Akt Attenuation of the Serine Protease Activity of HtrA2/Omi through Phosphorylation of Serine 212

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The serine protease HtrA2/Omi is released from the mitochondria into the cytosol following apoptosis stimuli, leading to the programmed cell death in caspase-dependent and -independent manners. The function of HtrA2/Omi closely relates to its protease activity, which is required for cleavage of its substrate such as the members of the X-linked inhibitor of apoptosis protein family. However, the regulation of HtrA2/Omi by signaling molecule has not been documented. Here we report that serine/threonine kinases Akt1 and Akt2 phosphorylate mitochondria-released HtrA2/Omi on serine 212 in vitro, which results in attenuation of its serine protease activity and pro-apoptotic function. Abolishing HtrA2/Omi phosphorylation by Akt, also named protein kinase B, represents a family of phosphoinositide 3-kinase-regulated serine/threonine kinases (1–3). Three members of Akt have been identified: Akt1/protein kinase Ba, Akt2/protein kinase Bβ, and Akt3/protein kinase Bγ (4–9), all of which are activated by growth factors in a phosphoinositide 3-kinase-dependent manner (10). Accumulated evidence shows that Akt and its downstream targets constitute a major cell survival pathway. Akt inhibits the programmed cell death in a number of cell types induced by a variety of stimuli through regulation of molecules at both pre-mitochondrial and post-mitochondrial levels (11–13). Several Akt targets have been identified at pre-mitochondrial level. The first Akt target identified was the pro-apoptotic protein BAD (14, 15). BAD is a member of the Bcl-2 family that initiates apoptosis by binding to Bcl-xL and Bcl2 on the outer mitochondrial membrane, causing the release of cytochrome c into the cytosol. Akt phosphorylates BAD on serine 136, which promotes the association of BAD with 14-3-3 proteins in the cytosol, thus inactivating its pro-apoptotic function. In addition, Akt reduces the transcription of a subset of pro-apoptotic genes by phosphorylation of Forkhead transcription factors, which causes their nuclear exclusion and inactivation (16).

Akt phosphorylates HtrA2/Omi on serine 212 within serine protease domain in vitro and in vivo and inhibits HtrA2/Omi protease activity. Although the association between HtrA2/Omi and XIAP was not interfered, the ability of HtrA2/Omi to cleave XIAP was attenuated after the phosphorylation by Akt. In addition, Akt also inhibits HtrA2/Omi release from the mitochondria. These data identify HtrA2/Omi as a substrate of Akt and a potentially important mediator of Akt pro-survival function at post-mitochondrial level.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—Hemagglutinin epitope (HA)-tagged wild-type, constitutively active (Myr-Akt), and domi-

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3 The abbreviations used are: IAP, inhibitor of apoptotic protein; XIAP, X-linked IAP; HA, hemagglutinin; GFP, green fluorescence protein; HEK, human embryonic kidney cells; shRNA, short hairpin RNA; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; GST, glutathione S-transferase; z, benzylxoxycarbonyl; FMK, fluoromethyl ketone.

This article has been withdrawn by the authors. The same data were used to represent different experimental conditions. Specifically, the Myc-M-HtrA2 panel from Fig. 3B was reused as actin in Figs. 5A and 6C. The HtrA2-P panel from mitochondria in Fig. 3E was reused as Akt in the same set of panels. In Fig. 5A, a band was pasted into the PARP immunoblot. The authors state that they stand by the overall conclusions of the work.
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**Figure 1.** Akt inhibits the apoptosis induced by mature HtrA2/Omi. A, schematic diagram of the domain structure of premature and mature HtrA2/Omi proteins. The premature HtrA2/Omi has an N-terminal putative transmembrane domain (TM) and a serine protease domain adjacent to a PDZ domain at the C terminus. A putative Akt phosphorylation serine residue is located in the serine protease domain. B, mature HtrA2/Omi-induced apoptosis is partially reduced by caspase inhibitor (z-VAD-FMK, ZVAD, 100 μM), the percentages of GFP-positive apoptotic cells were determined by fluorescent microscopy after staining with 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI). C, constitutively active Akt inhibited apoptosis induced by mature HtrA2/Omi similar to that of protease-inactive mutant M-HtrA2/Omi-S306A. Hela cells were co-transfected with GFP-M-HtrA2/Omi or GFP-M-HtrA2/Omi-S306A and WT-Akt or constitutively active Akt (myr-Akt). Forty-eight hours after transfection, apoptosis was measured as described in B.

**Summary:**

Akt negatively regulates HtrA2/Omi-induced apoptosis. The inhibitory effect of Akt is mediated by phosphorylation of the serine protease domain. Akt phosphorylation of HtrA2/Omi-TM is reduced in the presence of Akt activity.

**Cell Culture and Transfection**

- Human embryonic kidney (HEK) 293, HeLa, OV2008, A2780S, and OVCAR-3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cell transfection was performed with LipofectAMINE Plus (Invitrogen).

**Immunoprecipitation, Immunoblotting, and in Vitro Kinase Assay**

- For immunoprecipitation, cell lysate was incubated with the appropriate antibodies as noted in the figure legends in the presence of protein A-protein G (2:1)-agarose beads.

**In Vivo [32P]Orthophosphate Cell Labeling**

- HEK293 cells were transfected with pcDNA3-Myc-HtrA2/Omi together with or without wild-type and constitutively active Akt. Following incubation for 36 h, the cells were labeled with [32P]orthophosphate (0.5 mCi/ml) in minimal Eagle’s medium without phosphate for 4 h. HtrA2/Omi was immunoprecipitated with anti-Myc antibody. The immunoprecipitates were separated on SDS-PAGE and transferred to membranes. Phosphorylated HtrA2/Omi was detected by autoradiography.

**Subcellular Fractionation**

- Subcellular fractions were prepared by digitonin-based permeabilization buffer as described (33). The relative purity of the subcellular fractions was confirmed by Western blot using anti-actin (cytosolic marker) and anti-Cox-4 (mitochondrial marker) antibodies.

**Apoptosis Assay**

- Cells were transfected with GFP-fused mature HtrA2/Omi, HtrA2/Omi-S212A, or HtrA2/Omi-S212D and GFP alone as a control. After 48 h of transfection, the cells were stained with 4',6-diamidino-2-phenylindole and apoptosis was examined using fluorescence microscopy in GFP-expressing cells. The percentage of apoptotic cell was expressed as the mean number of apoptotic cells in a fraction of the total number of GFP-expressing cells. For ovarian cancer cells, the cells were infected with shRNA of Akt (Sigma) and then treated with cisplatin and Akt/PKB inhibitor-2 (API-2). Apoptotic cells were detected with TUNEL assay (12).

**In Vitro Proteolytic Cleavage Assay for HtrA2/Omi**

- Mature GST-HtrA2/Omi, -HtrA2/Omi-S212A, -HtrA2/Omi-S212D, and HtrA2/Omi-A144G mutant plasmids were created using a QuikChange site-directed mutagenesis kit (Stratagene). A plasmid encoding green fluorescent protein (GFP)-fused HtrA2/Omi was prepared by cloning HtrA2/Omi cDNA into pEGFP-C3 (Clontech) at KpnI/XbaI sites. pLKO.1-Puro-shAkt2 was purchased from Sigma. Antibodies against Akt substrate, XIAP, Akt, poly(ADP-ribose) polymerase, and actin were purchased from Cell Signaling. Anti-HtrA2 antibody was from R&D Systems. Recombinant Akt was obtained from Upstate. Cisplatin and β-casein were ordered from Sigma.
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RESULTS AND DISCUSSION

Akt Inhibits the Apoptosis Induced by Mature HtrA2/Omi—The serine protease HtrA2/Omi is synthesized as a precursor (premature HtrA2/Omi) that normally localizes at the mitochondria in healthy cell. Following apoptotic stimuli, the N-terminal amino acids preceding alanine 134 are cleaved and the resulted mature HtrA2/Omi is released from the mitochondria into the cytoplasm where it induces apoptosis (24–29). It has been shown that mature HtrA2/Omi functions as an antagonist of members of IAP family proteins that normally bind to and inhibit caspase-3, -7, and -9 (24–27). However, accumulated studies indicate that serine protease activity of HtrA2/Omi plays a more critical role than its antagonizing IAPs in inducing apoptosis (27–29), whereas the cleaving targets remain to be fully characterized (25, 26). Mutation of serine 306 within protease domain abolishes its serine protease and pro-apoptotic activity. Thus, HtrA2/Omi pro-apoptotic function is mediated through caspase-dependent and -independent mechanisms and totally relies on its serine protease activity (24–29, 34–36).

Because Akt exerts anti-apoptotic function at both pre- and post-mitochondrial levels, its one or more targets at post-mitochondrial level remain elusive, and the serine protease domain of HtrA2/Omi contains an Akt phosphorylation consensus site (Fig. 1A), we examined if HtrA2/Omi pro-apoptotic function is regulated by Akt. HeLa cells were transfected with GFP-fused premature or mature HtrA2/Omi (Fig. 1A) together with or without wild-type and constitutively active Akt and then treated with or without pan-caspase inhibitor z-VAD-FMK. Apoptotic cells were examined and accounted under fluorescent microscopy after staining with 4’,6-diamidino-2-phenylindole and propidium iodide. As expected, ectopic expression of premature HtrA2/Omi did not increase apoptosis compared with GFP vector control, whereas expression of mature HtrA2/Omi considerably induced apoptosis (Fig. 1B) together with or without wild-type and constitutively active Akt and then treated with or without pan-caspase inhibitor z-VAD-FMK.
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**A**

- M-HtrA2-S306A:
  - +
- M-HtrA2-S212D:
  - −
- M-HtrA2-S212A:
  - +
- M-HtrA2-Akt:
  - +

**B**

- HA-Myr-Akt:
  - +
- M-HtrA2:
  - −
- M-HtrA2-S212A:
  - +
- M-HtrA2-S212 D:
  - +

**C**

- IP: α-Myc
  - α-Myc
- IP: α-Myc
  - α-Myc

**D**

- IP: α-Flag
  - α-Flag
- FLAG-co-IP
  - FLAG

**FIGURE 4.** Phosphorylation of serine 212 of HtrA2/Omi by Akt abrogates its pro-apoptotic function. A, Akt inhibition of HtrA2/Omi was determined by Western blot analysis of recombinant forms of mature HtrA2/Omi incubated with constitutively active Akt. B, Akt inhibition of HtrA2/Omi down-regulation of XIAP, and the cleavage was attenuated by constitutively active Akt but not dominant negative Akt (Fig. 2B). These results indicate that the serine protease activity of HtrA2/Omi is abrogated by Akt through a kinase-dependent manner.

Akt Inhibits HtrA2/Omi via Phosphorylation by Akt in Vitro and in Vivo—To define the underlying mechanism of Akt inhibition of serine protease activity of HtrA2/Omi, we performed co-immunoprecipitation assays and observed no interaction between HtrA2/Omi and Akt (data not shown). Because HtrA2/Omi contains an Akt phosphorylation consensus site within its protease domain (Fig. 1A), which is conserved between human, rat, and mouse (Fig. 3A), we next investigated whether Akt phosphorylates HtrA2/Omi in vitro and in vivo [32P]orthophosphate cell-labeling experiment was performed in HEK293 cells. Fig. 3B shows that basal phosphorylation level of mature HtrA2/Omi is low. Expression of constitutively active Akt1 and Akt2 considerably induced HtrA2/Omi phosphorylation. To define Akt phosphorylation site of HtrA2/Omi, in vitro kinase assay was carried out using GST-fused wild-type HtrA2/Omi and mutant HtrA2/Omi-S212A as substrates. Repeated experiments revealed that both constitutively active Akt1 and Akt2 highly phosphorylate the wild-type GST-HtrA2/Omi but not HtrA2/Omi-S212A (Fig. 3C), indicating Akt phosphorylation of HtrA2/Omi on serine 212 in vitro.

We next examined if Akt phosphorylates serine 212 of HtrA2/Omi in vivo. HEK293 cells were transfected with wild-type and S212A-mutant HtrA2/Omi together with or without constitutively active Akt1 and Akt2. Immunoblotting analysis with the antibody that specifically recognizes Akt substrate revealed that wild-type HtrA2/Omi, but not HtrA2/Omi-S212A, was phosphorylated by Akt (Fig. 3D). These data indicate that Akt phosphorylates serine 212 of HtrA2/Omi in vitro and in vivo.

Akt Phosphorylates Endogenous Cytoplasmic HtrA2/Omi—To demonstrate if phosphorylation of HtrA2/Omi by Akt occurs at endogenous protein level and if Akt is a bona fide kinase for HtrA2/Omi, we knocked down Akt by infection of OV2008 ovarian cancer cells with lentiviruses expressing shRNA of Akt1. Following incubation for 72 h, the cells were treated with or without cisplatin and Akt inhibitor API-2 (37) and then fractionated. Total and phosphorylated HtrA2/Omi

constitutively active and dominant negative Akt. Similarly, mature HtrA2/Omi effectively cleaved XIAP, and the cleavage was attenuated by constitutively active Akt but not dominant negative Akt (Fig. 2B). These results indicate that the serine protease activity of HtrA2/Omi is abrogated by Akt through a kinase-dependent manner.

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FIGURE 5. Akt inhibition of HtrA2/Omi pro-apoptotic function depends on the phosphorylation of serine 212. A and B, Akt inhibits poly(ADP-ribos)e polymerase (PARP) cleavage induced by mature HtrA2/Omi by non-phosphorylatable HtrA2/Omi-S212A. HeLa cells were transfected with M-HtrA2/Omi-S212A (B) and constitutively active Akt. After 48 h of transfection, poly(ADP-ribose) polymerase cleavage was detected by Western blot (upper panel). Expression of transfected HtrA2/Omi was confirmed by Western blot using anti-Myc (panel 2) and anti-HA (panel 3) antibodies. The blot was rehybridized with anti-actin antibody (panel 4). C and D, constitutively active Akt inhibits wild-type but not non-phosphorylatable HtrA2/Omi cleavage (Fig. 5B). As expected, HtrA2/Omi was released from the mitochondria into the cytoplasm in response to cisplatin treatment in OV2008 cells. Moreover, cytoplasmic level of HtrA2/Omi was further increased by knockdown of Akt or treatment with Akt inhibitor API-2 (Fig. 3E), suggesting that Akt not only phosphorylates endogenous HtrA2/Omi in the cytoplasm but also inhibits its release from the mitochondria.

Akt Inhibits HtrA2/Omi Serine Protease Activity through a Phosphorylation-dependent Manner—Because Akt phosphorylates HtrA2/Omi and inhibits its serine protease activity, we next asked whether the phosphorylation of serine 212 is required for inhibition of HtrA2/Omi protease activity. In vitro protease assay was performed by incubation of different forms of HtrA2/Omi with β-casein or recombinant XIAP in the presence or the absence of Akt. Fig. 4A shows that β-casein and XIAP were largely cleaved by HtrA2/Omi-S212A, a mutant that could not be phosphorylated by Akt. Unlike wild-type HtrA2/Omi whose activity was inhibited by Akt (Fig. 2), the ability of HtrA2/Omi-S212A to cleave β-casein and XIAP was not affected by Akt. In contrast, HtrA2/Omi-S212D, a mutant mimicking phosphorylation, failed to cleave β-casein and XIAP, suggesting that inhibition of HtrA2/Omi protease activity by Akt depends on the phosphorylation of serine 212. A recent study showed that HtrA2/Omi forms the homotrimeric structure that is indispensable for executing its serine protease activity (38). Thus, Akt phosphorylation of serine 212 within the serine protease domain of HtrA2/Omi (Fig. 1A) might interfere with the homotrimeric formation. However, the detailed mechanism needs to be further investigated.

Further, we investigated the effects of Akt phosphorylation of HtrA2/Omi on XIAP expression in living cells. HeLa cells were transfected with different forms of HtrA2/Omi together with or without constitutively active Akt. Immunoblotting analysis revealed that the protein level of XIAP was considerably reduced in the cells expressing mature HtrA2/Omi and XIAP co-precipitates with anti-Akt substrate antibody revealed that the phosphorylation level of XIAP was further increased by knockdown of Akt or treatment with Akt inhibitor API-2 (Fig. 3E). As expected, HtrA2/Omi was released from the mitochondria into the cytoplasm in response to cisplatin treatment in OV2008 cells. Moreover, cytoplasmic level of HtrA2/Omi was further increased by knockdown of Akt or treatment with Akt inhibitor API-2 (Fig. 3E), suggesting that Akt not only phosphorylates endogenous HtrA2/Omi in the cytoplasm but also inhibits its release from the mitochondria.

Furthermore, expression of constitutively active Akt overrode the inhibitory effects of wild-type but not non-phosphorylatable HtrA2/Omi phosphorylation of serine 212 on XIAP expression (Fig. 4B).

Previous studies have demonstrated that the cleavage of XIAP by HtrA2/Omi is also dependent on the interaction between HtrA2/Omi and XIAP (27–29, 32, 35). Therefore, we further examined if Akt phosphorylation of HtrA2/Omi interferes with HtrA2/Omi-XIAP complex formation. A co-immunoprecipitation experiment showed that phosphomimetic and non-phosphorylatable HtrA2/Omi had similar capability of binding to XIAP. Expression of constitutively active Akt had no effect on the interaction (Fig. 4, C and D), indicating that the phosphorylation of serine 212 of HtrA2/Omi does not interfere with its N-terminal conserved IAP-binding motif, V134AVPS137 (Fig. 1A) (25). As a control, we also created HtrA2/Omi-A144G mutant by converting alanine 144 glycine. Consistent with previous finding (27), HtrA2/Omi-A144G failed to bind to XIAP (Fig. 4C). Taken collectively, these data indicate that Akt phosphorylation of HtrA2/Omi at serine 212 is a critical event for attenuation of HtrA2/Omi cleaving XIAP, although it does not affect HtrA2/Omi-XIAP complex formation.
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A

shAkt2: shControl: OVCA3
Akt2  +  +  +
HtrA2  +  +  +
Actin  -  -  -

B

shControl  shAkt2
CDDP: 0 12 24 0 12 24
M-HtrA2  +  +  +  +  +
HtrA2  +  +  +  +  +
Actin  +  +  +  +  +

C

HA-my-Akt: pcDNA:
HtrA2  +  +  +
Akt  +  +  +
Actin  +  +  +

D

Survival factors  Apoptotic stimuli
PKA  HtrA2  IAPs  Caspases
Mitochondria

FIGURE 6. Regulation of endogenous HtrA2/Omi by Akt. A, knockdown of AKT2 expression of Akt by transfection of a sc plasmid into OVCA3 cells was evaluated by Western blot analysis. B, knockdown of Akt2 in OVCA3 cells was accomplished by transfection with a control shRNA or shAkt2 lentivirus. C, expression of Akt inhibitors HtrA2/Omi by transfection with a control shRNA or shAkt2 lentivirus. The bottom panel represents the result from TUNEL assay. D, schematic illustration of Akt regulation of HtrA2/Omi.

different forms of mature HtrA2/Omi. Constitutively active and dominant negative Akt, the cells were transfected with wild-type or mutant Akt alone. Immunoblotting and TUNEL assay demonstrate that ectopic expression of wild-type HtrA2/Omi increased the phosphorylation level of HtrA2/Omi. Cells were treated with cisplatin and/or immunoprecipitated and/or immunoblotted with the indicated antibodies. D, schematic illustration of Akt regulation of HtrA2/Omi.

Accordingly, cisplatin-induced apoptosis was increased in AKT2-knockdown cells (Fig. 6B). To further demonstrate the effect of Akt on HtrA2/Omi release from the mitochondria in response to apoptotic stimulation, A2780S cells, which express low level of Akt and are sensitive to cisplatin, were transfected with constitutively active Akt or pcDNA3 and then treated with or without cisplatin. Immunoblotting analysis of cytoplasmic fraction revealed that expression of constitutively active Akt reduced HtrA2/Omi release from the mitochondria into the cytosol more than 1 fold (Fig. 6C), which is consistent with a recent report (40) showing inhibition of HtrA2/Omi release by expression of constitutively active Akt2 in A2780S cells. Taken collectively, we conclude that Akt abrogates HtrA2/Omi pro-apoptotic function through phosphorylation of serine 212 in cytoplasm and inhibition of HtrA2/Omi release from the mitochondria (Fig. 6D).

HtrA2/Omi as an apoptosis inducer has recently been challenged by a recent study that used HtrA2/Omi knock-out or mutant mice. HtrA2/Omi knock-out mice showed no reduced rate of cell death but suffered loss of a population of neurons in the striatum (41). Furthermore, a mutation in the protease domain of HtrA2/Omi has been identified as a cause of lethal neuromuscular wasting disorder in mnd2 (motor neuromuscular degeneration 2) mice (42). However, a number of reports have shown that, following apoptotic stimuli, premature HtrA2/Omi is cleaved, and the resulted mature HtrA2/Omi is released from the mitochondria into the cytosol where it mediates the programmed cell death through a serine protease-dependent mechanism (24–29, 39, 43–50). Based on those findings, HtrA2/Omi could act on maintenance of mitochondrial homeostasis in normal cells and mitochondrial HtrA2/Omi could have different function from the one in the cytoplasm. In fact, premature HtrA2/Omi localizes at the mitochondria in healthy cells, whereas apoptotic stimuli-induced mature HtrA2/Omi, which is 134 amino acids shorter than its premature form, is detected in the cytosol. The data from others and ours also show that expression of mature but not premature HtrA2/Omi provokes the programmed cell death (Fig. 1). Nevertheless, the data presented in this study demonstrate for the first time that HtrA2/Omi is regulated by phosphorylation. Akt phosphorylates HtrA2/Omi on serine 212 in vitro and in vivo. The phosphorylation of HtrA2/Omi by Akt results in abrogation of its serine protease and pro-apoptotic activities. Future investigations are required for determining the in vivo
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effect of Akt phosphorylation of HtrA2/Omi using an HtrA2/Omi-S212D or/and -S212A knock-in mouse model.

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