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Type 1 Inositol-1,4,5-Trisphosphate Receptor is a Late Substrate of Caspases During Apoptosis

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ABSTRACT

Apoptosis is characterized by the proteolytic cleavage of hundreds of proteins. One of them, the type 1 inositol-1,4,5-trisphosphate receptor (IP_3R-1), a multimeric receptor located on the endoplasmic reticulum (ER) membrane that is critical to calcium homeostasis, was reported to be cleaved during staurosporine (STS) induced-apoptosis in Jurkat cells. Because the reported cleavage site separates the IP_3 binding site from the channel moiety, its cleavage would shut down a critical signaling pathway that is common to several cellular processes. Here we show that IP_3R-1 is not cleaved in 293 cells treated with STS, TNFα, Trail, or ultra-violet (UV) irradiation. Further, it is not cleaved in Hela or Jurkat cells induced to undergo apoptosis with Trail, TNFα, or UV. In accordance with previous reports, we demonstrate that it is cleaved in a Jurkat cell line treated with STS. However its cleavage occurs only after poly(ADP-ribose) polymerase (PARP), which cleavage is a hallmark of apoptosis, and p23, a poor caspase-7 substrate, are completely cleaved, suggesting that IP_3R-1 is a relatively late substrate of caspases. Nevertheless, the receptor is fully accessible to proteolysis in cellulo by ectopically overexpressed caspase-7 or by the tobacco etch virus (TEV) protease. Finally, using recombinant caspase-3 and microsomal fractions enriched in IP_3R-1, we show that the receptor is a poor caspase-3 substrate. Consequently, we conclude that IP_3R-1 is not a key death substrate. J. Cell. Biochem. 113: 2775–2784, 2012.

KEY WORDS: INOSITOL-1,4,5-TRISPHOSPHATE RECEPTOR; CALCIUM; APOPTOSIS; CASPASE; TEV PROTEASE

During apoptosis, a plethora of intracellular proteins (>1,000 till date, CASBAH database at http://bioinf.gen.tcd.ie/casbah/ [Luthi and Martin, 2007]) are cleaved by caspases. Among these proteins are caspases themselves and proteins that are accessory to their activation pathways such as Bid and other Bcl-2 family members and the X-linked inhibitor of apoptosis protein (XIAP) [Fischer et al., 2003]. Another group of proteins are proteolyzed as part of the dismantling of the cell and the appearance of apoptotic hallmarks. For example, the high energy-consuming enzyme poly(ADP-ribose) polymerase 1 (PARP), which is involved in DNA repair, is inactivated by caspases, which proteolytically separates the catalytic domain from the binding domain, [Tewari et al., 1995] and the inhibitor of caspase-activated DNAse (ICAD) is cleaved and rapidly degraded thus releasing the active DNAse that is responsible for genomic DNA fragmentation [Enari et al., 1998; Sakahira et al., 1998]. The mitochondrial protein p75 subunit complex I of the electron transport chain is also cleaved by caspase-3 [Ricci et al., 2004], which permanently shut down the

Abbreviations: Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; Bcl-2, B-cell lymphoma 2; Bid, BH3 (baculovirus homology 3) interacting domain death agonist; ER, endoplasmic reticulum; ICAD, inhibitor of caspase-activated DNAse; IP_3R, inositol 1,4,5-trisphosphate receptor; PARP, poly(ADP-ribose) polymerase; PE, phycoerythrin; PLC, phospholipase C; ROCK, Rho-associated protein kinase; STS, staurosporine; TEV, tobacco etch virus; TNFα, tumor necrosis factor α; XIAP, X-linked inhibitor of apoptosis protein; UV, ultra-violet; Z-VAD-fmk, tert butoxycarbonyl-Val-Ala-Asp-fluoromethylketone.

Conflicts of interest: None.

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mitochondrial potential and energy generation. Nuclear lamins are proteolysed by caspase-6 [Orth et al., 1996; Takahashi et al., 1996] to help in fragmenting the nucleus, and protein kinase Cδ [Emoto et al., 1995] and Rho-associated protein kinase I (ROCK I) [Coleman et al., 2001; Sebbagh et al., 2001] are cleaved and lose their regulatory domains causing the characteristic blebs and apoptotic bodies. These proteins and scant others define apoptosis or are core to this process.

Surprisingly, very few among all the cleavage events operated by caspases have been demonstrated to be essential for apoptosis. Indeed, in most cases cleavage of a given protein has been well documented using biochemical or proteomic approaches but its relevance to apoptosis has been mostly inferred from the biological role of the native protein in healthy cells. For example, it is tempting to ascribe a genuine role for a caspase-mediated cleavage event when the target protein is implicated in a fundamental process such as Ca^{2+} signaling. Indeed, Ca^{2+} is crucial to many cellular processes ranging from fertilization to cell death [Berridge et al., 2000]. Cytosolic Ca^{2+} is controlled by plasma membrane and endoplasmic reticulum (ER) pumps that keep its concentration low (50–100 nM [Hajnoczky et al., 2003]) in the resting state and by various types of plasma membrane and ER channels that are responsible for cytosolic Ca^{2+} elevations (1–10 μM [Hajnoczky et al., 2003]) in the activated state of the system.

Based on the current literature, at least three proteins pertaining to cytosolic Ca^{2+} regulation are cleaved during apoptosis. (1) The plasma membrane Ca^{2+}-ATPase isoform 4b is cleaved in ischemic rat neurons [Schwab et al., 2002], in COS-7 cells after anoikis and in Hela cells treated with staurosporine (STS) [Paszty et al., 2002]. (2) The Na^{+}/Ca^{2+} exchanger is proteolysed during STS-induced SH-SY5Y neuroblastoma cell apoptosis [Bano et al., 2007]. (3) Finally, the type 1 inositol 1,4,5-trisphosphate receptor (IP_3R-1), has been shown by Hirota et al. [1999] to be cleaved by caspase-3 during STS-induced Jurkat cell apoptosis. The latter finding was corroborated in STS-treated SH-SY5Y neuron-like cells [Assefa et al., 2004] and other cell types using the same agent [Verbert et al., 2008].

IP3R-1 is a 260 kDa, 6-ER membrane spanning protein assembled in homo- or hetero-tetramers with IP_3R-2 and/or IP_3R-3 homologues. IP_3R-1 is the best characterized of the three isoforms and is predominantly expressed in many cell types such as Purkinje cells, neurons, vascular smooth muscle cells, and endothelial cells [Worley et al., 1987; Furuichi et al., 1993]. Many studies have addressed its importance in development, reproduction, secretion, muscle contraction, and many more cellular processes [Mikoshiba, 2007]. This channel assembly senses cytosolic IP_3 released from phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC). Because dozens of receptors, principally Gq protein-coupled receptors, can lead to PLC activation, IP_3Rs are key to cellular signaling and cell physiology. Consequently, the report of IP_3R-1 cleavage during apoptosis [Hirota et al., 1999] suggests that shutting down IP_3-induced Ca^{2+} signaling is important for the proper stoppage of cellular activity. This hypothesis is supported by the fact that the cleavage site is located in a regulatory region between the N-terminal IP_3 binding domain and the channel part of the receptor thus uncoupling IP_3 binding from channel activation. The ensuing channel is predicted to leak Ca^{2+} thus favoring cytosolic build up or, to the least, would apply stress on the Ca^{2+} pumping machinery [Assefa et al., 2004]. Furthermore, high and sustained cytosolic Ca^{2+} was shown to cause apoptosis [McConkey et al., 1989; Berridge et al., 1998; Nicotera and Orrenius, 1998; Berridge et al., 2000]. Thus, cleavage of the IP_3R-1 would have the dual purpose of disturbing key signaling processes and promoting apoptosis.

To our knowledge, the relevance of IP_3R-1 as a key death substrate as not been thoroughly assessed despite studies that have characterized the impact of cleavage on the receptor's function and showed the effect of a cleaved protein [Assefa et al., 2004; Verbert et al., 2008]. In this study, we attempted to determine whether or not IP_3R-1 cleavage is a general phenomenon occurring during apoptosis. We show that the cleavage only occurs at supra-physiological caspase-3 concentrations or in apoptotic conditions in which the entire pool of caspase-3 is activated, which is rarely the case. Finally, using a mutant IP_3R-1 that incorporates a tobacco etch virus (TEV) protease cleavage site in the vicinity of the reported caspase-3 motif or ectopically expressed caspase, we demonstrated that the cleavage site region is readily accessible for proteolysis in cellulo. Therefore, we conclude that the cleavage of IP_3R-1 by caspases is, compared to the proteolysis of other substrates, a relatively late apoptotic event and that the receptor is not a key death substrate, at least in the cellular paradigms we tested.

MATERIALS AND METHODS

CHEMICALS, REAGENTS, AND CELL CULTURE

Fetal bovine serum, DMEM (Dulbecco's modified Eagle medium) RPMI (Roswell Park Memorial Institute) 1640 media were from Multicell (Woonsocket, RI). Chicken serum was from Invitrogen (Burlington, ON). All DNA modifying enzymes and nucleotides were from New England Biolabs (Pickering, ON) or Roche Diagnostics (Mississauga, ON). Mouse anti-chicken IgM (clone M-4) was from Southern Technologies (Birmingham, AL). Annexin V-PE (phycoerythrin) was from MBL International Corp. (Woburn, MA). STS was from Enzo Life Sciences (Plymouth Meeting, PA). Recombinant human soluble Trail and TNFα were from Alexis Biochem (San Diego, CA). Cycloheximide, and Fura2-AM were from Calbiochem (San Diego, CA). All protease inhibitors were from Sigma-Aldrich (Oakville, ON). Other reagents were from Sigma-Aldrich or Mat Laboratories (Québec, QC).

DNA CONSTRUCTS

The rat IP_3R-1 cDNA [generous gift from Dr. Suresh Joseph (Thomas Jefferson University School of Medicine, Philadelphia, PA)], in a CMV promotor-driven expression plasmid, was used as a template for all constructs. This plasmid is maintained in JM109 *Escherichia coli* strain. IP_3R-1 mutants (Fig. 1A) were generated using overlapping PCR using *Expand Hi-fidelity long template* (Roche Diagnostics) with a pair of external primers and the appropriate primer pair to introduce the desired mutations (Table I). A 4.7 kb fragment obtained by *Apa* I digest of the PCR product was substituted for the equivalent fragment in the original plasmid. For the IP_3R-1 TEV mutant, the IP_3R-1 DEVA mutant was used as DNA template instead of the WT cDNA.

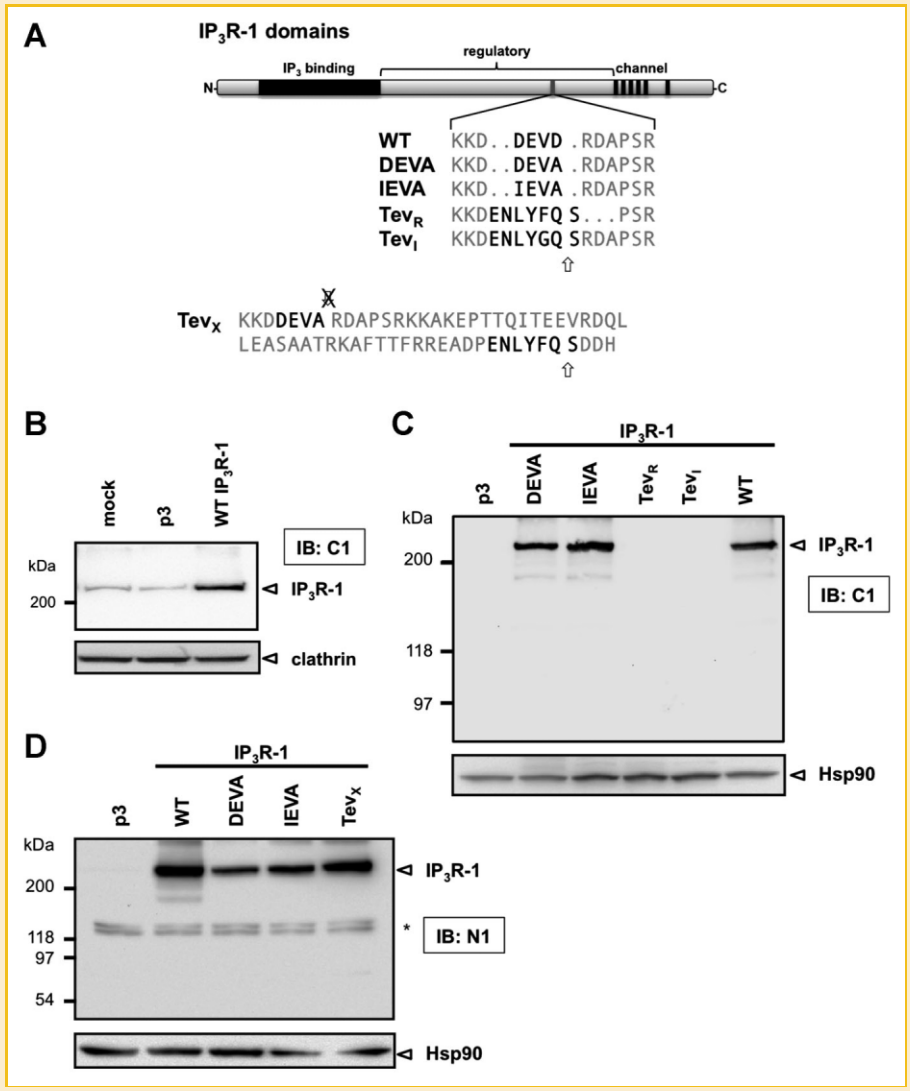

Fig. 1. Ectopic expression of wild-type IP_3R-1 and its mutants. A: Schematic representation of the IP_3R-1 receptor with its three main domains, and the primary sequence surrounding the putative caspase-3 cleavage site of the wild-type protein, and the engineered mutant proteins. The arrow represents the reported caspase-3 cleavage site or the theoretical TEV protease cleavage site. B–D: 293Ad cells were transfected or not (mock) with an empty plasmid (p3), or the indicated IP_3R-1 plasmids. Lysates were analyzed by immunoblotting (IB) with the indicated antibodies. Clathrin and Hsp90 were alternatively used as loading controls. In (C), extra bands are detected that may constitute minor secondary transcripts or non-specific proteolytic fragments caused by the overexpression. In (D), the doublet (*) detected at 130 kDa was considered non-specific because it was also detected in the empty plasmid sample. These experiments are representative of at least two independent datasets.

The TEV protease cDNA is comprised of residues 2038–2279 of the TEV polyprotein preceded by a V5 epitope sequence (MGKPIPNPLLGLDST). All TEV protease mutants were obtained by standard overlapping PCR using pcDNA3-derived primers and an appropriate primer pair to introduce the desired mutations (Table I). Both mutations L2038V and S2162G were reported to increase protein solubility [Cabrita et al., 2007] whereas mutation C2200A (amino acid numbering of the protease precursor) is the catalytic cysteine mutation, which is used as control.

Wild-type and catalytic mutant caspase-7 expression plasmids were previously described [Denault and Salvesen, 2003]. Both have a C-terminus flag epitope (ELDYKDDDDK). Sequence integrity of all amplified fragment was confirmed by automated DNA sequencing.

CELL CULTURE AND TRANSFECTION

293Ad, a derivate line of human embryonic kidney 293 (HEK 293) cells, and Hela (cervix carcinoma) were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine in 60 or 100-mm dishes at 37°C in humidified air with 5% CO_2. Cells were sub-cultured every 3 days. 293Ad cells at ~80% confluence were transfected using 6 μg of plasmid DNA and 15 μl of Lipofectamine 2000 (Invitrogen). Jurkat cells were cultured the same way but in RPMI 1640 media and cell density was kept at 10^5–10^6 cells/ml.

DT40-KO cells (chicken lymphoma cells), which do not express any IP_3R [Sugawara et al., 1997], were provided by Dr. Tomohiro Kurosaki via the RIKEN Institute (Ibaraki, Japan). They were cultured

TABLE I. Primers Used to Generate IP_3R-1 and TEV Protease Mutants

Name/mutants	Sequences (5′ to 3′)
IP3R-1	
External 5′ PCR	CCCAGTCATCAGAAACATCCTCCGG
External 3′ PCR	CGTTCTGTTCCCCTTCAGAGTCACTGC
DEVAf	GAAAGACGATGAGGTAGCCCGGGATGCCCCATCACGG
DEVAr	CCGTGATGGGGCATCCCGGGCTACCTCATCGTCTTTC
IEVAf	GGGAAATAAAAAGAAAGACATCGAGGTAGCCCGGGATGCCCCATCACGG
IEVAr	CCGTGATGGGGCATCCCGGGCTACCTCGATGTCTTTCTTTTTATTTCCC
Tev_Rf	GGGAAATAAAAAGAAAGACGAAAACCTGTACTTTCAGTCGCCATCACGGAAAAAAGC
Tev_Rr	GCTTTTTTCCGTGATGGCGACTGAAAGTACAGGTTTTCGTCTTTCTTTTTATTTCCC
Tev_If	GGGAAATAAAAAGAAAGACGAAAACCTGTACTTTCAGTCCCGGGATGCCCCATCACGG
Tev_Ir	CCGTGATGGGGCATCCCGGGACTGAAAGTACAGGTTTTCGTCTTTCTTTTTATTTCCC
Tev_Xf	CGGAGGGAGGCCGACCCTGAGAACCTGTACTTCCAGTCCGATGACCATTACCAATCTGGG
Tev_Xr	CCCAGATTGGTAATGGTCATCGGACTGGAAGTACAGGTTCTCAGGGTCGGCCTCCCTCCG
TEV protease	
L2038Vf	CACTTGTTTAGAAGAAATAATGGAACACTAGTGGTCCAATCACTACATGGTG
L2038Vr	CACCATGTAGTGATTGGACCACTAGTGTTCCATTATTTCTTCTAAACAAGTG
S2162Gf	CACTAGTTGCACATTCCCTTCAGGCGATGGCATATTCTGGAAGCATTGG
S2162Gr	CCAATGCTTCCAGAATATGCCATCGCCTGAAGGGAATGTGCAACTAGTG
C2200Af	CCAAGGATGGGCAGGCCGGCAGTCCATTAGTATCAAC
C2200Ar	GTTGATACTAATGGACTGCCGGCCTGCCCATCCTTGG

in RPMI 1640 media supplemented with 10% fetal bovine serum, 2% chicken serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 55 μM β-mercaptoethanol. Cells were maintained in 150-mm dishes and kept at 40°C in humidified air with 5% CO_2. DT40-KO cells were kept between 1.5×10^5–2.4×10^6 cells/ml and counted regularly to prevent cell death. Ten million cells were electroporated with 15 μg of plasmid DNA using Amaxa Nucleofector II and the Amaxa cell line nucleofector kit T (Lonza Laboratories, Allendale NJ). Cells were used 24 h post-transfection.

In cellulo IP_3R-1 cleavage was performed by co-transfecting cells (~80% confluence) with 4 μg of plasmid DNA encoding caspase-7 or TEV protease and 2 μg of IP_3R-1 plasmid cDNAs. Cells were harvested 24 or 48 h post-transfection for caspase-7 or TEV protease, respectively.

APOPTOTIC TREATMENTS

293Ad cells, Jurkat cells, or Hela cells were grown in 60-mm dishes to ~60–70% confluence. Fresh media containing Trail (250 ng/ml), STS (2 μM), or a mixture of TNFα (30 ng/ml) and cycloheximide (10 μg/ml) was added to the cells. For ultra-violet (UV) treatment, media was removed and cells were exposed to 100 J/m^2 of 254 nm light in a Spectrolinker XL-1000, then fresh media was added. Cells were harvested 24 h later or as indicated.

ANNEXIN V BINDING ASSAY

Cells (2×10^5 cells/ml) were incubated in annexin V binding buffer (10 mM Hepes pH 7.0, 137 mM NaCl, 2.5 mM $CaCl_2$) containing 1 μl/100 μl of Annexin V-PE. After 15 min in the dark, the cell suspension was analyzed using a Guava EasyCyte mini flow cytometer (Millipore, Billerica, MA). Cells were initially sorted based on forward and side scattering to eliminate cellular debris and aggregate, and then on fluorescence intensity. An arbitrary value was set for PE fluorescence to separate the negative and positive cell populations from the control sample, and this arbitrary value was used for all the treated samples of an individual experiment.

IN VITRO CASPASE-3 CLEAVAGE ASSAY

293Ad cell membrane proteins were obtained by harvesting PBS-washed cells using a cell scraper in 0.5 ml of 10 mM Hepes pH 7.3 and 20–30 passages through a Teflon-glass homogenizer. Unbroken cells and large debris were cleared by centrifugation (1,000*g* for 5 min.) and membranes were recovered at 100,000*g* for 1 hr. Pellet was resuspended in 0.1 ml of caspase buffer (50 mM Hepes pH 7.4, 1 M sodium citrate, 50 mM NaCl, 0.01% Chaps). Protein concentration was normalized using the *BCA Protein Assay Kit* (Thermo Scientific, Burlington, ON). Then, 20 μl of extract were mixed with 20 μl of caspase buffer containing the indicated concentration of recombinant active site-titrated caspase-3 (made in-house as previously described [Denault and Salvesen, 2002, 2007]) and 4 mM DTT. After 1 h at 37°C, 20 μl of SDS–PAGE loading buffer were added to the reaction and samples were analyzed by immunoblotting (IB).

SDS–PAGE AND IB

Cell extracts were prepared in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100 or NP-40) containing protease inhibitors [1 mM EDTA, 1 mM 1,10-orthophenantroline, 50 μM 3,4-dichlor-oisocoumarine, 10 μM E64, 10 μM leupeptin and 1 μM MG-132] for 45 min at 4°C. Polycaspase inhibitors were added when required (100 μM Z-VAD-fmk and 100 μM Ac-DEVD-CHO) to avoid post-lysis caspase activity. Insoluble material was cleared by centrifugation at 18,000*g* for 30 min. Supernatant was collected and protein concentration normalized using a Bradford assay (BioRad, Mississauga, ON). Proteins were resolved on a 8–18% (6% continuous for IP_3R-1 IB) ammediol (2-amino-2-methyl-1,3-propanediol) polyacrylamide gradient gel [Bury, 1981]. Then, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) pH 11 and 10% methanol at constant current (0.4 A for 40–60 min) as previously described [Matsudaira, 1987]. Ensuing membranes were blocked with 5% non-fat dry milk in PBS-T (137 mM NaCl, 8.7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 2.7 mM KCl, and 0.1% Tween-

20), washed and blotted in PBS-T containing 5% BSA with the indicated primary antibodies and corresponding horseradish peroxidase-coupled secondary antibodies (GE Healthcare; Waukesha, WI). Blots were developed by chemiluminescence using Westpico SuperSignal (Thermo Scientific) and a VersaDoc 4,000 mp imaging system (BioRad). The antibodies used were: Mouse anti-V5 (V-8012; Sigma-Aldrich), mouse anti-flag (F-3040; Sigma-Aldrich), mouse anti-caspase 3 (40924; Active Motif), mouse anti-PARP (556362; BD Biosciences, Mississauga, ON), mouse anti-p23 (MA3-414; Thermo scientific), mouse anti-Hsp90 (610419; BD Biosciences). The rabbit anti-IP_3R-1 antibody (PA1-901), recognizing the epitope NKKKDDEVDRDAPSRKKAKE located in the N-terminal portion of IP_3R-1 and named N1 in this article, was obtained from BD Biosciences. The rabbit anti-IP_3R-1 antibody recognizing the epitope MNVNPQQA located in the C-terminus of IP_3R-1 and named C1 in this article, was developed in-house [Poitras et al., 2000].

CYTOSOLIC Ca^{2+} MEASUREMENT IN DT40-KO CELLS

Cytosolic Ca^{2+} was measured in DT40-KO cells loaded with the Ca^{2+} probe Fura2 (EMD inc., Mississauga, ON). Ten million cells were washed and incubated for 20 min at room temperature in 10 ml HBSS (20 mM Hepes pH 7.4, 120 mM NaCl, 10 mM dextrose, 5.3 mM KCl, 1.8 mM $CaCl_2$, and 0.8 mM $MgSO_4$) containing 0.7 μM Fura2-AM. Then, cells were collected by centrifugation and incubated in 0.4 ml HBSS for 20 min at room temperature to allow for the probe de-esterification. Cells were placed on a glass coverslip in a circular chamber mounted on an Olympus IX71 inverted microscope coupled to a photometric videomicroscopy camera [EMCCD Evolve (Photometrics, Tucson AZ)] and a MetaFluor digital imaging systems (Olympus Canada, Markham ON). Loaded cells were selected and cytosolic Ca^{2+} analyzed (512 × 512 pixel images; 1 image/s). The emitted fluorescence ratio at 510 nm from the two excitation wavelengths (F_{334}/F_{380}) was calculated, and cytosolic Ca^{2+} concentration was determined using the MetaFluor version 7.5.6.0 software according to the method of Grynkiewicz et al. [1985].

RESULTS

EXPRESSION AND ACTIVITY OF IP_3R-1

Ectopic expression in 293Ad cells of full-length (2,749 residues) wild-type (WT) IP_3R-1 (see schematic in Fig. 1A) produced a 260-kDa band readily detected by IB using a polyclonal antibody (C1; described in Materials and Methods section) directed against the C-terminus of the receptor (Fig. 1B, lane 3). Quantification of the IP_3R-1 signal, using clathrin as the normalizing protein, revealed a greater than 10-fold overexpression compared to mock or empty plasmid transfection sample containing only the endogenous IP_3R-1 (lanes 1 and 2). To study the relevance of the caspase cleavage site in the regulatory domain of IP_3R-1, we generated two types of mutants (Fig. 1A). First, we mutated the previously reported caspase-3 recognition motif ($D^{1888}EVD\downarrow$) into DEVA or IEVA to prevent its cleavage. The latter mutant was made to prevent cleavage at the P4 aspartate residue when the original P1 residue is mutated to alanine (cleavage occurs at the P1-P1' junction). A second pair of mutants that incorporate a TEV protease cleavage site were produced either by substituting a 7-amino acid stretch encompassing the DEVD motif for ENLYFQ↓S (Tev_R) or by inserting the motif in lieu of DEVD (Tev_I). IB of lysates from transfected cells showed that the mutants IP_3R-1-DEVA and IP_3R-1-IEVA were efficiently expressed whereas the mutants IP_3R-1-Tev_R and IP_3R-1-Tev_I were not expressed (Fig. 1C). This suggests that the incorporation of the ENLYFQ↓S sequence at this precise location within the regulatory domain of IP_3R-1 dramatically altered its expression. Consequently, we inserted the TEV protease motif 44 residues downstream of the caspase cleavage site (Fig. 1A) that we chose based on the absence of predictable secondary structure. This mutant, named IP_3R-1-Tev_X, also carries the DEVA mutation to prevent its cleavage by caspases. Figure 1D shows that the mutant IP_3R-1-Tev_X was efficiently expressed in 293Ad cells.

To test the functionality of IP_3R-1 mutants we took advantage of the DT40-KO cells lacking all three IP_3R isoforms [Sugawara et al., 1997]. Stimulation of WT DT40 cells with anti-IgM gives rise to a robust increase in cytosolic Ca^{2+} whereas DT40-KO cells do not respond to this stimulus [Sugawara et al., 1997; Broad et al., 2001; Venkatachalam et al., 2001; Vazquez et al., 2003]. Figure 2 shows single cell analysis of anti-IgM-induced cytosolic Ca^{2+} increase. As expected, no cytosolic Ca^{2+} increase was observed in any of the

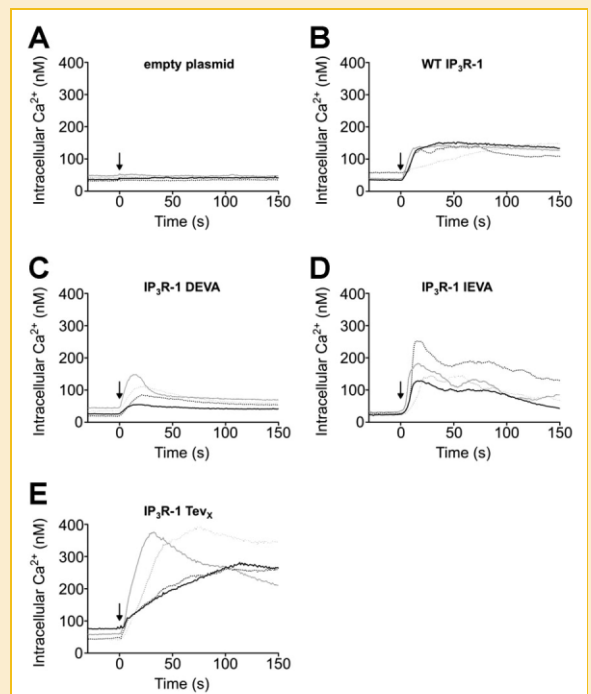

Fig. 2. IP_3R-1 mutants are functional. A–E: DT40-KO cells were nucleofected with an empty plasmid (A) or a plasmid encoding the various IP_3R-1 cDNAs (B–E). After 24 h, cells were loaded with Fura2-AM and mounted on a videomicroscopy system to analyze their cytosolic Ca^{2+} (see Materials and Methods section). The medium was replaced for a nominally Ca^{2+}-free medium and anti-IgM (400 ng/ml) was used to stimulate IP_3 production and Ca^{2+} release from ER store. Four typical single cell responses are reported as cytosolic Ca^{2+} concentration. Ca^{2+} increase was observed in about 5% of cells, which matches the low transfection efficiency (<10%) of these cells.

control DT40-KO cells upon stimulation with anti-IgM (Fig. 2A). However, significant cytosolic Ca^{2+} increases ranging from ~30 nM to ~100 nM were observed in DT40-KO cells expressing the WT or the mutant IP_3R-1, upon stimulation with anti-IgM (Fig. 2B–E). It is important to note that due to the low transfection efficiency (usually <10%), the representative responses shown in panels B–E were from selected cells representing only about 5% of the cells within a field. Nonetheless, these rises in cytosolic Ca^{2+} clearly demonstrated that all the mutants could sustain ER Ca^{2+} release, and were functional.

IP_3R-1 IS NOT CLEAVED DURING APOPTOSIS

Hirota et al. [1999] showed that IP_3R-1, but not the other two IP_3Rs, is cleaved during STS-induced apoptosis. We tried to reproduce these results by challenging Hela (Fig. 3A) and Jurkat cells (Fig. 3B) with several apoptotic stilmuli (Trail, a combination of TNFα and cycloheximide, or UV). However, we did not detect the expected C-terminal 95 kDa fragment of IP_3R-1 despite the engagement of the apoptotic machinery as shown by the production of cleaved caspase-3. It is important to note that apoptosis does not require complete caspase-3 activation. In this study, we often observed

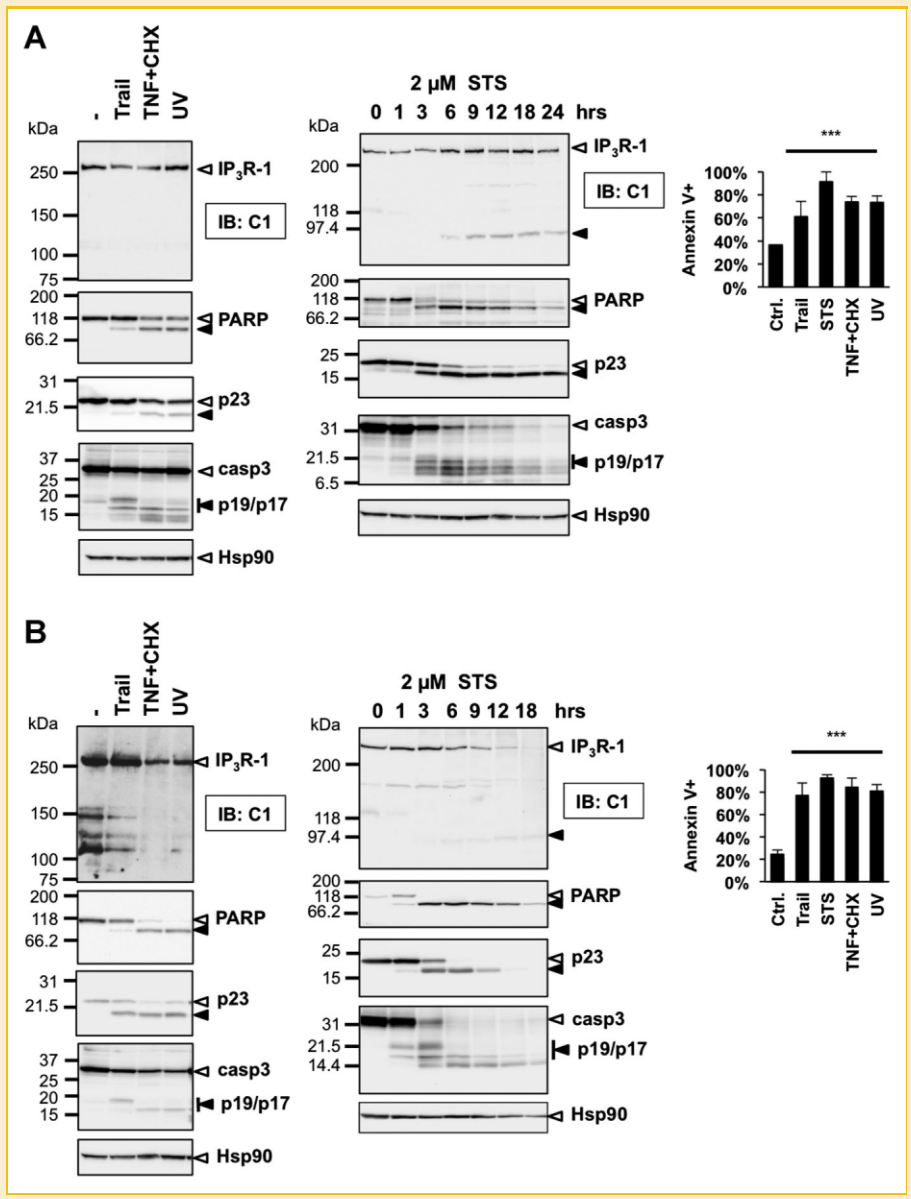

Fig. 3. IP_3R-1 receptor is not cleaved in many apoptosis paradigms. Hela (A) or Jurkat (B) cells were left untreated or incubated with Trail (250 ng/ml) for 18 h, a mixture of TNFα (30 ng/ml) and cycloheximide (CHX; 10 μg/ml) for 18 h, or exposed to UV light (UV; 100 J/m^2 of 254 nm wavelength), then incubated for 18 h (left). Middle panels show a time-course of STS (2 μM) treatment. Lysates were analyzed by immunoblotting with the indicated antibodies. Of notice, as part of the activation process of caspase-3, multiple bands are detected including p19/p17 (large subunit with/without the N-terminal 28-residue peptide), and some others. Hsp90 was used as a loading control. White arrowheads indicate full-length proteins whereas cleavage fragments are marked with a black arrowhead. For each condition, annexin V binding was assessed by flow cytometry (right). Means ± SD from five independent experiments are presented. STS treatment was analyzed at 18 h. The gate was arbitrarily set between the two cell peaks. All datasets were significantly different ($P < 0.001$) from control based on a Bonferroni multiple comparison test.

>60% annexin V positive cells with less than 50% procaspase-3 conversion, with the exception of apoptosis induced by STS, which results in complete procaspase-3 processing. Finally, when Jurkat or Hela cells were challenged with STS (Fig. 3, middle panels), a strong inducer of caspase-3 activation, cleaved IP_3R-1 appeared only after 6 h, a time at which PARP, p23, and caspase-3 were almost completely cleaved. From these results, we concluded that IP_3R-1 is a relatively late substrate during apoptosis in the paradigms we tested.

IP_3R-1 IS CLEAVED BY CASPASES IN VITRO

To verify whether the ectopically expressed IP_3R-1 receptor can be cleaved by caspase-3, we prepared crude microsomal preparations from transfected 293Ad by hypo-osmotic lysis and differential centrifugation. The ensuing microsomes were incubated with serial dilutions of active site-titrated recombinant caspase-3 (Fig. 4). Under these conditions, we detected a C-terminal 95 kDa fragment, analogous to the one previously reported by Hirota et al. [1999]. The generation of this fragment required relatively high concentrations of caspase-3, in the micromolar range. We also observed a 180–190 kDa C-terminal fragment that presumably is due to a secondary cleavage site. Indeed, alternative caspase cleavage sites exist in the N-terminal domain of the receptor. These results clearly show that ectopically expressed IP_3R-1 is susceptible to cleavage at high concentrations of caspase-3.

IP_3R-1 IS CLEAVED BY ECTOPICALLY EXPRESSED PROTEASES

We also tested the ability of ectopically expressed proteases to cleave IP_3R-1. Caspase-7 has the same substrate specificity as caspase-3 [Thornberry et al., 1997; Stennicke et al., 2000], but, contrary to the latter, it self-activates efficiently upon transfection in mammalian cells [Duan et al., 1996; Denault and Salvesen, 2003]. Cotransfection of WT IP_3R-1 and WT caspase-7 cDNAs (Fig. 5A, lane 5) resulted in the production of the cleaved 95 kDa C-terminal fragment along with the cleavage of p23, a known caspase-7 substrate [Walsh et al., 2008]. The 95-kDa fragment of IP_3R-1 was not obtained when the catalytic mutant of caspase-7 was transfected (lane 6). The 95-kDa C-terminal fragment of IP_3R-1 was neither obtained when caspase-7 was cotransfected with a mutant IP_3R-1 whose caspase cleavage site had been mutated (lanes 7–9). Coexpression of the mutant IP_3R-1-Tev_X and TEV protease (Fig. 5B, lane 10) generated a 165-kDa N-terminal fragment that did not appear when the mutant receptor was coexpressed with a catalytically dead TEV protease (mutation C2200A, last lane). Taken together, these results clearly show that ectopically expressed IP_3R-1 is available to proteolysis in cellulo at the DEVD site or in its vicinity. Importantly, the TEV protease, which has no substrate in the human proteome, could cleave the mutant receptor bearing the ENLYFQ↓S recognition motif. It thus represents a useful approach to provoke a controlled proteolysis of a caspase substrate in cellulo.

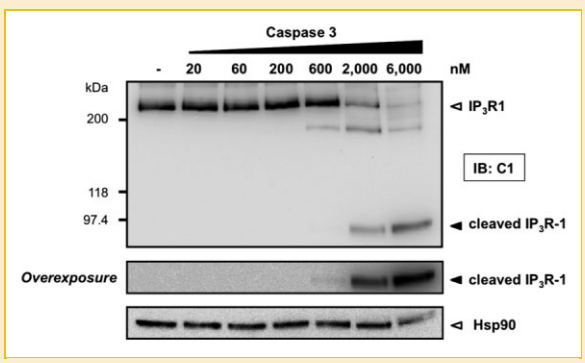

Fig. 4. In vitro cleavage of IP_3R-1 by recombinant caspase-3. Microsomes (0.5 mg/ml) from 293Ad cells overexpressing wild-type IP_3R-1 were incubated with the indicated concentration of active site-titrated recombinant caspase-3 for 1 h at 37°C. IP_3R-1 was analyzed by immunoblotting with the C1 antibody. A fourfold longer exposure time showing the cleaved IP_3R-1 fragment is also presented. Hsp90 was used as a loading control. These results are representative of two independent experiments.

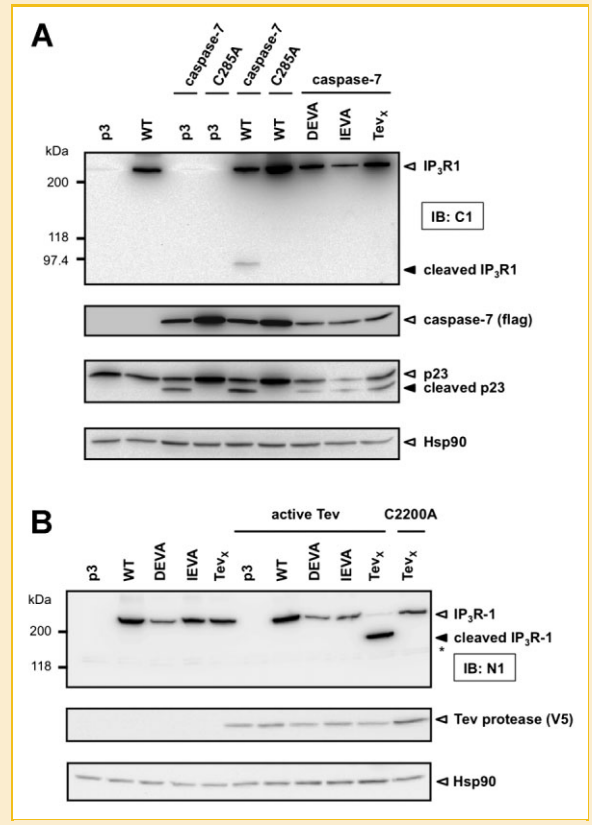

Fig. 5. In cellulo cleavage of IP_3R-1 by overexpressed proteases. A: 293Ad cells were co-transfected with empty plasmid (p3), or the indicated IP_3R-1 plasmid, and active or inactive (C285A) flag-tagged caspase-7 plasmid. Lysates were analyzed by immunoblotting with the indicated antibodies. White arrowheads indicate full-length proteins whereas cleavage fragments are marked with black arrowheads. Hsp90 was used as a loading control. B: 293Ad cells were co-transfected as indicated with empty plasmid (p3), or with the indicated IP3R-1 plasmid, and active or inactive (C2200A) V5-tagged TEV protease plasmid. Lysates were analyzed as in (A). In (B), the doublet (*) detected at ~130 kDa was considered non-specific because it was also detected in the empty plasmid sample. These results are representative of three independent experiments.

DISCUSSION

During apoptosis, hundreds of proteins are cleaved by caspases. Although one can rationalize a role for a particular cleavage event, often, no attempt is made to establish the likelihood for a particular protein to be proteolyzed. The end result is that true death substrates are not distinguished from bystander substrates, i.e., substrates whose cleavage is irrelevant to the apoptotic process. However, this information is critical because it reduces the number of players that need to be taken into account to understand apoptosis.

The IP_3R-1 is central to intracellular Ca^{2+} signaling. Therefore, the report by Hirota et al. [1999] that IP_3R-1 is cleaved during STS-induced apoptosis is quite interesting, as it would perturb many cellular processes that depend on Ca^{2+}. Remarkably, the reported cleavage occurred at an unusual DEVD motif baring an arginine residue C-terminal to the scissile bond (P1' position). Nonetheless, extensive cleavage was observed in Jurkat cells and this unusual motif is present in the human, mouse, and rat orthologs of IP_3R-1, but absent from IP_3R-2 and IP_3R-3, clearly identifying the type 1 as a potential target during apoptosis. Therefore, we sought to determine how important was IP_3R-1 cleavage during apoptosis. Surprisingly, we did not observe IP_3R-1 cleavage in several cell lines treated with established "physiologic" apoptosis inducers (Trail, TNFα, UV). However, we did observe the generation of the cleavage fragment in Hela and Jurkat cells treated with the non-specific kinase inhibitor STS, as seen by Hirota et al. [1999]. Interestingly, the fragment was barely detectable at a time at which PARP, a key death substrate, p23, a relatively poor caspase-7 substrate, and procaspase-3 were almost completely cleaved. We can speculate that this dichotomy is due to excessive caspase-3 activation that allows the cleavage of less optimal substrates such as IP_3R-1. Indeed, STS was the only inducer that resulted in full procaspase-3 conversion, which is rarely the case with other apoptotic agents. Furthermore, our results using overexpressed caspase-7 support this hypothesis since abnormally high caspase activity is reached in such experiments. Also, in vitro assays using recombinant caspase-3 demonstrated that IP_3R-1 is cleaved only at a protease concentration above 600 nM, which is higher than the assumed endogenous caspase-3 concentration (100–300 nM) at which all the true death substrates are cleaved [Stoka et al., 2001]. Taken together, these results demonstrate that IP_3R-1 is a poor caspase-3 substrate. It is noteworthy that other groups have detected cleavage of the receptor [Assefa et al., 2004; Verbert et al., 2008]. In both instances, either STS or extended exposure to an apoptotic stimulus was used to produce cleaved IP_3R-1, which is in agreement with our hypothesis that the receptor is a relatively late caspase substrate. Amongst the many possibilities that may explain this, we propose that the presence of a P1' arginine residue at the cleavage site may be the main obstacle to efficient proteolysis. Indeed, work by Stennicke *et al.* demonstrated that compared to small residues that are found in the P1' position of most other caspase substrates, arginine decreases catalytic specificity of a short peptide by 11–14-folds [Stennicke et al., 2000]. Other factors may also play a role in hampering proteolysis including presence of secondary structural elements and occlusion by surrounding domains or even interacting partners.

Importantly, we demonstrated that the lack of IP_3R-1 cleavage observed in 293Ad cells was not due to inaccessibility of the cleavage site. First, we showed that overexpression of caspase-7, which self-activates upon overexpression and generates strong caspase activity [Duan et al., 1996; Denault and Salvesen, 2003], resulted in IP_3R-1 cleavage. Secondly, we used an engineered IP_3R-1 carrying a TEV protease recognition site that can be cleaved at will by ectopic expression of TEV protease. Using this method, we showed that IP_3R-1 could be cleaved in a controlled manner. Actually, because there is no cleavage site for this protease in the human proteome, this approach is ideal to post-translationally modify a protein including decoupling a cleavage event occurring during apoptosis from full-blown apoptosis. For example, we previously used this approach to demonstrate that caspase-8 activation does not lead to apoptosis unless it is cleaved [Oberst et al., 2010].

Few caspase substrates other than caspases themselves are critical for the proper execution of apoptosis. Genetic studies in mice on the necessity for caspase-mediated DNA fragmentation by CAD [Liu et al., 1997; Enari et al., 1998; Sakahira et al., 1998], a well-recognized apoptotic hallmark, showed that this endonuclease is dispensable for apoptosis and cell removal by professional phagocytes [McIlroy et al., 2000; Nagata et al., 2003]. On the contrary, mice with a caspase resistant PARP, whose proteolysis by caspases is also a hallmark of apoptosis, are resistant to endotoxic shock and to ischemia-reperfusion [Petrilli et al., 2004]. Consequently, it is difficult to establish what constitutes a bona fide death substrate. This critically important issue has been recently addressed by Timmer and Salvesen [2007]. IP_3R-1 fulfills many of the proposed requirements for a bona fide substrate. Indeed, the receptor is cleaved by recombinant caspase in vitro, fragments were identified in cellulo and mutation of the critical P1 aspartate residues abrogates cleavage. However, cleavage is inefficient enough to assign IP_3R-1 as a general substrate for apoptosis, and it has not been shown that lack of cleavage seriously impedes on apoptosis. Nevertheless, it clearly promotes it [Verbert et al., 2008], and we do not rule it out as key player in some as yet unknown apoptotic paradigm.

Many other studies suggested that IP_3Rs play a significant role in the apoptotic process [Khan et al., 1996; Jayaraman and Marks, 1997; Sugawara et al., 1997; Chen et al., 2004]. These studies proposed that an intact and functional IP_3R is necessary to sustain apoptosis. Interestingly, because the caspase-cleaved form of IP_3R-1 exhibits increased leakiness, it was suggested that it enhances Ca^{2+} release from the ER during apoptosis [Assefa et al., 2004; Nakayama et al., 2004]. It was also shown that the full length IP_3R-1 and the caspase-truncated IP_3R-1 can sustain apoptosis even when the IP_3R-1 is mutated to inactivate the pore of the Ca^{2+} channel [Sugawara et al., 1997]. Further results suggested that although the intrinsic ion-channel function of IP_3R-1 is dispensable for apoptosis induced by STS in DT-40 lymphocytes, the C-terminal tail of IP_3R appears to be essential, possibly reflecting key protein–protein interactions with this domain [Chen et al., 2004]. The mechanism by which IP_3R sustains apoptosis is not clear presently. Independently of its possible role in supporting the apoptotic process, our results suggest that IP_3R-1 is not part of the critical set

of death substrates because it is not cleaved in most cells and apoptotic paradigms.

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