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# HtrA2 cleaves Apollon and induces cell death by IAP-binding motif in Apollon-deficient cells ☆

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**Abstract**

Apollon/BRUCE is a giant IAP protein that has BIR and UBC domains in its amino- and carboxy-terminals, respectively. Apollon binds and ubiquitylates SMAC/DIABLO and caspase9, and regulates apoptosis by facilitating proteasomal degradation of these proteins. Apollon overexpression inhibits apoptosis, while its downregulation sensitizes cells to apoptosis, suggesting that Apollon level is important for apoptosis regulation. Here we show that HtrA2/Omi catalytically cleaves Apollon with its serine protease activity. Conversely, Apollon ubiquitylates and facilitates proteasomal degradation of HtrA2 that binds to Apollon through IAP-binding motif. Thus, Apollon and HtrA2 mutually downregulate each other. Expression of catalytically active, but not inactive, HtrA2 induced apoptosis in Apollon-expressing cells. In Apollon-deficient cells, however, expression of catalytically inactive HtrA2 mutant with IAP-binding motif also induced apoptosis. These results indicate that HtrA2 induces apoptosis in two different mechanisms, one with serine protease domain and the other with IAP-binding motif, in Apollon-deficient cells.


*Keywords:* IAP; Apollon; HtrA2; Apoptosis; Ubiquitin

Apoptosis is a type of cell death that is evolutionarily conserved among all animals [1–3]. Deregulation of the apoptotic pathway is implicated in the pathogenesis of cancer, autoimmune diseases, and neurodegenerative disorders [4,5]. The IAPs (inhibitor of apoptosis proteins) are a family of proteins that have one to three baculoviral IAP repeat (BIR) domains, and inhibit apoptosis by direct binding and inhibition of caspases [6–8]. Apollon (also known as BRUCE) is a huge IAP that has BIR and ubiquitin-conjugating enzyme (UBC) domains at amino- and carboxy-terminals, respectively [9–12]. Apollon ubiquitylates and facilitates proteasomal degradation of caspase9 and SMAC [12]. Overexpression of Apollon inhibits apoptosis [11–13], and deficiency in Apollon sensitizes cells to apoptosis induced by various stimuli [10,12,14], suggesting an important role of Apollon in apoptosis regulation.

The anti-apoptotic IAP function is counteracted by SMAC [15,16] and HtrA2 [17–21] that dislodge caspases from IAPs. HtrA2 has IAP-binding motif (IBM) and

☆ *Abbreviations:* HtrA2, high temperature requirement A2, IAP, inhibitor of apoptosis protein; IBM, IAP-binding motif; BIR, baculoviral IAP repeat; XIAP, X-chromosome-linked IAP; cIAP, cellular IAP; BRUCE, BIR repeat containing ubiquitin-conjugating enzyme; SMAC, second mitochondria-derived activator of caspases; DIABLO, direct IAP-binding protein with low p*I*; UBC, ubiquitin-conjugating enzyme; ZVAD, benzyloxycarbonyl-valinyl-alanyl-aspartate-fluoromethyl ketone; MEF, mouse embryonic fibroblast; DEVD-MCA, acetyl-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide; HA, hemagglutinin.

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serine protease domain, both of which are involved in apoptosis regulation [18,21–24]. We reported in this paper that HtrA2 catalytically cleaved Apollon with its serine protease activity, and conversely, Apollon facilitated proteasomal degradation of HtrA2 by ubiquitylation that required IBM-mediated HtrA2 binding to Apollon. Thus, HtrA2 and Apollon mutually downregulated each other. We also reported that expression of catalytically inactive HtrA2 with IBM induced apoptosis in Apollon-deficient MEFs, as did catalytically active HtrA2, which suggests the multiple mechanism of apoptosis induction by HtrA2 in the context of Apollon deficiency.

## Materials and methods

*Plasmid construction, transfection, and immunoblot analysis.* Wild-type and various mutants of Apollon [12] and HtrA2 [17,22] cDNAs were constructed as described previously. Cells ($2 \times 10^5$) were seeded in 6-well plates and transfected with a total of 2 μg of plasmid DNA for 24 h. The cells were harvested and lysed in 60 μl lysis buffer (0.1 M Tris, pH 8.0/1% SDS/10% glycerol). Cell lysates were separated by 4–20% gradient polyacrylamide gel electrophoresis, transferred onto PVDF membranes (Perkin-Elmer Life Sciences), and analyzed by Western blotting using appropriate antibodies. Protein bands were detected using Enhanced Chemiluminescence detection (ECL) kits (Amersham-Pharmacia). Antibodies against Apollon [12] and HtrA2 [17] were generated as described. Other antibodies were: anti-BRUCE monoclonal antibody (mAb) (Transduction Laboratories), anti-XIAP mAb (MBL), anti-Survivin mAb (Santa Cruz), anti-SMAC polyclonal antibody (pAb) (Chemicon), anti-tubulin pAb (Cosmobio), anti-Myc pAb (MBL), and HRP-conjugated anti-FLAG mAb (Sigma), HRP-conjugated anti-HA mAb (Roche).

*Coimmunoprecipitation.* A total of $10^6$ cells were lysed in 1% Nonidet P-40 (NP-40) buffer (10 mM Hepes, pH 7.4/142.5 mM KCl/5 mM $MgCl_2$/1 mM EGTA/1% NP-40), containing protease inhibitors (1:100 v/v dilution of PMSF and Aprotinin) (Sigma). Lysates cleared with 20 μl Protein G–Sepharose (Zymed Laboratories) at 4 °C for 1 h were immunoprecipitated by incubation with 1 μg of antibodies and 20 μl Protein G–Sepharose for 2 h. After extensively washing with 500 μl wash buffer (10 mM Hepes, pH 7.4/142.5 mM KCl/5 mM $MgCl_2$/1 mM EGTA/0.1% NP-40) without protease inhibitor, the precipitates were boiled in 2 × SDS buffer and Western blotted. Normal IgG was the negative control for immunoprecipitation.

*Generation of recombinant HtrA2 and in vitro protease assay.* Recombinant mature HtrA2 protein was produced as described [17]. Apollon protein immunoprecipitated from transiently transfected HT1080 cells or casein was incubated with graded concentrations of recombinant HtrA2 protein at 37 °C for 1 h. The reactions were subjected to SDS–PAGE and analyzed by Western blotting with anti-Apollon, or the gel was stained with Coomassie brilliant blue to visualize casein fragments.

*Ubiquitylation assay.* 293T cells were transfected for 24 h with p3xFLAG-CMV-10 vectors encoding Apollon, pcDNA3-HA-ubiquitin, and pcDNA3-HtrA2-myc. The cells were then incubated with MG132 (10 μM) for 4 h before harvested and lysed in 1% NP-40 buffer. The cell lysates were immunoprecipitated with anti-myc antibody and the precipitates were analyzed by Western blotting using HRP-conjugated anti-HA mAb (Roche).

*Cell death assay and caspase assay.* Wild-type and Apollon-deficient MEFs were transiently transfected for 48 h with various HtrA2 constructs using LipofectAMINE Plus (Gibco-BRL) according to the manufacturer's instructions and then cell viability was determined by Trypan blue exclusion. Caspase activities were measured by cleavage of DEVD-MCA as described previously[12].

## Results

### *HtrA2 binds Apollon*

HtrA2 bound Apollon and XIAP, but not survivin, as did SMAC when IAPs were overexpressed (Fig. 1A). Fig. 1B shows that endogenous HtrA2 bound endogenous Apollon, although it was less than the binding to XIAP. To examine the role of IBM in the binding to Apollon, various mutants of HtrA2 were transfected into cells and examined for their binding to Apollon (Fig. 1C). Full-length HtrA2 that is processed to mature form in mitochondria to expose IBM bound Apollon (lanes 2 and 3), while ΔN HtrA2 that has an additional methionine at amino terminal of IBM did not (lanes 4 and 5). These results indicate that the IBM plays a crucial role in the binding to Apollon.

We further examined the domains of Apollon required for binding to HtrA2 (Fig. 1D). The 1–1648 Apollon fragment, which binds SMAC and caspase9 [12], bound HtrA2 (lane 4). The 1–677 Apollon fragment, which binds caspase9 but not SMAC [12], did not bind HtrA2 (lane 5). A point mutation (C327A) in a conserved cysteine residue in BIR domain of Apollon (lanes 6 and 10), but not another point mutation (C4638A) in the conserved cysteine in UBC domain (lane 9), abolished the binding to HtrA2, indicating that the BIR domain of Apollon plays an important role in the binding to HtrA2. These results suggest that HtrA2 binds Apollon in a manner similar to SMAC.

### *Apollon ubiquitylates HtrA2 and facilitates degradation of HtrA2*

Apollon has a UBC domain in its carboxy terminal and ubiquitylates associated proteins such as SMAC and caspase9 [11,12]. Therefore, we next examined the ubiquitylation of HtrA2 by Apollon. Cells were transfected with HtrA2-myc, HA-ubiquitin along with Apollon, and the cell lysates were immunoprecipitated with anti-myc (HtrA2) followed by Western blot analysis with anti-HA antibody to detect ubiquitylated HtrA2 (Fig. 2). A smear of protein bands which migrated slowly in the gels was greatly enhanced by Apollon, indicating that Apollon ubiquitylates HtrA2.

We next measured degradation of HtrA2 in the cells overexpressing Apollon. To prevent the cleavage of Apollon by HtrA2 (described below), we expressed catalytically inactive HtrA2 mutants in the cytoplasm by a ubiquitin fusion method [25], and cells were analyzed by Western blot after treated with protein synthesis

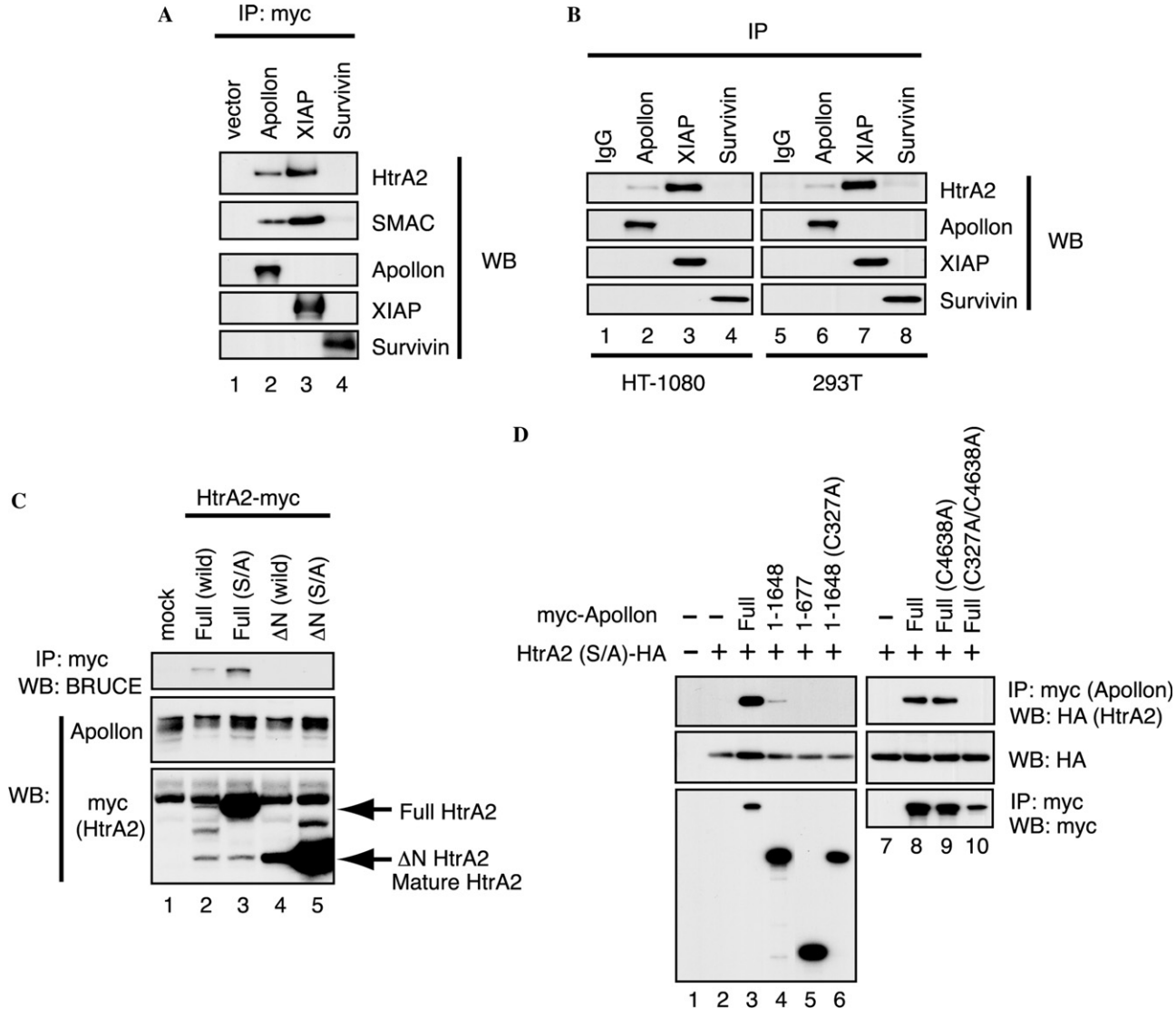


Fig. 1. HtrA2 binds Apollon. (A) HrtA2 and SMAC bind Apollon and XIAP but not survivin. Cells were transfected with plasmids encoding myc-tagged Apollon, XIAP, and survivin. Cell lysates were immunoprecipitated with anti-myc antibody and the precipitates were analyzed by Western blotting with indicated antibodies. (B) Endogenous HtrA2 binds endogenous Apollon and XIAP. Lysates from HT1080 and 293T cells were immunoprecipitated with control IgG (lanes 1 and 5) or antibodies against Apollon (lanes 2 and 6), XIAP (lanes 3 and 7), and survivin (lanes 4 and 8). Precipitates were Western blotted using indicated antibodies. (C) IAP-binding motif of HtrA2 is required for binding to Apollon. Catalytically active (wild) and inactive (S/A) myc-tagged full-length and ΔN HtrA2 were transfected into HT1080 cells. ΔN HtrA2 has an additional methionine at amino terminal of mature HtrA2. Immunoprecipitates of anti-myc antibody were Western blotted using anti-BRUCE (upper panel). Endogenous Apollon (middle panel) and expression of HtrA2-myc proteins (lower panel) were confirmed. (D) BIR domain of Apollon is involved in the binding to HtrA2. Myc-tagged Apollon mutants and catalytically inactive HA-tagged full-length HtrA2 were transfected into 293T cells. Immunoprecipitates of anti-myc antibody were Western blotted using HRP-conjugated anti-HA (upper panel). Expression of HtrA2 (middle panel) and Apollon mutants (lower panel) was confirmed.

inhibitor cycloheximide. Apollon facilitated degradation of HtrA2 with intact (AVPS) IBM (Fig. 3A, lanes 1–8; 3B, left panel), while not the HtrA2 with mutated (MVPS) IBM (Fig. 3A, lanes 9–16; 3B, right panel). The facilitated degradation was suppressed by proteasome inhibitors, MG132 and lactacystin (Fig. 3C). These results indicate that Apollon ubiquitylates and facilitates proteasomal degradation of HtrA2, which requires IBM-mediated HtrA2 binding to Apollon.

*HtrA2 cleaves Apollon with its serine protease activity*

HtrA2 is reported to cleave IAPs such as XIAP and cIAP1. Therefore, we next examined if HtrA2 cleaves Apollon. Expression of catalytically active full-length and ΔN HtrA2 proteins reduced the level of Apollon protein in the cells (Fig. 4A, upper panel), which coincided with appearance of additional protein bands detected by anti-Apollon antibody (Fig. 4A, lanes 2 and

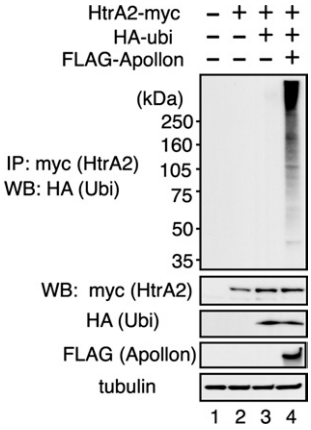


Fig. 2. Apollon ubiquitylates HtrA2. 293T cells were transfected with myc-tagged HtrA2, HA-tagged ubiquitin, and FLAG-tagged Apollon. Immunoprecipitates of anti-myc (top panel) or lysates (lower panels) were Western blotted using indicated antibodies.

4, arrows in the right). Expression of catalytically inactive HtrA2s (S/A), in which the conserved serine residue at 306 was substituted to alanine [17], did not give the additional protein bands (lanes 3 and 5). Addition of a caspase inhibitor ZVAD did not affect the appearance of the bands (lanes 6–8). These results suggest that HtrA2, but not caspases, catalytically cleaves Apollon to give the fragments detected by the anti-Apollon antibody. IBM of HtrA2 is not required for this cleavage, because catalytically active HtrA2 without IBM (ΔN wild) also produced the fragments.

To confirm that HtrA2 cleaves Apollon, we incubated Apollon with recombinant HtrA2 protein in vitro (Fig. 4B). Western blot analysis with anti-Apollon antibody showed that HtrA2 cleaved full-length Apollon to produce fragments with similar molecular mass that were observed in cells expressing catalytically active HtrA2 proteins. Cleavage of casein serves a control of the catalytic activity of the recombinant HtrA2 protein.

We next carried out Western blot analysis of cells undergoing apoptosis to examine if the Apollon fragments were found in the cells undergoing apoptosis. The level of Apollon protein decreased when cells were treated with staurosporine and etoposide (Fig. 4C, top panel, lanes 1–3), which coincided with the appearance of a 95-kDa fragment of Apollon (arrow in middle panel). ZVAD treatment did not abrogate the appearance of the 95-kDa fragment (lanes 5 and 6). These results suggest that HtrA2 cleaves Apollon upon apoptosis induction.

### *Induction of cell death in Apollon-deficient cells by HtrA2*

To study further the role of IBM and serine protease domain of HtrA2 in apoptosis regulation, we expressed

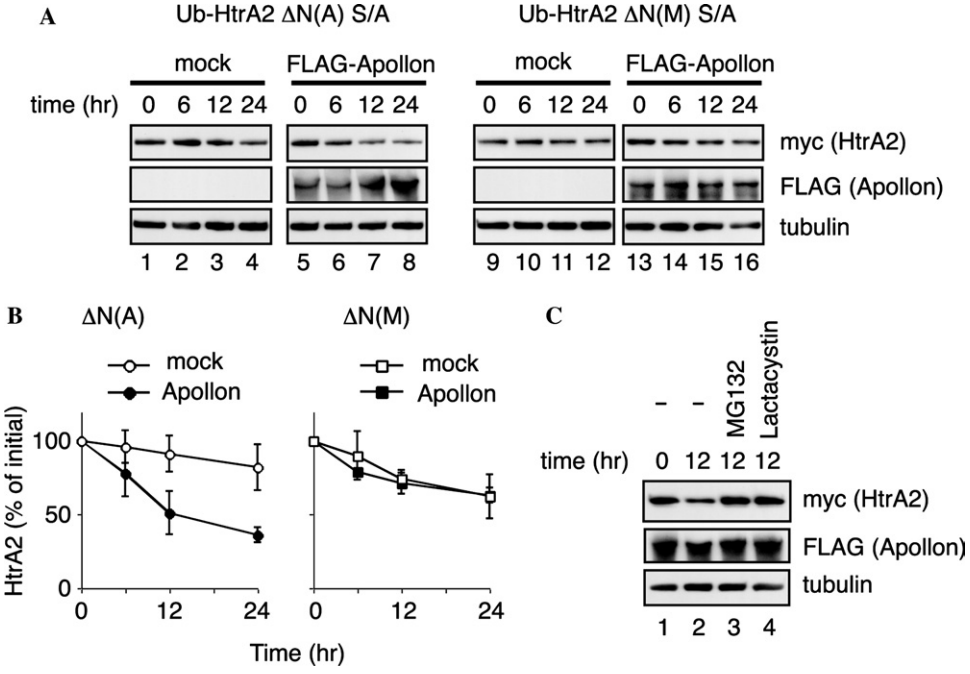


Fig. 3. Apollon facilitates proteasomal degradation of HtrA2. (A,B) Apollon facilitates degradation of HtrA2 with intact IAP-binding motif. Catalytically inactive myc-tagged HtrA2 with intact (Ub-HtrA2 ΔN(A) S/A) and mutated (Ub-HtrA2 ΔN(M) S/A) IAP-binding motif were expressed in cytosol by a ubiquitin-fusion method with or without FLAG-tagged Apollon. Cells were incubated with 20 μg/ml cycloheximide for indicated periods, and cell lysates were analyzed by Western blot using indicated antibodies (A). Protein bands in triplicate experiments were measured and plotted against time (B). Bars, SD. (C) Proteasome inhibitors suppressed the degradation of HtrA2. Cells were treated with 10 μM MG132 or 20 μg/ml lactacystin.

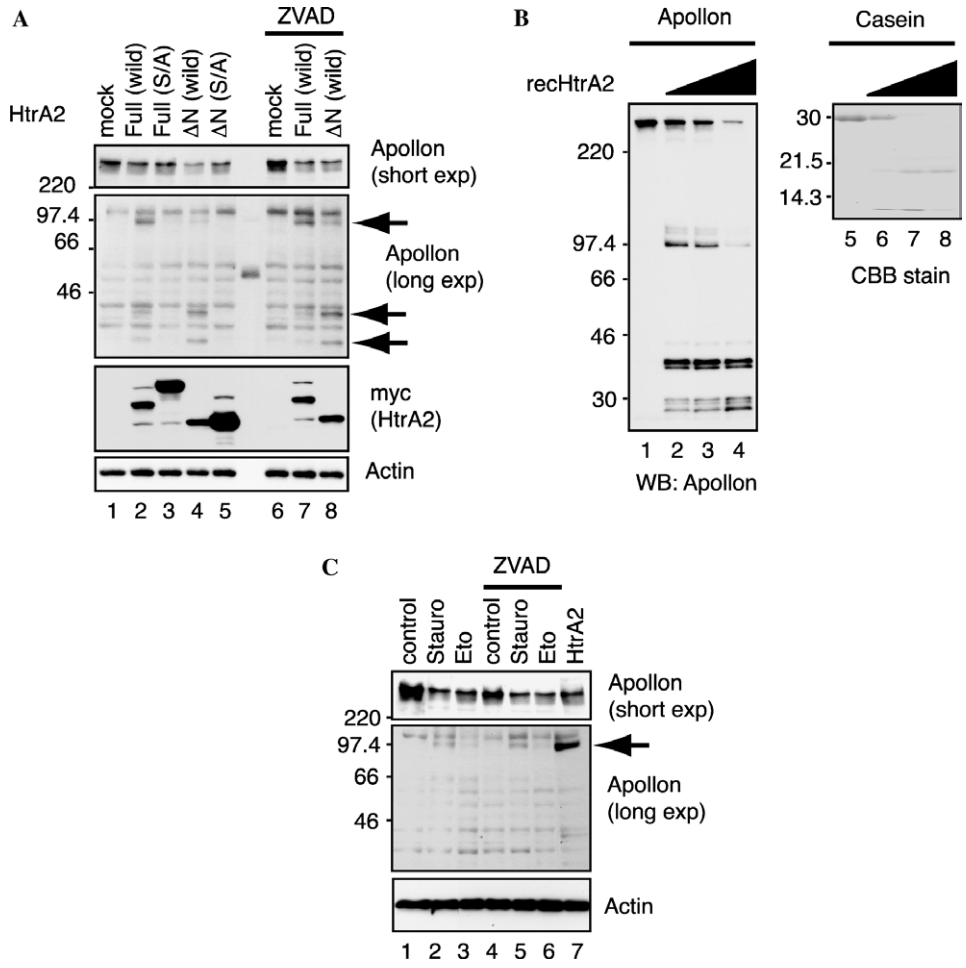


Fig. 4. HtrA2 cleaves Apollon. (A) Expression of catalytically active HtrA2 proteins generates fragments detected by anti-Apollon antibody. Catalytically active (wild) and inactive (S/A) myc-tagged full-length and ΔN HtrA2 were transfected into cells in the presence or absence of a caspase inhibitor ZVAD. Cell lysates were analyzed by Western blotting with indicated antibodies. Arrows in the right show the protein bands appeared in the cells transfected with catalytically active HtrA2 plasmids. (B) Cleavage of Apollon by recombinant HtrA2 in vitro. Apollon protein immunoprecipitated from transiently transfected HT1080 cells or casein was incubated with 0, 50, 100, and 200 nM recombinant HtrA2 protein. (C) Apollon fragments appeared in apoptotic cells. HT1080 cells were treated with 0.1 μg/ml staurosporine or 100 μg/ml etoposide in the presence or absence of ZVAD for 24 h. Catalytically active full-length HtrA2 was transfected into cells (lane 7). Cell lysates were analyzed by Western blotting with indicated antibodies. An arrow in the right shows the protein band appeared in apoptotic cells and HtrA2 transfected cells.

various HtrA2 mutants in wild-type and Apollon-deficient MEFs (Fig. 5A), and measured the extent of apoptosis (Figs. 5B and C). The catalytically active Ub-HtrA2s ((A) wild, (M) wild), but not catalytically inactive Ub-HtrA2s ((A) S/A, (M) S/A), induced apoptosis in wild-type MEFs (Fig. 5B, open bars). In Apollon-deficient MEFs, however, the catalytically inactive Ub-HtrA2 with intact IBM ((A) S/A), as well as catalytically active Ub-HtrA2s ((A) wild, (M) wild), induced apoptosis, while not the catalytically inactive Ub-HtrA2 with mutated IBM ((M) S/A) (Fig. 5B, filled bars). Caspase activation by expression of these HtrA2 proteins was consistent with the induction of apoptosis (Fig. 5C). These results indicate that HtrA2 induces apoptosis in multiple mechanisms, one with serine protease domain and the other with IBM, in the cells deficient in Apollon.

## Discussion

IAPs including Apollon regulate caspases by direct binding and ubiquitylation [6–8,12]. IAP antagonists such as HtrA2 and SMAC block the IAP function [15–18,20,21], and the balance between IAPs and IAP antagonists could determine cellular sensitivity to apoptosis. Among the IAPs, Apollon could play an important role in regulating apoptosis sensitivity, since upregulation and downregulation of Apollon both affect the cellular sensitivity to apoptosis [10–12,14]. This contrasted the role of XIAP, whose upregulation highly desensitizes cells to apoptosis [12,26,27], whereas no sensitization was observed by downregulation or gene targeting studies [28]. This may be due to a potent caspase-inhibitory activity of residual XIAP, and/or compensatory overexpression of close homologues

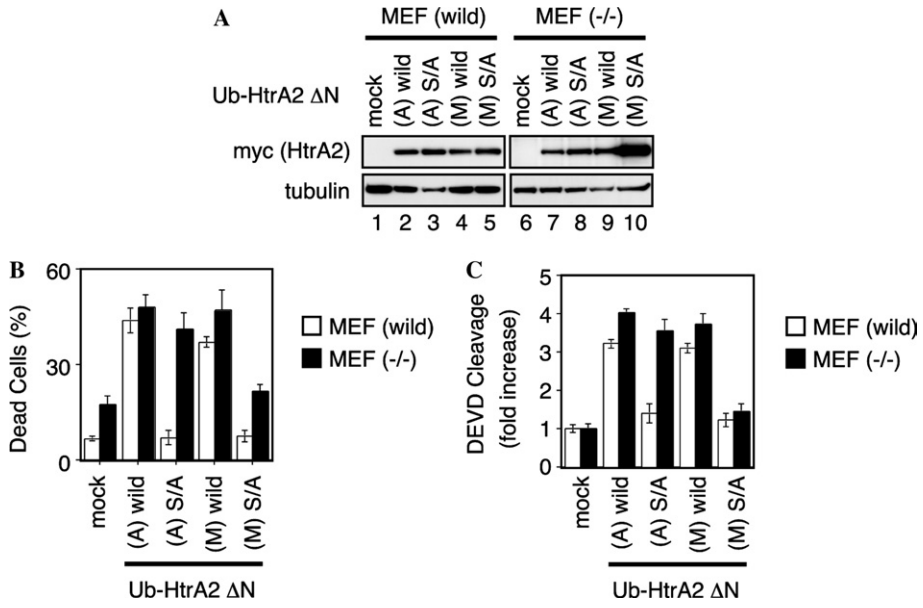


Fig. 5. Catalytically inactive HtrA2 with IBM induces apoptosis in Apollon-deficient MEFs. (A) Expression of various HtrA2 mutants in wild-type and mutant MEFs. Catalytically active (wild) and inactive (S/A) myc-tagged HtrA2 with wild-type (A) and mutated (M) IBM were expressed in cytosol by an ubiquitin-fusion method in wild-type (left panels) and Apollon-deficient (right panels) MEFs. (B) Induction of apoptosis by expression of HtrA2. (C) Caspase activation by expression of HtrA2. Wild-type (open bars) and Apollon-deficient (closed bars) MEFs were transfected with indicated HtrA2 plasmids for 48 h and cell viability was examined by Trypan blue exclusion (B) or caspase activity in cell lysates was determined (C). Bars, SD.

cIAP1 and cIAP2 as reported in XIAP-deficient mice [28]. Thus, regulation of Apollon level could be an important event to promote apoptosis. Nrdp1-mediated downregulation of Apollon was reported in various cells during progression of apoptosis [14].

We reported in this paper that HtrA2 catalytically cleaved Apollon during the progression of apoptosis. HtrA2 has IBM and catalytically active serine protease domain in its mature form. With the IBM, HtrA2 stably bound to BIR domain of Apollon. However, the IBM-mediated stable binding is not required for Apollon cleavage by HtrA2, since ΔN HtrA2 that lacks IBM also cleaved Apollon into fragments detected by anti-Apollon antibody. Probably, the serine protease domain of HtrA2 directly recognizes Apollon as a substrate, though this interaction is not stable enough to be detected by co-immunoprecipitation experiments. It is possible, however, that IBM-mediated stable binding of HtrA2 to Apollon enhances the Apollon cleavage as reported for the cleavage of XIAP and cIAP1 by HtrA2 [23,24].

Expression of catalytically active, but not catalytically inactive, HtrA2 induces apoptosis in wild-type MEFs as reported previously [17,21,22,24]. The catalytic cleavage of IAPs, including Apollon as described above, by HtrA2 could play a role in irreversibly inactivating IAPs and promoting apoptosis. Interestingly, expression of catalytically inactive HtrA2 with intact IBM induced apoptosis in Apollon-deficient MEFs. These results suggest a sequential mechanism of apoptosis induction by HtrA2, first irreversible cleavage of Apollon (and other IAPs) with serine protease domain, and then induction of apoptosis with IBM in Apollon-depleted cells. Mature SMAC that has IBM at amino terminal may cooperate with HtrA2 in the induction phase after depletion of Apollon. These IBM proteins are relatively stable in Apollon-deficient cells and may induce apoptosis in a manner similar to Reaper, Hid, and Grim in *Drosophila* [2,3,7,12].

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