not shown). Other cysteine protease inhibitors tested, E-64d for cathepsin B/H/L and calpain and N-acetyl-Leu-Leu-norleucinal for calpain, did not block IP₃R1 degradation during apoptosis (data not shown).

**Specific Digestion of IP₃R1 by Recombinant Caspase-3—**To confirm that IP₃R1 is cleaved by caspase-3, digestion of IP₃R1 was tested by treatment with the purified recombinant caspase-3 in vitro. Fig. 3 shows cleavage of IP₃R1 by recombinant caspase-3, in a concentration-dependent manner, and the fragmentation pattern was consistent with that seen in Jurkat cells undergoing apoptosis. In the presence of the caspase-3-like protease inhibitor, Ac-DEVD-CHO, this specific cleavage was inhibited (Fig. 3).

**Cleavage Site of IP₃R1 by Caspase-3—**To determine the cleavage sites, the purified fragments of 215,000, 160,000, and 95,000 were subjected to N-terminal amino acid sequencing. The N-terminal amino acid sequence of the major fragment of 95,000 is RDAPXR (X is not determined), consistent with 1892RDAPSR of mouse IP₃R1, indicating that IP₃R1 is cleaved at just after DEVD consensus sequence for caspase-3 (Fig. 1A). N-terminal amino acid sequences of two minor fragments of 215,000 and 160,000, however, could not be determined, because they were too faint to examine the sequence or N termini blocking.

**Caspase-3 is Essential for the Specific Degradation of IP₃R1—**To determine whether IP₃R1 is cleaved by caspase-3 or is proteolyzed in its absence by other caspases, we examined the degradation of IP₃R1 during apoptosis in the caspase-3 deficient cell line, MCF-7 cells (breast carcinoma cell) (17). As noted by other investigators (17), pro-caspase-3 was not detected in MCF-7 cells, where we used an anti-caspase-3 antibody that recognizes pro-caspase-3 but not the active form of caspase-3 (Fig. 4). Although TNF-α or staurosporine induced apoptosis in MCF-7 cells, caspase-3-deficient MCF-7 cells failed to demonstrate cleavage of IP₃R1 (Fig. 4). To confirm that caspase-3 is essential for IP₃R1 degradation during apoptosis, we established MCF/Casp-3 cells that were stably transfected to express caspase-3. Fig. 4 shows that two independent stable transformants of MCF/Casp-3 cells, 2B1 and 2B5 (two representative clones out of seven tested), express pro-caspase-3. In these cell lines, no spontaneous activation of caspase-3 and no morphological changes were observed (data not shown). Treatment of MCF/Casp-3 cells, 2B1 and 2B5, with TNF-α/cycloheximide or staurosporine resulted in decreases in pro-caspase-3 because of processing into an active form. In contrast to the caspase-3-deficient MCF-7 cells, MCF/Casp-3 2B1 and 2B5 cells showed IP₃R1 degradation upon apoptotic stimuli, and the fragmentation patterns were the same as seen in Jurkat cells and in vitro cleavage.

**Caspase-3 Digestion of IP₃R1 Resulted in Inhibition of IP₃-induced Ca²⁺ Release Activity—**Effects of the cleavage of IP₃R1 by caspase-3 on channel activity were then investigated using mouse cerebellar microsome fractions, in which IP₃R1 is dominantly expressed. Fig. 5A shows typical profiles of ATP-induced Ca²⁺ uptake and IP₃-induced Ca²⁺ release (ICR) in microsome fractions treated with various concentrations of caspase-3. Control microsomes or caspase-3-digested microsomes were loaded with Ca²⁺ by adding 2 mM of ATP, and then 1 μM of IP₃ was added to induce Ca²⁺ release. Although the ATP-induced Ca²⁺ uptake was not affected by caspase-3, ICR was inhibited by caspase-3 in a dose-dependent manner. To quantify the degree of digestion, control microsomes and caspase-3-treated microsomes were subjected to Western blots, and amounts of intact IP₃R1 were measured by densitometric analysis as described under “Experimental Procedures.” The percentage of digested IP₃R1 and the channel activity are summarized in Fig. 5B. Increasing caspase-3 concentrations (0, 10, 20, 50, and 100 μg/ml) resulted in increase in the percentage of...
MCF-7, resulted in IP3R1 degradation upon apoptotic stimuli. Pro-caspase-3 was not detected in MCF-7 cells, as reported (17). Specific degradation of IP3R1 during apoptosis was induced by a combination of TNF-α (30 ng/ml) and cycloheximide (10 μg/ml), was not observed in caspase-3-deficient, MCF-7 cells. On the contrary, MCF7/Casp-3 2B1 and 2B5, which stably express caspase-3, showed activation of pro-caspase-3 and IP3R1 degradation upon apoptotic stimulation.

**DISCUSSION**

**Specific Degradation of IP3R1 during Apoptosis**—In the IP3R1 primary amino acid sequence, there is the DEVD consensus sequence for caspase-3 cleavage at 1888–1891 amino acids of mouse IP3R1 (Fig. 1A), which is conserved among mice, rats, and humans. In Jurkat cells, IP3R1 was cleaved during apoptosis. One major fragment of 95,000 and two minor fragments of 215,000 and 160,000 of IP3R1 were detected using an mAb against the C terminus of IP3R1, indicating that IP3R1 is cleaved at three sites during apoptosis (Fig. 1, B and C). The different degrees of degradation of IP3R1 seen with use of staurosporine and anti-Fas IgM would be attributed to different caspases activated or to different activities of these proteases. In both cases, the main fragment of 95,000 was observed, and the molecular size was consistent with that of the expected fragment if IP3R1 is cleaved at DEVD consensus sequence for caspase-3. Therefore, IP3R1 is probably degraded by caspase-3 or caspase-3-like proteases. IP3R2 and IP3R3 were resistant to specific degradation. In IP3R2 and IP3R3, there is no DEDX sequence and no potential cleavage site, which resembles the tetrapeptide sequences identified in various substrates for caspase-3.

Khan et al. (13) reported down-regulation of IP3R1 and up-regulation of IP3R3 during apoptosis in WEHI-B cells induced by IgM, thymocytes, and splenic cells, as induced by dexamethasone. In the present study, there was no apparent increase in IP3R3 expression. These differences may relate to different cells used and to the stimuli used to induce apoptosis. We found the time course of cell death induced by staurosporine or anti-Fas IgM to be more rapid (10–20 h) than that seen with dexamethasone (24–96 h).

**IP3R1 Is Cleaved by Caspase-3 (like Protease)—**Caspase-3 (like protease) inhibitor or a caspase inhibitor of broad specificity but not caspase-1 inhibitor inhibited the degradation of IP3R1 during apoptosis, thereby indicating that IP3R1 is cleaved by caspase-3 or caspase-3-like protease (Fig. 2). It was reported that IP3R1 was down-regulated in response to chronic activation of cell surface receptors, an event that was caused by IP3R1 degradation by the cysteine protease, calpain (24). In the case of apoptosis, caspase-3 cleaves IP3R1, as described under “Experimental Procedures.” IICR activity and the percentages of digestion were normalized against control (3% = 0; values are means ± S.D., n = 3).

**Caspase-3 Is Essential for Cleavage of IP3R1 during Apoptosis**—Junicke et al. (17) demonstrated that MCF-7 breast carcinoma cells have genetically lost caspase-3 and that caspase-3 is essential for DNA fragmentation and for blebbing. However, MCF-7 cells do respond to certain apoptotic stimuli, suggesting...
that caspase-3 is not essential for apoptosis and that other caspases may be activated. Actually, caspase-2, -7, -8, -9, and -10 were detected in MCF-7 cells, and caspase-8 is activated in MCF-7 during the apoptosis induced by TNF-α (25). These same authors reported that most substrates of caspase-3 were cleaved during apoptosis in caspase-3-deficient MCF-7 cells, and they stated that caspase-3 is essential for cleavage of α-fodrin but dispensable for the cleavage of poly(ADP-ribose) polymerase, Rb, p21-activated kinase 2, DNA-dependent protein kinase catalytic subunit, gelsolin, and DNA fragmentation factor 45-kDa subunit, which suggested that caspasess other than caspase-3 are activated and cleaved these substrates in MCF-7 cells (25). It is, however, uncertain whether cleavage of these substrates by other caspases in caspase-3-deficient cells is functional for apoptosis, because other groups reported that DNA fragmentation factor 45-kDa subunit and gelsolin require caspase-3 for proper cleavage and its function (3, 4, 26, 27), despite the finding that they were cleaved in the absence of caspase-3, possibly by other caspases.

In the case of IP3R1, caspase-3-deficient MCF-7 cells failed to demonstrate cleavage of IP3R1 (Fig. 4), indicating that IP3R1 was not cleaved by remaining caspases, such as caspase-8. In MCF/Casp3 cells, pro-caspase-3 became the active form, as induced by apoptotic stimuli (Fig. 4), thereby indicating that the caspase-3 activation pathway was functional. Upon apoptotic stimuli, IP3R1 was cleaved when MCF-7 cells were stably transfected with caspase-3, indicating that caspase-3 is essential for the cleavage of IP3R1. Thus, our data show that IP3R1 is a specific substrate for caspase-3 and that this cleavage cannot occur with other caspases in MCF-7 cells.

Caspase-3 Inactivates IP3-induced Ca2+ Release Activity—Effects of the cleavage of IP3R1 by caspase-3 on channel activity were also investigated using mouse cerebellar microsome fractions, in which IP3R1 is dominantly expressed (Fig. 5). The time course of Ca2+ uptake was not affected by caspase-3 treatment, indicating that the Ca2+-ATPase function is resistant to cleavage by caspase-3 (Fig. 5A). On the contrary, the IIICR was inhibited by caspase-3 in a dose-dependent manner (Fig. 5A).

Digestion of up to 50% did not significantly inhibit the channel activity, suggesting that even partially digested IP3R1 can function as a Ca2+ channel (Fig. 5B), as was observed in trypsinated IP3R1 (20). Therefore 90% of the digested IP3R1 has 25% IIICR activity. Alternatively, IIICR is not highly cooperative, because if the Hill coefficient of IIICR is 4, the digested IP3R subunit could have a dominant negative effect on IP3R channel activity. Moreover, inhibitory effects on the IIICR were observed in over 50% of the digested IP3R1, suggesting that cleavage of at least two subunits of IP3R is needed to inactivate the IP3R channel. These results are in accord with our previous studies on kinetics of the purified IP3R1, in which the Hill coefficient of IIICR was 2 (28).

In conclusion, IP3R1 is a newly identified caspase-3 substrate, and caspase-3 is essential for cleavage of IP3R1 during apoptosis. One of the cleavage sites of IP3R1 is the DEVD consensus sequence for caspase-3. Cleavage of IP3R1 by caspase-3 resulted in inhibition of IP3-induced Ca2+ release activity, in a digestion-dependent manner, an event that may possibly interfere with the IP3/Ca2+ signaling pathway and intracellular Ca2+ homeostasis within cells undergoing apoptosis.

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