Apoptosis Inhibitor 5 is an endogenous inhibitor of caspase-2

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Abstract

Caspases are key enzymes responsible for mediating apoptotic cell death. Across species, caspase-2 is the most conserved caspase and stands out due to unique features. Apart from cell death, caspase-2 also regulates autophagy, genomic stability and aging. Caspase-2 requires dimerization for its activation which is primarily accomplished by recruitment to high molecular weight protein complexes in cells. Here we demonstrate that Apoptosis Inhibitor 5 (API5/AAC11) is an endogenous and direct inhibitor of caspase-2. API5 protein directly binds to the caspase recruitment domain (CARD) of caspase-2 and impedes dimerization and activation of caspase-2. Interestingly, recombinant API5 directly inhibits full length but not processed caspase-2. Depletion of endogenous API5 leads to an increase in caspase-2 dimerization and activation. Consistently, loss of API5 sensitizes cells to caspase-2-dependent apoptotic cell death. These results establish API5/AAC-11 as a direct inhibitor of caspase-2 and shed further light onto mechanisms driving the activation of this poorly understood caspase.
Two Sentence Summary

Apoptosis Inhibitor 5 (API5) has been identified to be a direct and endogenous inhibitor of initiator caspase-2. API5 directly binds to the CARD domain and prevents caspase-2 dimerization.

Key findings of the study

- API5 is an endogenous inhibitor of caspases-2
- API5 directly binds to the CARD domain of caspase-2 and prevents dimerization
- API5 loss sensitizes cells to caspase-2-dependent apoptotic cell death
Introduction

Normal development and tissue homeostasis are regulated by apoptosis, a form of Programmed Cell Death (PCD) defined by unique morphological and biochemical features [1]. Caspases are key executioner enzymes of apoptosis and depending on the chronology of activation, they are classified into initiator and effector caspases [2]. Caspases cleave hundreds of substrates to elicit an apoptotic phenotype and thus the activation of caspases is tightly controlled. Inhibitor of Apoptosis proteins (IAPs) are thus far the only known endogenous inhibitors of caspases as they can directly bind to caspases and prevent their activation [3]. Initiator caspases are activated by dimerization, which is accomplished by recruitment of caspase monomers to multimeric protein complexes assembled in cells in response to apoptotic stimuli [4]. For instance, caspase-8 is recruited and activated in the Death Inducing Signaling Complex (DISC) and caspase-9 is recruited to the apoptosome for activation [5]. Caspase-2 is a unique member of the caspase family as it exhibits features of both initiator and effector caspases [6]. More recent studies revealed other functions of this caspase: it suppresses tumours, plays a role in autophagy and regulates metabolism and aging (for reviews see [7,8]). In response to DNA damage, caspase-2 is recruited to a high molecular weight complex called PIDDosome which contains PIDD (p53-induced protein with a death domain DD) and the adapter protein RAIDD (receptor-interacting protein-associated ICH-1/CED-3 homologous protein with a DD) [9]. However, recent evidence suggests that caspase-2 can be activated in a PIDDosome-independent manner [10]. We have previously shown that caspase-2 functions as an initiator caspase during pore forming toxin (PFT)–mediated apoptosis in a variety of cell types [11]. In these settings, the activation of caspase-2 is PIDDosome-independent and critically dependent on the intracellular potassium ion concentration. Depletion of intracellular potassium ions by PFTs led to oligomerization and recruitment of caspase-2 into a high molecular weight complex thus leading to its activation.
In our attempts to uncover the molecular determinants that drive the activation dynamics of caspase-2 in PFT-mediated apoptosis, we identified API5/AAC-11/FIF (Anti-apoptosis clone 11 and Fibroblast growth factor-2 interacting factor) as a novel inhibitor of caspase-2. API5 was originally discovered as a protein responsible for protecting cells against growth factor deprivation-induced cell death [12]. API5 can also bind to acinus, ALC1 and FGF2 regulating cell death and tumorigenesis [13-16]. Structurally, API5 comprises of multiple helices constituting HEAT (Huntington, Elongation Factor 3, PR65/A, TOR)-like and ARM (Armadillo)-like repeats that mediate protein-protein interactions [17]. API5 also inhibits E2F1-dependent apoptosis in a transcription-independent manner [18]. Recent studies revealed that API5 is highly expressed in various cancers and associated with poor prognosis especially in NSCLCs and cervical cancer, however, the molecular mechanisms behind API5-mediated cell survival remains poorly understood [19,20]. Here, we reveal that API5 directly binds to the CARD domain of caspase-2 and prevents its dimerization and activation. Loss of API5 thus sensitizes cells to caspase-2 dependent apoptotic cell death.

**Results and discussion**

Caspase-2 functions as an initiator caspase during PFT-mediated apoptosis in multiple cell types [11]. To identify proteins that regulate caspase-2 activation in these settings, we have performed a mass spectrometry-based analysis of active caspase-2-containing protein complexes. HeLa cells treated with biotin-VAD-fmk were subjected to α-toxin treatment and proteins bound to biotin-VAD-fmk were precipitated by streptavidin beads. We subjected the proteins bound to the streptavidin beads to trypsin digestion and performed mass spectrometry analysis. As expected, caspase-2 peptides were identified and among the other co-precipitated proteins, we identified four unique peptides with six peptide spectrum matches for Apoptosis Inhibitor 5 (Fig. 1a). We confirmed the presence of API5 in active caspase-2-containing complexes by
immunoblots (Fig. 1b). As shown before [14,19], API5 antibodies detect a double band in HeLa cells recognising both the 55 and 57 KDa isoforms (Fig.1b). Gel filtration analysis revealed that API5 and caspase-2 are shifting towards high molecular weight fractions in PFT-treated cells (Fig. 1c). Together, these results confirmed that API5 is probably associated directly with the activation of caspase-2. We then explored if API5 plays a role in the regulation of cell death mediated by PFTs. Immunoblot analysis of PFT-treated cells revealed that API5 tends to be degraded in apoptotic cells (Fig. 2a). To decipher the physiological role of API5 in regulating caspase-2 activation, we resorted to both siRNA and shRNA-mediated loss of function approaches. As expected, stable knockdown of API5 sensitized HeLa cells to PFT-mediated cell death (Fig. 2b and 2c). These results are recapitulated with two different siRNAs targeting API5 (Fig. 2d and Supplementary Fig. S1a and S1b). Consistently, enhanced PARP cleavage is detected in API5-depleted cells as early as 9 h post induction (Fig. 2d). As expected, enhanced caspase-3/7 activity was detected in API5-depleted cells treated with PFT (Supplementary Fig. S1c). Long-term clonogenicity and cell survival assays with API5-specific shRNAs further confirmed that depletion of API5 significantly reduced the survival of PFT-treated HeLa cells (Figs. 2e-2g). API5-depleted cells were sensitized to PFT but not to TNFa/CHX, staurosporine, camptothecin, etoposide, cisplatin and brefeldin A (Figs. 3a and 3b). To avoid any cell type-specific phenotypes, we performed similar experiments in NCI-H1650 lung carcinoma cell lines. Depletion of API5 strongly sensitized these cells to PFT- but not to TNFa/CHX-, camptothecin- or etoposide-induced cell death (Supplementary Fig. S2a-c). We then tested whether API5 directly influences caspase-2 activation in vivo. Caspase-2 possesses a CARD domain at its N-terminus and CARD-mediated dimerization is required for its initial activation
This initial activation step is followed by auto-processing leading to a mature caspase-2 dimer containing two P19 and P12 subunits [7]. We tested whether caspase-2 dimerization and/or activation are enhanced in response to PFTs in API5-depleted cells by employing the “in situ active caspase trapping” approach [22,23]. As expected, experiments employing biotin-VAD revealed that more active, dimerized caspase-2 was detected in API5-depleted cells after PFT treatment (Fig. 4a). Caspase-2 activity assays employing a fluorogenic substrate (VDVAD-AFC) also confirmed increased caspase-2 activation in API5-depleted cells in response to PFTs (Supplementary Fig. S3a). Further, we also detected enhanced caspase-2 processing in response to PFT in API5-depleted cells (Fig. 3a, 4b and Supplementary Fig. S3b). As caspase-2 processing can also happen downstream of effector caspase activation [24], we have reconfirmed whether caspase-2 activation is triggered in the absence of caspase-3/7 in response to depletion of potassium ions as with PFTs [25]. To perform these experiments, we have employed valinomycin as MEFs are unresponsive to α-toxin. As expected, caspase-2 activity was triggered in Caspase3/7 double knockout MEFs in response to valinomycin (Supplementary Fig. S4).

We then tested if the sensitization to cell death observed in API5-depleted cells is dependent on caspase-2. Consistently, co-knockdown of caspase-2 reduced the sensitization to apoptosis in API5-depleted cells suggesting a role for API5 in inhibiting caspase-2-mediated cell death (Figs. 4c and 4d). In addition, we explored the potential mechanisms by which API5 can regulate caspase-2 dimerization. As API5 possesses HEAT repeats and ARM-like repeats, we tested if API5 can directly interact with caspase-2. Flag pulldown experiments employing recombinant API5 and in vitro-translated caspase-2 produced from rabbit reticulocyte lysates revealed a direct
interaction between API5 and caspase-2 (Fig. 5a). Interestingly, deletion of 50 amino acids at the C-terminus of API5 (API5 1-454=API5ΔC) abrogated this interaction (Fig. 5a). As API5 directly binds to caspase-2 and regulates caspase-2 dimerization, we hypothesized that API5 can directly bind to the CARD domain, which is responsible for driving this dimerization event. *In vitro* binding experiments, employing recombinant proteins confirmed a direct interaction between the CARD domain of caspase-2 and API5 (Fig. 5b). As expected, API5ΔC failed to interact with the CARD domain of caspase-2. As CARD domains are also present in other caspases like caspase-9 and caspase-1, we tested if API5 can bind to them. Interestingly, we failed to detect any interaction between API5 in caspase-9 or caspase-1 (Supplementary Fig. S5a and S5b). Consistently, loss of API5 failed to sensitize cells to caspase-9-dependent cell death (Fig. 3b). Next, we tested if recombinant API5 can directly inhibit the activity of fully processed recombinant caspase-2, which is devoid of CARD domains. Incubation of increasing concentrations of API5 failed to inhibit the activity of fully processed caspase-2 (Fig. 5c). To further confirm the role of API5 in inhibiting dimerization of the CARD domains directly, we performed *in vitro* reconstitution experiments employing full-length caspase-2 purified from rabbit reticulocyte lysates. As expected, presence of API5 directly prevented the dimer formation between Flag-tagged caspase-2 and His-tagged caspase-2 (Fig. 5d). To further confirm these observations, we have performed additional gain of function experiments. Consistently, overexpression of API5 inhibited the activity of co-expressed caspase-2 in a concentration-dependent manner (Fig. 5e). Taken together, these data suggest that API5 is a direct inhibitor of caspase-2 and that API5 directly binds and impedes CARD-mediated dimerization and activation (Fig. 5f).
IAPs are known to inhibit caspases (casp-8/9 and casp-3/-7) directly through their BIR (Baculoviral IAP Repeat) domains and RING domains and direct inhibitors of caspase-2 are thus far unknown [26]. Caspase-2 was cloned in 1994 and despite intense research, the molecular machinery driving the activation of this caspase remains unclear [27,28]. Though the PIDDosome serves as an activation platform during DNA damage-mediated apoptosis, caspase-2 can also be activated in the absence of PIDD and RAIDD [10]. We have demonstrated that caspase-2 can be recruited to a high molecular weight complex in a PIDD- or RAIDD-independent manner in response to PFTs in human epithelial cells [11]. Here, by employing mass spectrometry, we identified API5 as a novel inhibitor of caspase-2. While cleavage is essential for effector caspase activation, initiator caspases depend on dimerization of caspase monomers but not on cleavage for activation [5]. Initiator caspases are brought to close proximity in multimeric protein complexes leading to their activation and auto-processing. CARD domain-containing proteins exhibit homophilic interactions with other CARD domain-carrying proteins leading to the formation of high order multimers in cells [29]. Thus, binding of CARD domain-containing caspases with adaptor molecules like RAIDD or APAF1 leads to an increased local concentration of the initiator caspases resulting in their dimerization and activation. Here, we present one of the first inhibitors of caspases that directly interferes with the CARD-CARD interaction both in vitro and in vivo. Our in vitro binding experiments with recombinant proteins reveal that the C-terminus of API5 is required for interaction with the caspase-2 CARD domain. However, it is currently unclear if the C-terminal fragment drives the interaction or if the deletion of the API5 C-terminus leads to an altered conformation of API5 that fails to interact with CARD domains. Interestingly, we could not detect any interaction with the CARD domain of caspase-9
or caspase-1. Thus, further biochemical and structural analyses are clearly warranted to characterize the interaction between API5 and caspase-2. API5 has also been shown to be upregulated in several cancers and our studies raise the possibility that inhibition of caspase-2 activation could possibly contribute to the pro-tumorigenic role of API5 and chemoresistance. Interestingly, API5 is degraded in apoptotic cells (Fig 2a and 3a) and further studies are warranted to uncover the mechanisms driving this degradation. Further, caspase-2 was originally identified to contribute to growth factor deprivation-induced apoptosis, a process directly inhibited by API5 overexpression [30]. Our preliminary data suggest that loss of API5 also sensitizes tumour cells to starvation-induced cell death (GI and KR unpublished observations). However, it is currently unclear if caspase-2 is directly involved in this process. The role of caspase-2 in mediating DNA damage- or ER-stress-mediated apoptosis remains contentious and we detect that loss of API5 primarily sensitizes cells to PFT but not to other inducers of apoptosis (Fig. 3 and Supplementary Fig. S2). API5 has also been shown to undergo phosphorylation and it would be interesting to explore if posttranslational modifications determine the binding of API5 to caspase-2 in cells [31]. Taken together, our study reveals a novel mechanism (direct disruption of CARD-mediated dimerization) regulating initiator caspase activation and identifies one of the first direct inhibitors of caspase-2. The study sheds further light on the mechanisms driving the activation of caspase-2, a poorly defined member of the caspase family.

Materials and methods

Cell culture and apoptosis induction

HeLa cells were cultured in RPMI-1640 medium and A549, NCI-H1650, Caspase-2/− and Caspase-3/7 DKO MEFs were cultured in DMEM (both Gibco BRL), both
supplemented with 10% FCS (Gibco BRL), and 0.2% penicillin (100 U/ml) / streptomycin (100 µg/ml) (Gibco BRL) and 2 mM L-glutamine at 37 °C in 5% CO2.

The cell death experiments were performed with purified alpha-toxin (α-haemolysin from *Staphylococcus aureus*) (Sigma) for various time points. Staurosporine (Cell Signaling), cisplatin (Uniklinik Mainz), etoposide (Sigma), camptothecin (Selleckchem), TNFα (R&Dsystems), cycloheximide (Sigma), brefeldin A (Selleckchem), valinomycin (Sigma).

**shRNA-mediated RNA interference by lentiviral particles**

In order to achieve a stable knockdown, HeLa cells were seeded in 96-well plate format at 40,000 cells/100 µl medium. The next day, the medium was changed to polybrene (Hexadimethrine bromide, final concentration 8 µg/ml)-containing medium and the cells were infected with shRNA-carrying lentiviral particles (SIGMA) at an MOI of 5. The medium was changed 24 hours later. At 48 h post infection the cells were trypsinized and re-suspended in puromycin containing medium and seeded into 12 well plates. To avoid clonal-specific effects, a pool of infected cells was used for the subsequent experiments after validating the knockdown of the respective genes by western blot analysis. The following sequences were employed for accomplishing knockdown:

Caspase-2 #3 (TRCN0000003507):

5’CCGGGTTGAGCTGTGACTACGACTTCTCGAGAAGTCGTAGTCACAGCTCA
ACTTTTT-3’

API-5 #5 (TRCN0000294026):

5’CCGGGGAGAAGAGTAATGGTCAATCCTCGAGGATTGACCATTACTCTTTCT
CCTTTTTG-3’

ShControl (SHC002V): non-target shRNA control transduction particles.
**SiRNA transfection**

Cells were seeded into 12-well plates to obtain 50–60% confluency the next day. *Lipofectamine RNAiMax* (Invitrogen) transfection reagent was used to achieve efficient siRNA transfection (final DNA concentration: 60 nM). The next day, the medium was changed and cells were treated with α-toxin. Approximately a day later, the transfected samples were subjected to various treatments (like apoptosis induction) as specified in the figure legends. The following siRNAs provided efficient knockdowns: siAPI5#1 (SI02225580, QIAGEN), siAPI5#4 (Hs02_00341902, SIGMA).

**Cell death assays**

After the respective treatments, the cells (0.5x10⁶ cells) were collected by trypsinization. Samples were washed once with PBS and then resuspended in 100 μl of 1X annexin binding buffer provided by the manufacturer (ENZO), and 5 μl of Annexin-V-FITC stock solution and 1 μg/ml (final concentration) propidium iodide (PI) (Sigma) were added to the cells and incubated for 15 min. After incubation, the stained samples were measured by flow cytometry. The cell debris was excluded from analysis. For the flow cytometry analysis, the following channels were used: FITC-FL1 channel (488-nm blue laser/530-nm band-pass filter), PI-FL2 channel (488-nm blue laser/585-nm band-pass filter; BD FACSCanto I and II, Becton Dickinson). For long-term clonogenicity assays, 1x10⁵ Shcontrol and shAPI5 knockdown cells were seeded into 12-well plates and treated with 300 ng/ml of α-toxin for 24 h. The dead cells were washed away and the surviving cells were allowed to replicate for 2 days to test their clonogenic capacity. The cells were labelled using crystal violet solution and pictures of the clones were
taken from various fields. For testing cell viability, 1x 10^4 control and API5 knockdown cells were seeded into 96-well plates and treated with 300 ng/ml α-toxin for 24h. The dead cells were washed away and the surviving cells were labelled with crystal violet solution. The crystal violet was then eluted using 33% acetic acid and the absorbance at 590 nm was measured. The relative cell viability was calculated by setting the cell viability of the untreated cells as 1.

**Biotin-VAD pull-down of activated caspases**

HeLa cells were seeded into 6-well plates and were grown until 80–90% confluency. They were treated with 50 μM biotin-VAD (MP Biomedicals, Enzyme System Products) for 1 h and then 300 ng/ml of α-toxin was added to the cells. The cells were incubated for various time points and then collected by scraping. The collected cells were washed with PBS and resuspended in 500 μl KPM buffer (50 mM KCl, 50 mM HEPES, 10 mM EGTA, 1.92 mM MgCl₂, pH 7.0, 1 mM DTT and 1X protease inhibitor cocktail (Roche)). The cells were lysed by repeated freezing and thawing (3X) and centrifuged for 10 min (15,000 g, 4°C). 50 μl of the supernatant was collected as loading control. The rest of the sample (450 ml) was incubated with 30 μl of streptavidin agarose beads (Invitrogen) at 4°C on a vertical rotator overnight. The next day, samples were centrifuged (2,300 rpm for 3 min at 4°C) and the supernatant was thoroughly discarded. The beads were resuspended in 500 μl of KPM buffer. This step was repeated three times. Finally, the beads were resuspended in 60 μl of 5X Laemmli buffer with 5% beta-mercaptoethanol, were boiled and subjected to SDS–PAGE for subsequent immunoblot or mass spectrometric analysis. The mass spectrometry analysis was essentially performed as described earlier [11].
**Immunoblot analysis**

Cells were lysed directly in 5X Laemmli (with 5% beta-mercaptoethanol) buffer and the proteins were separated by SDS–PAGE. The proteins were transferred to nitrocellulose membranes (90 min, 860 mA, RT) and the presence of proteins was monitored by immunoblot analysis following standard procedures. The following primary antibodies were employed: anti-caspase-2 (clone 11B4, Alexis), anti-API5, anti-Caspase-9, anti-PARP, anti-His (Cell Signaling Technologies) and anti-Actin (Sigma).

**Fluorescence time-lapse microscopy**

HeLa cells were plated into 12-well plates. Immediately after treatment, the cells were stained with either Caspase 3/7 reagent (Essen Bioscience), alternatively with YOYO-1 (Life Technologies) and Magic Red Caspase-3/7 Detection Kit (Immunochemistry Technologies) following the manufacturer’s instructions. The plates were incubated in the IncuCyte Zoom automated microscopy system (Essen BioScience), which enabled us to conduct the measurements inside a cell culture incubator (37°C, 5% CO2) in order to ensure stable conditions for long term measurements. Images were taken with a digital camera attached to the microscope at various time points (green channel: Caspase-3/7 and YOYO-1; red channel: Magic Red Caspase-3/7; phase contrast channel) with 10x objective.

**In vitro translation**

*In vitro* translation assays were performed using the *TNT Coupled Reticulocyte Lysate* (Promega) by following the manufacturer’s instructions. Briefly, 1 µg of pCDNA3-
caspase-2-Flag(C) (Addgene #11811, kind gift of Prof. Guy Salvesen), pET-53-DEST-
caspase-2-His(N) or pCDNA3-API5-T7 (kind gift from Dr. Jean Luc Poyet), was mixed
with T7 polymerase, rabbit reticulocyte lysate (25 µl), amino acid cocktail, RNase
inhibitor and buffer provided by the manufacturer. The samples were incubated 30°C
for 90 minutes and subsequently frozen at -80°C.

**In vitro pulldown experiments**

The DNA encoding the CARD domain (amino acid 15-104) of human caspase-2 was
amplified with PCR and cloned into pGEX4T vector (GE Healthcare) using BamHI and
XhoI restriction sites generating a N-terminal GST-fusion construct. The recombinant
protein was overexpressed in *E. coli* BL21_pLysS (Novagen). The cells were grown at
37°C in LB medium supplemented with 50 µg ml⁻¹ ampicillin. Protein expression was
induced by adding 0.5 mM isopropyl-B-D-1-thiogalactopyranoside (IPTG) when the
cells reached an optical density at 600 nm of about 0.5 and cell growth continued for 20
h at 18°C.

The pellet was resuspended in buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 1 mM
EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and disrupted by sonication
on ice. The lysate was centrifuged at 18,000 rpm for 1 h at 4°C. The supernatant was
loaded onto GST-binding resin (Novagen) using an open column which had previously
been equilibrated with buffer A (20 mM HEPES pH 7.5, 200 mM NaCl, 1 mM EDTA,
1 mM DTT) and subsequently was washed with buffer A. The protein was eluted with
buffer A containing 20 mM reduced L-glutathione. Product homogeneity of the purified
protein was determined by SDS-PAGE under denaturing conditions using 12% (v/v)
polyacrylamide gels. The protein concentration was determined using the Bradford method with bovine serum albumin as a standard.

API5 His-tag protein was purified by a Ni\(^{2+}\)-chelated NTA Agarose (Qiagen) using an open column. GST-CARD protein and GST protein itself were purified by GST resin (Novagen) using an open column. The elution buffer of the purified proteins (API5, CARD and GST) was changed to buffer B (20 mM HEPES pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.0025% tween-20) using an Amicon Ultra-15 ultrafiltration device. The proteins were concentrated to 1 mg ml\(^{-1}\). The GST-CARD tag protein and GST protein were immobilized on GST resin by incubating with GST resin which had previously been equilibrated with buffer B for 90 min at 4\(^{\circ}\)C. After incubation, these columns were washed 3 times with buffer B using open columns. The API5 protein was loaded onto GST-CARD tag protein-bound GST resin and GST protein-bound GST resin, and washed 3 times with buffer B. The bound proteins were eluted with buffer B containing 20 mM reduced L-glutathione. The eluted proteins were visualized using coomassie blue staining or Western blot analysis.

The \textit{in vitro} translated samples were mixed with recombinant API5 and re-suspended in 300 µl lysis buffer (50 mM TRIS HCl, 150 mM NaCl pH 7.4, 1 mM EDTA, 0.5% triton x-100). First, 30 µl of the lysate was taken as loading control. Subsequently, 20 µl of equilibrated \textit{ANTI-Flag M2 Magnetic Beads} (SIGMA) were pipetted into tubes containing 270 µl of lysate. The samples were incubated overnight at 4\(^{\circ}\)C on a vertical rotator. Next day, the samples were placed in a magnetic separator and washed 3 times with TBS buffer (50 mM TRIS HCl, 150 mM NaCl pH 7.4). The washed magnetic beads were resuspended in 5X Laemmli sample buffer and boiled for 3 minutes. Finally, the eluates were collected into a separate tube and stored at -20\(^{\circ}\)C until analysed by SDS
PAGE and subsequent Western blotting. For testing the interaction between Caspase-1/9 and API5, 293T cells were transfected with FLAG tagged Caspases (Caspase-9 and Caspas-1 obtained from Sinobiological). The caspases thus produced were precipitated by FLAG beads and the interaction with API5 is tested with recombinant API5 proteins as described above.

**Caspase-9 immunoprecipitation**

Hela cells were harvested at 80% confluency (2 wells of a 6-well plate/sample) and resuspended in 500 μl RIPA buffer (50 mM Tris HCl, pH 7.5, 250 mM NaCl, 10% Glycerol, 1% Triton X-100 with protease inhibitor cocktail (ROCHE) and 1 mM DTT. The samples were lysed by repeated freeze-thaw cycles (3) and centrifuged for 10 min at 15,000 g and 4 °C. The supernatant was collected and 50 μl was taken as input. The rest of the samples was mixed with 4.5 μl of caspase-9 antibody (Cell Signalling) and incubated overnight at 4 °C in a vertical rotator. On the next day 20-30 μl of agarose A and G beads (Roche) were mixed with the samples and incubated at 4 °C for 2 h on a vertical rotator. The beads were washed three times in RIPA buffer and re-suspended in 5x Laemmli sample buffer (with 5% beta-mercaptoethanol).

**Caspase-2 activity assay**

Depending on the experiment, either 1x10⁶ cells or purified recombinant proteins (human recombinant caspase-2 (ENZO) with human recombinant API5) were resuspended in 50 μl ice cold lysis buffer and incubated on ice for 10 minutes. Next, 50 μl 2x Reaction buffer (with freshly added DTT, 1mM) was pipetted and finally 5 μl of caspase-2 fluorometric caspase substrate (VDVAD-AFC, final concentration 50 μM)
was mixed with the sample (*Caspase-2 Fuorometric assay kit, ENZO*). The mixtures were incubated in a flat bottom 96-well plate at 37 °C for various time points and the fluorescence intensity increase was detected using a Fluorometric Plate Reader (Victor X, Perkin Elmer).

**Size exclusion chromatography from endogenous samples**

HeLa cells (10x75 cm² flasks at 80% confluency) were treated with α-toxin and harvested by scraping at 4 hours post treatment. The control and treated samples were resuspended in hypotonic buffer and lysed by repeated freeze-thaw cycles (3x). Next, the lysates were centrifuged and the supernatant was collected in a separate tube. The samples were kept at 4°C and were subjected to size exclusion chromatography (column: sephacryl HR500 (GE)). The entire protocol was described previously[11].

**Statistical analysis**

Student’s t-test was performed to test for statistical significance of the results (*P=0.05, **P=0.01, ***P=0.005). The sample size (number of experiments) has been indicated in the figure as n.

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**Figure legends**

**Figure 1** (a) Mass spectrometry analysis of active caspase-2 complexes. HeLa cells were pre-incubated with biotin-VAD-fmk (B-VAD, 50 μM) for an hour and the cells were subsequently treated with 300 ng/ml of α-toxin as mentioned in the methods. The cells were then harvested and the active caspase-2 complexes were precipitated by streptavidin agarose beads. The entire sample was subjected to trypsin digestion and the proteins were identified by mass spectrometry. Shown is the MS/MS spectra of one of the API5 peptides identified. (b) The presence of API5 in caspase-2 complexes was verified by immunoblot analysis. (c) Gel filtration analysis of control and PFT-treated HeLa cell lysates. HeLa cells were treated with α-toxin (150 ng/ml) for 4 h. Then, the cells were harvested, lysed and the crude protein extract was prepared for gel filtration analysis. The proteins were separated by size exclusion chromatography as detailed in the methods section. The proteins from each collected fractions were precipitated and the presence of proteins of interest was tested by western blot analysis. The individual fractions are indicated.

**Figure 2.** (a) API5 is degraded during PFT-mediated apoptosis. HeLa cells were treated with α-toxin (300 ng/ml for 24 h) and the presence of API5, caspase-2, PARP was tested by immunoblots. Ponceau-stained membrane is presented below to verify the loading. (b) API5 depletion sensitizes HeLa cells to PFT-mediated apoptosis. ShControl and shAPI5 HeLa cells were treated with α-toxin (600 ng/ml for 24 h) and the percentage of cell death was analysed by FACS. The annexinV-PI staining pattern of control and toxin treated cells from a representative experiment is presented. Shown in (c) is the quantification of experiments presented in (b) (n=3, P=0.0286). (d) HeLa cells were
transfected with siRNAs (siControl or siAPI5#4) for one day prior to α-toxin treatment. The cells were harvested for Western blot analysis at different time points (as indicated). FL: full length, *: processed PARP (e) HeLa cells were treated for 24 h with α-toxin (300 ng/ml) and the dead cells were washed away. The surviving cells were allowed to replicate for 48 h to check for clonogenic survival. Shown are data from a representative experiment (f). For testing the viability of the cells, the crystal violet assay was performed on both control and shAPI5 treated with toxin and the surviving cells were quantified. The error bars represent the mean ± S.D. (n=3). (g) The efficiency of the shAPI5 was verified by Western blot and vinculin was employed as a loading control.

Figure 3. Depletion of API5 sensitizes HeLa cells to PFT, but not to other inducers of apoptosis. ShControl and shAPI5 cells were treated for 24h with α-Toxin (150 ng/ml), Staurosporine (125nM), TNFα (20ng/ml) +CHX, Camptothecin (4µM), Etoposide (50µM), Cisplatin (40µM), Brefeldin A (5µM) for 24H. Cells were harvested and labelled with propidium iodide for cell death assay (b) or lysed for Western Blot analysis (a). The Ponceau staining of the entire membrane is shown below. For (b) n=3 two way anova with a bonferroni test- p value<0,001.

Figure 4. (a) Depletion of API5 enhances caspase-2 dimerization and activation. shControl and shAPI5 HeLa cells were incubated with biotin-VAD-fmk (B-VAD, 50 µM) 1 h prior to α-toxin (300 ng/ml) treatment and incubated for 18 h. The active caspase-2 complexes were precipitated with Streptavidin-agarose beads as mentioned in the methods section. The samples were then tested for the presence of active caspase-2 and API5 by immunoblot analysis. The relative intensity of caspase-2 bands was measured by image J software and is indicated below the bands. (b) HeLa cells were
transfected with siRNAs and 24 hours later they were challenged with α-toxin at different concentrations and the processing of caspase-2 was monitored by immunoblots. FL: full length caspase-2. (c) Microscopy analysis of API-5 and caspase-2 depleted cells upon α-toxin treatment. shControl and shCaspase-2 HeLa cells were transfected with siRNAs and 24 h later, the cells were pre-incubated with fluorescent magic red caspase substrate and green fluorescent YOYO-1 for 30 min following the manufacturer’s instructions. The cells were treated with α-toxin (300 ng/ml) for 24 h. The images were acquired at 12 h post treatment in the IncuCyte imaging system. (d) ShControl and shCaspase-2 HeLa cells were transfected with siRNAs and one day later the cells were challenged with α-toxin (300 ng/ml). One set of samples was subjected to an in vitro caspase-2 activity assay measurement by plate reader (see S3) while the second set of samples were prepared for annexin-V/PI measurements by FACS as mentioned in the methods.

Figure 5. (a) API5 directly interacts with caspase-2. In vitro translated Flag-tagged caspase-2 was incubated with recombinant API5 and its truncated versions for 2 h and caspase-2 was pulled down with Flag-M2 beads. The samples were eluted and subjected to western blot analysis to check for binding between caspase-2 and API5. 11B4 refers to the antibody clone raised against caspase-2. The various constructs of API5 and their respective amino acid compositions are indicated above. FL: full length, aa: amino acid. (b) The interaction between recombinant API5 and the CARD domain of caspase-2 was tested by GST pulldown experiments as indicated in the methods section. The samples were eluted and separated via SDS-PAGE and stained with coomassie blue. The red arrow indicates the anticipated size of API5. The green arrow shows the anticipated size of caspase-2-GST-CARD. PD: pull down. (c) Human recombinant caspase-2 was
incubated with different concentrations of human recombinant API5 (1x=100 ng) for 1 h at 37°C and subjected to caspase activity measurement using a fluorescent plate reader as mentioned in the methods. (d) In vitro translated caspase-2-Flag and caspase-2-His were co-incubated with increasing concentrations of in vitro translated API5 protein for 30 min and caspase-2-Flag was immunoprecipitated. IP FLAG: co-immunoprecipitation, input: total sample before IP. FL: full length. (e) API5 overexpression inhibits caspase-2 activity. 293T were transected with caspase-2 with increasing amounts of API5 for 48H. Cells were then collected and treated with FAM-VDVAD-FMK-FLICA for measuring caspase 2 activity following manufacturer’s instructions (ImmunoChemistry). (f) Schematic view of API5-mediated inhibition of caspase-2 dimerization and activation. N: N-terminus, C: C-terminus of the protein.
Supplementary Figure legends

Figure EV1. (a) HeLa cells were transfected either with control or API5 siRNAs for one day and then treated with 300 ng/ml of PFT for 24 h. The cells were harvested and the dead cells were measured by FACS analysis after annexin v-PI staining as detailed in the methods. Shown in (b) are data from three (n=3; left panel) or four (n=4, right panel) independent experiments, the error bars represent ±SD of the mean. The dead cells include annexin-V positive early apoptotic as well as annexinV-PI double positive cells, indicating the late apoptotic/secondary necrotic populations as analysed by flow cytometry. The efficiency of the knockdown with siRNA#1 was monitored by immunoblots (upper panel insert). (c) Microscopy analysis of API-5 depleted cells upon α-toxin treatment. HeLa cells were transfected with siRNA and treated with PFT as mentioned before. The cells were treated with in situ Caspase-3/7 substrate (green) for 30 min as mentioned in the methods. The images were acquired after 6 h post toxin treatment.

Figure EV2. NCI-H1650 cells (lung adenocarcinoma) cells were transfected with control or API5 siRNAs employing Saint Red reagent. After 24 h, the cells were treated with α-Toxin (150 ng/ml), TNFα (20 ng/ml) + CHX, camptothecin (4μM) or etoposide (50μM) for 24 h. Cells were harvested and labelled with propidium iodide for analyzing cell death by (a and b) FACS analysis n=3 left, n=1 right. or for (c) western blot analysis.
Figure EV3. (a) Measuring caspase-2 activity. ShControl and shCaspase-2 HeLa cells were transfected with siRNAs and 24 hours later they were challenged with α-toxin (300 ng/ml). 24 hours post-treatment, the samples were subjected to *in vitro* caspase-2 activity measurement as indicated in the methods and following manufacturer’s instructions. (b) The cells were treated as above and 24 hours later were subjected to western blot analysis. FL- full length, *-processed form.

Figure EV4. Wild type or caspase 3/7 double KO MEFs were treated with valinomycin (30μM) for 24 h. Cells were then collected and treated with FAM-VDVAD-FMK-FLICA reagent. Caspase-2 activity was measured by FACS analysis following the manufacturer’s protocol (ImmunoChemistry). Shown are data from a single representative experiment.

Figure S5. (a) HeLa cells were incubated in serum-free EBSS (SI-starvation induced) media or treated with α-toxin and the samples were lysed and subjected for caspase-9 immunoprecipitation (IP) (see materials and methods) 24 hours post-treatment. The total lysates and the immunoprecipitated-eluted samples were analysed by western blot. (b) 293T cells were transfected with pCMV2-Flag-Caspase-1 or pCMV3-Flag-Caspase-9 for 48 h. The FLAG-tagged caspases were precipitated by anti-FLAG-magnetic beads. Magnetic beads were washed 5 times and then incubated overnight at 4°C with recombinant API5. After washing (3X) the beads were re-suspended in SDS buffer and subjected to western blot analysis.
Figure 1

(a) Mass spectrum showing relative abundance of API5, ion score 49, m/z 503.763, 2+. The peaks are labeled as y2, y3, y4, y5, and y6.

(b) Western blot analysis showing the effects of α-toxin on Caspase-2. The blot shows bands at 50 KDa for B-VAD and Input, with an additional band of 50 KDa for API5.

(c) Analysis of fractions 6 to 17 for Control and α-toxin treated samples. The Western blot shows bands at 63 KDa and 48 KDa for 150 ng/ml α-toxin treated samples, with bands at 35 KDa for Control samples. The bands are labeled as API5 and Caspase-2.
Figure 2

(a) Western blot analysis showing the expression levels of API5, Caspase-2 FL, Caspase-2*, PARP, PARP*, and Vinculin in the presence of α-toxin.

(b) Flow cytometry analysis of PI fluorescence and Annexin-V binding in cells treated with α-toxin.

(c) Bar graph showing the percentage of cell death for shCo and shAPI5 #5.

(d) Western blot analysis of API5, PARP FL, PARP*, and actin at different time points (4h, 9h, 24h).

(e) Microscopy images of cell morphology under shControl and shAPI5 conditions with and without α-toxin.

(f) Graph showing relative cell viability for shCo and shAPI5 #5.

(g) Western blot analysis of API5 and Vinculin at different molecular weights.
Figure 3

(a) Western blot analysis showing the expression of API5, PARP FL, PARP*, Caspase 2 FL, and Caspase 2* under different conditions.

(b) Bar graph depicting the relative cell death (fold) for shCo and shAPI5 #5 treatments with various compounds: α-Toxin, STS, TNFα/CHX, CPT, Eto, Cis, and BrefA.
Figure 4

(a) 

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<tr>
<td>-</td>
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B-VAD pull down

50 KDa

50 KDa

50 KDa

API5

(b) 

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50 KDa

35 KDa

Caspase-2 FL

Caspase-2 processed

Caspase-2

Ponceau

(c) 

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<thead>
<tr>
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| shControl | shCasp-2
| shCasp2 | shCasp2 |
| shControl | shCasp2
| shCasp2 | shCasp2 |
| YOYO-1 green FL |
| YOYO-1 green FL |
| YOYO-1 green FL |

Phase contrast

Phase contrast

Phase contrast

Phase contrast

Percentage of Cell death

α-toxin

n=5

*$p<0.0001$
Figure 5

(a) 

His-API5 FL:  + - - + - - 
His-API5 1-454:  + + - + + - 
His-API5 1-243:  - - + - + + 
Flag-Caspase 2:  - - - + + + 

(b) 

(c) 

(d) 

(e) 

(f) 

Caspase-2

N= CARD

dimerization

processing

+ API5

no dimerization

APIS

Caspase-2

Vinculin

FAM-VDUO-FLICA fluorescence
Fig. EV1

(a) Flow cytometry data showing Annexin-V-FITC fluorescence intensity and PI fluorescence intensity for control (−) and α-toxin-treated samples. Q1, Q2, Q3, and Q4 represent different quadrants of the flow cytometry plots.

(b) Bar graphs depicting the percent of cell death for siCo and siAPI5#4 (left) and siCo and siAPI5#1 (right) in the absence (−) and presence of α-toxin. Statistically significant differences are indicated by an asterisk (*).

(c) Immunofluorescence images showing caspase 3/7 activity (FL green) and phase contrast for siControl and α-toxin-treated samples. The overlay shows the combined caspase 3/7 activity and phase contrast images.
Fig. EV2

(a) Percentage of Cell Death

(b) Percentage of Cell Death

(c) 

siAPI5 - + - + - + - + - +

siControl - + - + - + - + - +

63 KDa API5

100 KDa PARP

48 KDa Caspase 2

Ponceau
Fig. EV3

(a) VD-VAE-AFC fluorescence intensity (arbitrary units)

- siAPI5#4: - - + +
- α-toxin: - - + +

(b) Western blot analysis

α-toxin 300 ng/ml
- shControl: - - - + + +
- shCaspase-2#4: - - + + - - +

α-toxin 100 ng/ml
- siAPI5#4: - - + + - - +

API5: 50 KDa
Caspase-2 FL: 50 KDa
Caspase-2*: 37 KDa
Ponceau
Fig.EV4

WT MEFs

Casp3/7 DKO

Control  
Valinomycin

Casp2 active 3.30

Casp2 active 44.3

Casp2 active 3.60

Casp2 active 32.6
Fig. EV5

(a) Input and IP caspase-9

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API5 low exposure

API5 high exposure

(b) Input and Pull down

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Flag