IAP-IAP Complexes Required for Apoptosis Resistance of *C. trachomatis*–Infected Cells

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Host cells infected with obligate intracellular bacteria *Chlamydia trachomatis* are profoundly resistant to diverse apoptotic stimuli. The molecular mechanisms underlying the block in apoptotic signaling of infected cells is not well understood. Here we investigated the molecular mechanism by which apoptosis induced via the tumor necrosis factor (TNF) receptor is prevented in infected epithelial cells. Infection with *C. trachomatis* leads to the up-regulation of cellular inhibitor of apoptosis (cIAP)-2, and interfering with cIAP-2 up-regulation sensitized infected cells for TNF-induced apoptosis. Interestingly, besides cIAP-2, cIAP-1 and X-linked IAP, although not differentially regulated by infection, are required to maintain apoptosis resistance in infected cells. We detected that IAPs are constitutively organized in heteromeric complexes and small interfering RNA–mediated silencing of one of these IAPs affects the stability of another IAP. In particular, the stability of cIAP-2 is modulated by the presence of X-linked IAP and their interaction is stabilized in infected cells. Our observations suggest that IAPs are functional and stable as heteromers, a thus far undiscovered mechanism of IAP regulation and its role in modulation of apoptosis.

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Introduction

The modulation of host cell apoptosis appears to be an important mechanism for many bacterial pathogens in order to establish an infection. Certain pathogens like *Salmonella* spp., *Shigellae* spp., and *Yersiniae* spp. induce apoptosis of macrophages to avoid eradication by professional phagocytes, the first line of defense of the immune system [1,2]. However, experimental data derived from infections in caspase-1 knockout mice demonstrate that host cell apoptosis as a consequence of bacterial infection may also serve as a defense strategy of the host [3]. In this context, it is important to note that pathogenic bacteria have also evolved strategies to efficiently prevent host cell apoptosis. Bacteria of this group may depend on the intact host cell not only to complete their life cycle but also to protect themselves from the host immune system like in the case of the obligate intracellular *Rickettsia* spp. and *Chlamydia* spp. [4–7].

Whereas some of the mechanisms underlying bacteria-induced apoptosis are established [8], relatively little is known about how bacterial infection actively interferes with host cell apoptosis. The study of cell culture models has gained more insights into possible principles of infection-induced apoptosis inhibition, which might also be relevant in vivo. One of these findings is that infection of host cells by obligate intracellular bacteria like *Rickettsia rickettsii* might initially start the intrinsic cell death program but is then counteracted by bacteria-induced antiapoptotic nuclear factor-κB (NF-κB) activation [5]. In addition to inhibition of apoptosis triggered as a consequence of infection, induction of apoptosis by external stimuli may also be prevented. Cells infected with *Chlamydia trachomatis* (Ctr) or *C. pneumoniae* resist receptor-, stress-, and granzyme B–induced apoptosis [4,6,7]. These findings suggested that inhibition of apoptosis may play a role in maintaining the integrity of the infected cell by resisting these cytotoxic T-cell responses.

Caspases, the executioners of the apoptotic program [9], are activated either by stimulation of surface-exposed death receptors [10] or by mitochondria, which respond to apoptosis triggers with the release of caspase-activating factors [11]. Caspases are synthesized as zymogens with low enzymatic activity. Initiator caspase-8 is activated upon ligation of death receptors by recruitment to the *death inducing signaling complex* [12], while caspase-9 activation is mediated by the formation of the apoptosome via mitochondrial pathways [11]. These initiator caspases cleave and activate the effector caspases, for example, caspase-3 and caspase-7.

The activation of effector caspases is considered to be a point of no return in apoptotic signaling and controlled at multiple levels [12–15]. Inhibitors of apoptosis protein (IAPs) constitute an important class of apoptosis regulators func-

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Abbreviations: BIR, baculovirus IAP repeat; CHX, cycloheximide; cIAP, cellular inhibitor of apoptosis; Ctr, *Chlamydia trachomatis;* IAP, inhibitor of apoptosis protein; MOI, multiplicity of infection; NF-κB, nuclear factor-κB; qRT-PCR, quantitative real-time PCR; RNAi, RNA interference; siRNA, small interfering RNA; XIAP, X-linked IAP; TNF, tumor necrosis factor

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Synopsis

Apoptosis is a kind of programmed cell death that plays a crucial role in normal development and homeostasis. Pathogens modulate host cell apoptosis to establish a successful infection. *Chlamydia trachomatis* (Ctr) are obligate intracellular bacteria that are shown to be a major causative of sexually transmitted diseases in humans. Here the authors unveil the molecular mechanisms behind resistance to apoptosis in the infected cells. They suggest that Ctr-mediated apoptosis resistance requires a special group of proteins called inhibitors of apoptosis protein (IAP). IAPs block the very last step in the apoptotic pathway by preventing the activation of effector proteases (caspases) responsible for killing the cells. Although Ctr infection leads to the up-regulation of one of the IAPs, cellular inhibitor of apoptosis (cIAP)-2, X-linked IAP (XIAP), and cIAP-1 are still required to maintain apoptosis resistance. These data suggested that IAPs work in unison to modulate cell survival. In addition, the authors identified that IAP proteins are constitutively organized into heteromeric complexes in tumor cells to modulate their stability and cell survival. In particular, the authors detected that cIAP-1, cIAP-2, and XIAP exist in a high-molecular-weight "IAPosome" complex, which interacts with caspases to resist apoptosis. These observations thus enhance our current understanding of *Chlamydia*–host interaction as well as IAPs, which are targeted by cancer therapeutics.

tioning at the level of caspases. The prototype IAP contains a baculovirus IAP repeat (BIR) and the RING domain. The BIR domains are essential for the antiapoptotic function of IAPs and are involved in the direct binding to caspases [13,16]. Some of the IAPs have been shown to contain ubiquitin transferase activity, which is associated with the C-terminal RING domain of these proteins. However, not all of the eight human IAP members identified so far contain a RING domain and their potency to prevent apoptosis also differs significantly [13]. In the case of X-linked IAP (XIAP), the RING domain contains E3 ligase activity involved in autoubiquitination but also in the ubiquitination of caspases [17].

Regulation of IAPs seems to involve different mechanisms and pathways. The proapoptotic Smac/DIABLO, an IAP-interacting and -inactivating protein, is released from the mitochondria together with cytochrome *c* [18,19]. Omi/HtrA2, another mitochondrial protein released during the onset of apoptosis, is a serine protease which inactivates IAPs by cleavage [20]. Some of the IAPs, like cellular inhibitor of apoptosis-2 (cIAP-2) and XIAP, are transcriptionally up-regulated by the antiapoptotic NF-κB [21]. However, the mechanisms underlying IAP regulation are still poorly understood.

Here we show that inhibition of apoptosis in cells infected with Ctr depends on cIAP-1, cIAP-2, and XIAP. Infection induced the up-regulation of cIAP-2, and the recruitment and stabilization of other IAPs leading to apoptosis resistance. Apart from cIAP-2 silencing, blocking the expression of cIAP-1 and XIAP also sensitized Ctr-infected cells to apoptosis induced by TNF receptor. In addition, depletion of IAPs affected the stability of other IAPs. We have detected that IAPs are constitutively organized in several heteromeric complexes and function in concert in regulating the stability of each other and the survival of the cell. In particular, we have detected cIAP-1, cIAP-2, and XIAP in a constitutive high-molecular-weight complex of around 400 kDa at endogenous levels in the HeLa cell cytosol, suggesting the presence of a constitutive IAP-IAP complex and revealing a thus far undiscovered mechanism of IAP regulation.

Results

Processing of Caspase-3 Is Blocked in Infected Cells

Chlamydia-infected cells exhibit an extensive resistance to various stimuli of the extrinsic and intrinsic apoptotic pathways [4,6,7]. It has been shown previously that apoptosis is blocked at the level of Bak and Bax activation in Ctr-infected cells [22,23]. However, apoptosis is efficiently induced via the TNF receptor in mouse embryonic fibroblast and in HeLa cells lacking Bak and Bax [24] (Oliver Kepp, K. Rajalingam, and T. Rudel, unpublished data), suggesting that TNF-induced apoptosis is independent of these mitochondrial effectors. To unravel the molecular basis for resistance of infected cells for TNF-induced apoptosis [6], the activation of caspases was investigated in detail. Caspase-3 is cleaved at aspartic acid residue D175 generating the small subunit and the p19 N-terminal fragment containing the prodomain. The prodomain is then cleaved autocatalytically to generate the p17 fragment indicative of fully active caspase-3 [25]. Standard caspase-3 blots revealed that activation of caspases is blocked at the level of caspase-3 as the processing of the p19 fragment of caspase-3 to the active p17 fragment is ablated in the infected cells treated with TNF and cycloheximide (CHX) (Figure 1A). However, *Chlamydia* infection did not block the activation of caspase-8 as the full-length caspase is processed into the mature p18 fragment and the caspase-8 substrate BID is similarly processed in the control and in the infected cells treated with TNF/CHX (Figure 1B). In addition, we made use of Biotin-VAD-fmk to detect active caspases present in treated and infected cells. Biotin-VAD-fmk binds to active caspase dimers in an irreversible manner and facilitates their precipitation using streptavidin resin. Infected and non-infected HeLa cells were treated with TNF/CHX and active caspases were precipitated with Biotin-VAD-fmk. To avoid the nonspecific degradation of host cell proteins observed in lysates of infected cells (unpublished data), a special protocol for sample preparation was used (see Protocol S1) and the amount of caspases in lysates of infected cells was always controlled (Figure 1C and 1D). Precipitates were then separated and tested by immunoblot analysis for the presence of active caspase-8 and caspase-3. Initiator caspase-8 was present in reduced amounts in the precipitate of infected cells (Figure 1D), suggesting only a partial activation in infected cells. The partial activation of caspase-8 was verified by a second assay that exploits the activation-dependent binding of fluorescently labeled substrates which can then be quantified under a fluorescent microscope (Figures 1E and S1; for details, see Protocol S1). Consistent with the previous observations, caspase-3 was not detected in biotin-VAD-fmk precipitates (Figure 1D), suggesting that effector caspase-3 downstream of caspase-8 is not activated in infected cells. Further, to evaluate the effects of infection time and apoptosis induction time on chlamydial inhibition of caspase-3, activation time-course experiments were performed. These analyses revealed that the appearance of the active p17 fragment of caspase-3 decreased with time post-

infection irrespective of the time of apoptosis induction (Figure 1F). As expected, caspase-8 processing was not altered at later time points postinfection, suggesting that caspase-8 is activated in the infected cells (Figure 1G).

Transcriptional and Posttranscriptional Regulation of IAPs in Infected Cells

The lack of active effector caspase-3 in infected cells indicated that Ctr infection may prevent caspase activation by IAPs, which inhibit caspases by direct binding and/or the

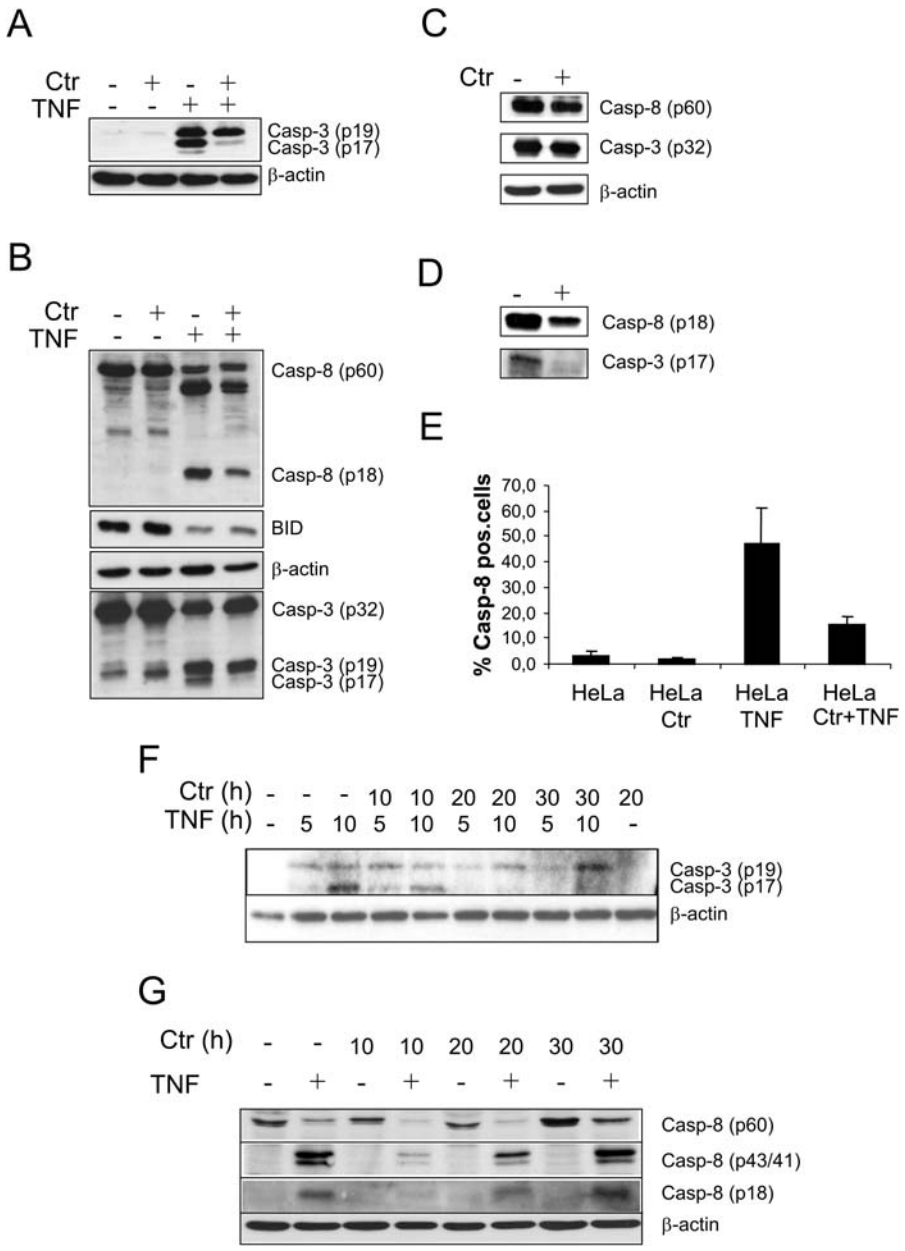

Figure 1. Reduced Activation of Caspase-3 in Infected Cells

(A) HeLa cells were infected (+) for 16 h or not (–) and treated for an additional 8 h with TNFα and CHX. Lysates of these cells were separated and probed with an antibody specifically recognizing the active p19/17 fragment of caspase-3 by immunoblot analysis.

(B) Blots from the same lysates used in (A) were probed for caspase-8 (casp-8 p18) and caspase-3 (casp-3 p19/17). The processing of BID as a specific substrate for caspase-8 is also shown.

(C) HeLa cells were infected and treated with TNF/CHX as mentioned before. Caspase-8 p60 (Casp-8) and –3 p32 (Casp-3) was detected by immunoblot analysis of the lysates. β-Actin staining was included as equal loading control.

(D) Active caspase-8 and caspase-3 were precipitated from the same lysates as in (A) using Biotin-VAD-fmk, and precipitated proteins were detected with antibodies for caspase-8 and caspase-3 as indicated.

(E) Active caspase-8 was monitored in living HeLa cells infected with Ctr and treated with TNFα/CHX (TNF) as described in Protocol S1 (Detection of active caspases). Shown is the mean ± SD of three experiments.

(F) Caspase-3 p19 and p17 fragments were detected by immunoblotting in lysates of cells infected for the indicated times with Ctr and treated for 5 or 10 h with TNFα/CHX.

(G) Cells infected for the indicated time points with Ctr were treated with TNFα/CHX for 10 h, and the full-size caspase-8 (p60) and the cleavage products (p41/43; p18) were analyzed by immunoblotting.

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induction of their proteasomal degradation [13]. To test whether chlamydial infection may cause increased expression of inhibitors of apoptosis, quantitative real-time PCR (qRT-PCR) was established for mRNA quantification of the IAPs. mRNAs of neither *cIAP-1* and *XIAP* nor *survivin* were significantly increased in cells infected with Ctr at 24 h postinfection (Figure 2A). Only the mRNA level of cIAP-2 was increased 6- to 8-fold (Figure 2A). The expression of IAPs was also monitored by immunoblot analysis (Figure 2B). The levels of cIAP-2 and, unexpectedly, that of survivin were clearly increased in infected cells (Figure 2B). Moreover, time-course experiments over 48 h revealed a steady increase in cIAP-2 and survivin protein levels in infected cells (Figure 2C). Since survivin has been shown to play a role in cell cycle control, up-regulation of survivin could depend on the cell cycle status of infected cells. FACS analysis, however, revealed no such difference between infected and noninfected cells (unpublished data). These data suggested that IAP expression is controlled by transcriptional and posttranscriptional mechanisms in infected cells.

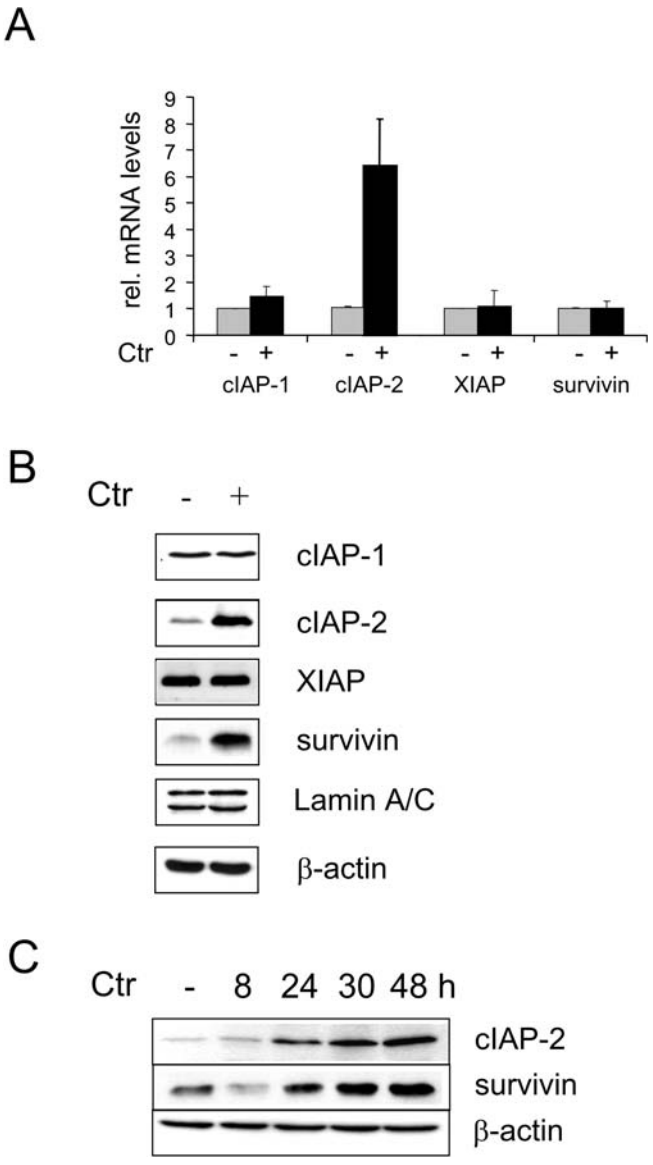

Figure 2. Infection-Induced Up-regulation of cIAP-2

(A) mRNA levels of different IAPs were monitored by qRT-PCR in HeLa cells infected (+) or not (–) with Ctr. The amount of cIAP-2 mRNA increases strongly in infected cells.

(B) The level of IAP expression was determined by immunoblot analysis in noninfected (–) and infected (+) cells. Note that cIAP-2 and *survivin* are strongly up-regulated in infected cells.

(C) Time course of cIAP-2 and *survivin* up-regulation was tested in infected cells by immunoblot analysis. Cells were infected and lysed at different time points postinfection as indicated.

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IAPs Are Required for Infection-Induced Apoptosis Resistance

To test if IAPs are required for the inhibition of apoptosis in infected HeLa cells, small interfering RNAs (siRNAs) were designed to specifically interfere with IAP expression. Silencing of the *cIAP-1, cIAP-2, XIAP,* and *survivin* genes was tested by qRT-PCR (Figure S2A) and immunoblotting (Figure S2B). Functional siRNAs were then transfected, and infected and treated cells were analyzed. Interestingly, interfering with cIAP-2 expression sensitized infected cells to TNF/CHX-induced apoptosis (Figure 3) as revealed by TUNEL analysis. We therefore analyzed whether suppression of cIAP-2 expression influenced the activation of effector caspases. The generation of the caspase-3 p17 fragment was reduced by 57.8% in infected treated cells compared to treated cells as was analyzed by densitometry (Figure 3B) suggesting that the processing of the caspase-3 p19 fragment to the p17 fragment is controlled by cIAP-2. This assumption is in agreement with only 28.8% reduction of the p17 fragment in infected treated cells versus treated cells in which cIAP-2 expression was reduced by siRNA transfection to the level of noninfected cells (Figure 3B), demonstrating a 50.17% rescue of p19 to p17 processing by cIAP-2 silencing.

When the expression of cIAP-1 and XIAP was suppressed by RNA interference (RNAi), the infected cells were sensitized to TNF/CHX-induced apoptosis despite the up-regulation of cIAP-2 (Figure 3C). To confirm these unexpected results, the same experiment was repeated with a second set of siRNAs to rule out unspecific off-target effects of the first set of siRNAs (Figure 3C). It is important to note that irrespective of the cell type and multiplicity of infection (MOI) used, sensitization depended on the size of the inclusions. Cells carrying very large inclusions were resistant to sensitization, suggesting different mechanisms of apoptosis inhibition during the early and late phases of infection, respectively (unpublished data). Sensitization did not correlate with the infection-induced changes in the expression levels of IAPs since the transfection of siRNA directed against *survivin* completely prevented the strong up-regulation but had no sensitizing effect on TNF/CHX-induced apoptosis (Figure 3C). These results suggested that multi-BIR–containing IAPs are involved in apoptosis resistance of cells infected with Ctr.

IAPs Interact with Each Other

A possible explanation for the sensitization of infected cells to TNF/CHX-induced apoptosis by silencing of *cIAP-1, cIAP-2,* or *XIAP* was that all IAPs whose ablation sensitized infected cells are organized in functional complexes. Therefore, we attempted to isolate endogenous IAP complexes from control and apoptotic cells. When we precipitated cIAP-2, XIAP was coprecipitated and vice versa (Figure 4A), suggesting the interaction of cIAP-2 and XIAP in a complex in these cells.

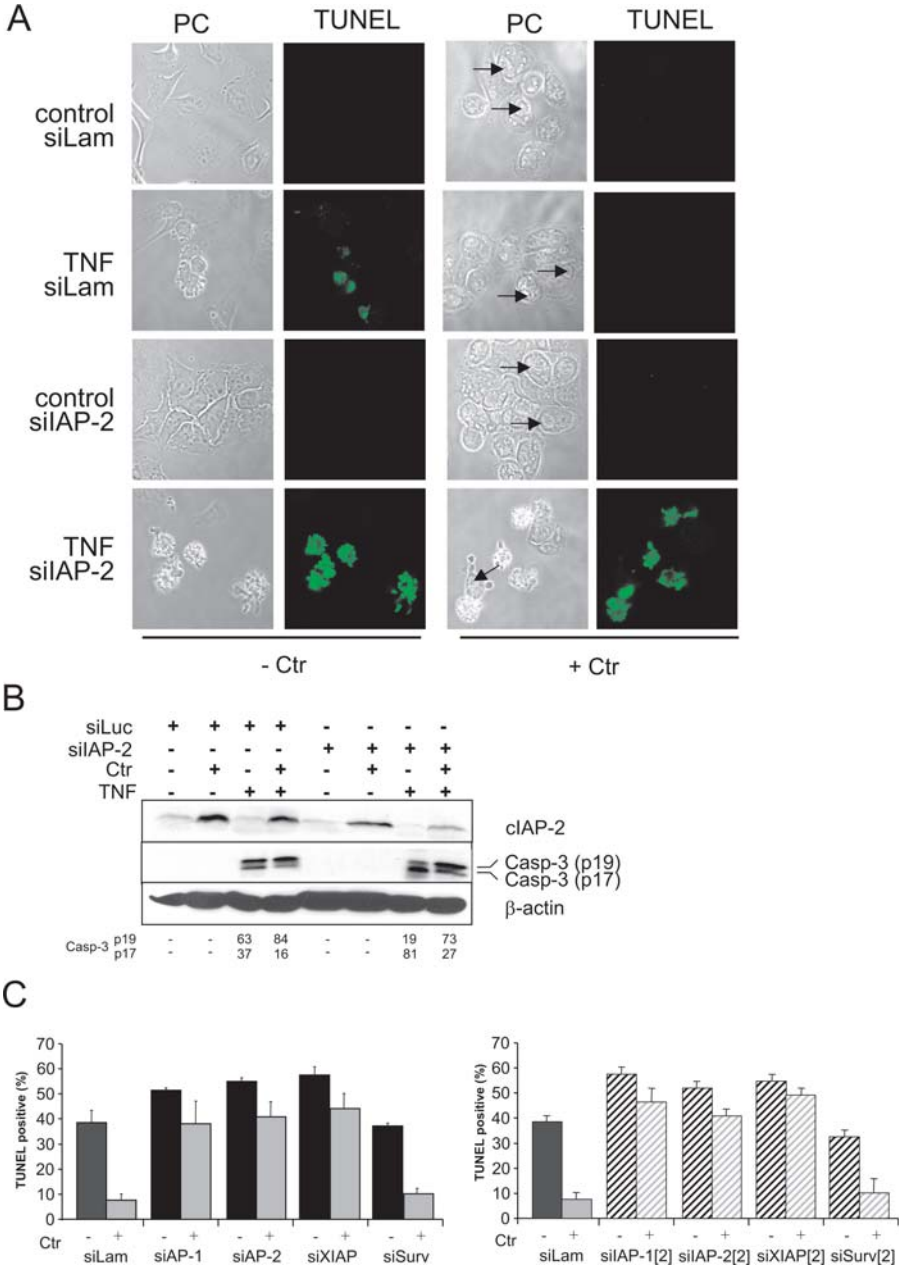

Figure 3. IAPs Required for Infection-Induced Apoptosis Resistance

(A) HeLa cells were transfected with control siRNA (siLam) or siIAP-2 and infected (+ Ctr) or not (– Ctr) with Ctr. Then, apoptosis was induced by treatment with TNF/CHX, and the apoptotic response was measured by phase contrast (PC) and TUNEL analysis. Arrows point to Ctr inclusions.

(B) Infection-induced up-regulation of cIAP-2 prevents the processing of the caspase-3 p19 to the p17 fragment. HeLa cells were transfected with siRNAs, infected, and treated with TNF/CHX as indicated. cIAP-2 and the p19 and p17 fragment of caspase-3 were monitored by immunoblotting on the same membrane. The level of cIAP-2 in the siIAP-2 and TNF/CHX-treated infected sample roughly equals that of the uninfected siLuc and TNF/CHX-treated samples. Note that the proportion of the p19 and p17 fragments in these samples is similar but is clearly shifted in the other TNF/CHX-treated samples. The proportion of caspase-3 p19 and p17 fragment was calculated by densitometric analysis and outlined below the immunoblot (in %).

(C) The apoptotic response was determined in cells transfected with the indicated siRNAs and either infected (+) or not (–) by TUNEL assay. Any one of the siRNAs directed against *cIAP-1*, *cIAP-2*, and *XIAP*, but not the control and the *survivin* siRNA, strongly sensitizes infected cells for TNF-induced apoptosis. Shown is the mean ± SD of three experiments.

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The amount of these IAPs was reduced in apoptotic cells probably due to cleavage or degradation during apoptosis (Figure 4A). Since silencing of cIAP-1 also sensitized infected cells to apoptosis, we investigated whether cIAP-1 was present in the cIAP-2–XIAP complex. cIAP-1 was detected in the cIAP-2–XIAP precipitates by immunoblot analysis (unpublished data) and immunoprecipitates of cIAP-1 contained cIAP-2 and XIAP (Figure S3), confirming cIAP-1 as part of the cIAP-2–XIAP complex. Consistent with our sensitization data, we could not detect survivin in the cIAP-2 and XIAP immunoprecipitates (Figure S3B). These data also suggested that the antibodies we used to immunoprecipitate IAPs did not exhibit cross-reactivity with all BIR domain–containing proteins. Active caspase-3 was detected in all IAP complexes

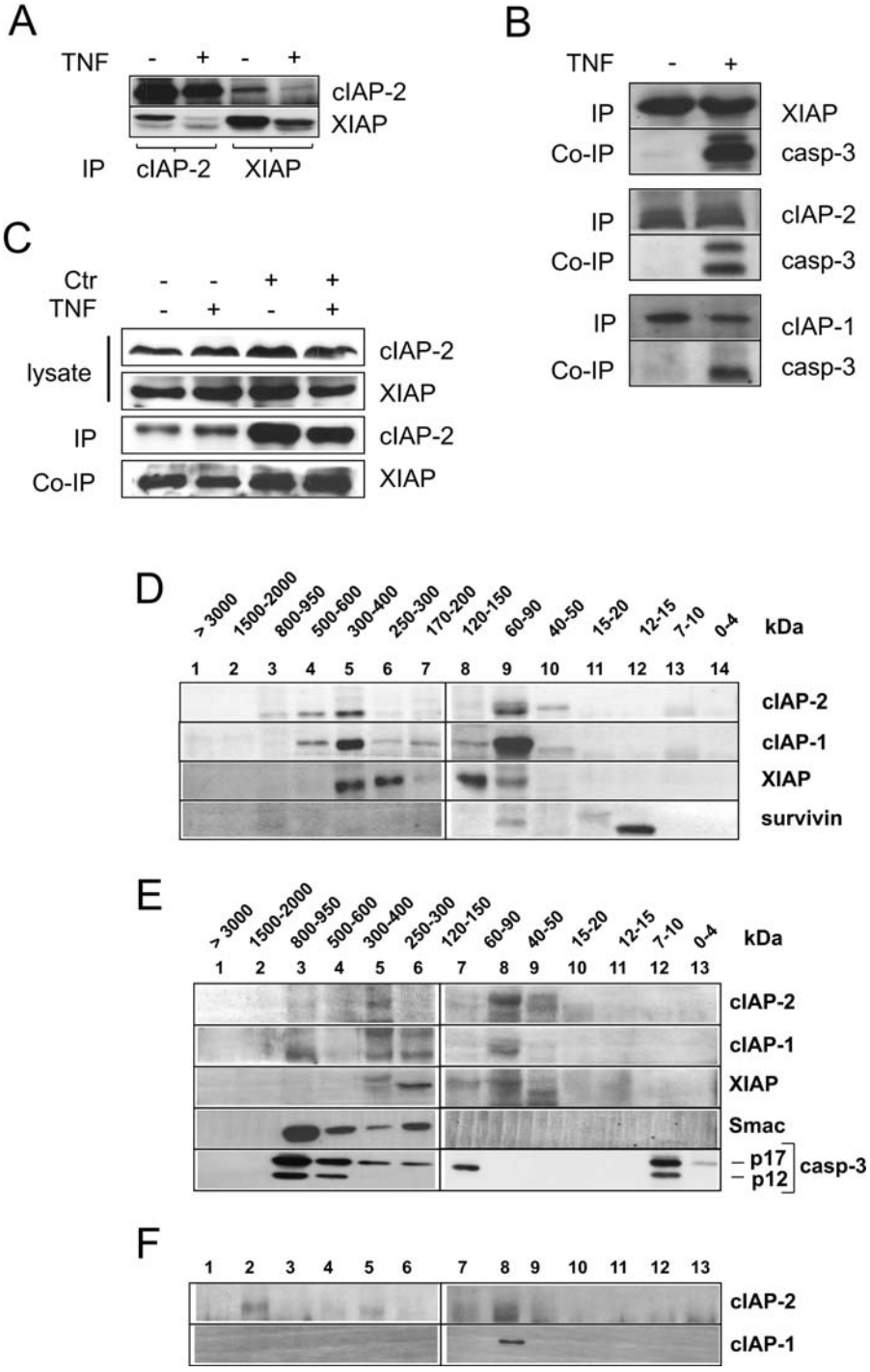

Figure 4. IAPs Are Organized in Heteromeric Complexes

(A) Endogenous cIAP-2 and XIAP were immunoprecipitated (IP) from control and apoptotic cells as indicated. The level of endogenous cIAP-2 and XIAP was determined in precipitates by immunoblotting.

(B) Endogenous IAPs were precipitated from control and TNF/CHX-treated cells and the coprecipitation of active caspase-3 was monitored by immunoblotting.

(C) Endogenous cIAP-2 was immunoprecipitated from control and infected cells treated or not with TNF/CHX as indicated. The level of endogenous cIAP-2 and XIAP was determined in precipitates (IP) and in lysates.

(D) The IAP complexes were isolated by gel filtration as mentioned in Materials and Methods. The proteins from different fractions were TCA-precipitated and resolved in SDS-PAGE, and the presence of IAPs was checked by immunoblot analysis as indicated. Note that high amounts of cIAP-1, XIAP, and cIAP-2 were present together in a protein complex of around 400 kDa. Shown are the fraction numbers (fractions) and the approximate size of proteins and complexes in the respective fractions in kDa.

(E) The cytosol from HeLa cells induced to apoptosis with TNF/CHX was isolated by subcellular fractionation, and gel filtration was performed as mentioned in Materials and Methods. The proteins were separated by SDS-PAGE, and the immunoblot analyses were performed as before.

(F) Gel filtration experiments were performed in XIAP-silenced cells and the presence of cIAP-1 and cIAP-2 in various fractions was tested by immunoblot analysis. Shown in (C), (D), and (E) are the data from one representative experiment.

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precipitated from apoptotic cells, indicating a role of these IAPs in the activation of caspase-3 (Figure 4B). These data suggested that the "sensitizing" IAPs interact with each other either directly or indirectly in a single or multiple complexes to modulate cell survival. Interestingly, increasing amounts of XIAP coprecipitated with endogenous cIAP-2 from infected cells compared to noninfected cells (Figure 4C). Since XIAP is not up-regulated in infected cells (Figure 2A and 2B), coprecipitation of XIAP and cIAP-2 indicated that both were recruited to the same complex. Thus, the infection-induced increase in cIAP-2 levels may initiate the recruitment of the strong caspases inhibitor XIAP to a complex regulating caspase-3 activity.

IAPs Are Organized in Large Heteromeric Complexes

To prove the existence of native IAP-IAP complexes, we isolated the cytosol of HeLa cells by subcellular fractionation and separated the proteins by gel filtration. cIAP-1, cIAP-2, and XIAP were found together in fractions containing protein complexes of around 400 kDa, and the fractions representing the monomeric forms (Figure 4D). While low amounts of cIAP-2, XIAP, and cIAP-1 were also present in several of the high-molecular-weight fractions, survivin was mainly detected in the low-molecular-weight fraction reflecting the monomeric size (Figure 4D). Similar high-molecular-weight IAP-IAP complexes could also be detected in the cytosol of HecIB cells and Jurkat T cells (Figures S4 and S5A). To check if the 400-kDa complex is disrupted during apoptosis, gel filtration experiments were performed with HeLa and Jurkat cells induced to apoptosis with TNF/CHX and anti-CD95 antibody, respectively (Figures 4E and S5B). Consistent with our previous observations (Figures 4A and S3), only low amounts of IAPs were detected in the fractions from apoptotic cells, suggesting that IAPs are possibly cleaved or degraded during cell death (Figures 4E and S5B). However, we have also detected a small amount of cIAP-1 in a high-molecular-weight fraction of around 850 kDa in HeLa cell fractions, suggesting that cIAP-1 is possibly recruited to a different complex during apoptosis. It was already known that Smac binds to IAPs and inactivates them during apoptosis. To investigate if the interaction of Smac with the IAP-IAP complex occurs during apoptosis, we probed the same blots for Smac. Interestingly, Smac was detected in all the IAP-containing fractions of high but not of low molecular weight (Figures 4E and S5B), suggesting an interaction and a possible inactivation of these IAP complexes by Smac during apoptosis. Similar results were obtained when these cells were induced to apoptosis with cisplatin (unpublished data). Consistent with a role of the heteromeric IAP complex for the activation of caspases, caspase-3 copurified with the Smac-enriched fractions in apoptotic cells (Figures 4E and S5B). This, and the exclusive presence of Smac and active caspase-3 in the high-molecular-weight fractions, may suggest that a heteromer rather than a monomer is probably the functional IAP unit. Taken together, these data confirmed that "sensitizing" IAPs are organized in heteromeric complexes.

IAPs Stabilize Each Other

In addition to transcriptional regulation, IAP stability is posttranslationally controlled by phosphorylation, polyubiquitination, and proteasomal degradation [13,26]. As we have detected IAP-IAP complexes at endogenous levels in these cells, we tested if loss of individual IAPs influences the stability of other IAPs. siRNAs were transfected in HeLa cells and the amount of IAPs was measured by immunoblot analysis. The specificity of the different siRNAs, e.g., the lack of any cross-inhibition on other IAPs, was confirmed by qRT-PCR (Figure S6). To avoid potential unspecific degradation of host proteins by chlamydial proteases during lysis, infected cells were resuspended in gel loading buffer on ice followed by boiling. This procedure preserved host proteins of lysed infected cells and was used in all experiments. Surprisingly, silencing of XIAP expression had a pronounced effect on the amount of up-regulated cIAP-2 and survivin in infected cells (Figure 5). Down-regulation of cIAP-2 led to a mild reduction of cIAP-1 and survivin but had no influence on XIAP levels. However, suppression of cIAP-1 expression had no negative effect on cIAP-2 or XIAP, and repression of survivin by RNAi did not alter cIAP-1, cIAP-2, or XIAP levels (Figure 5), suggesting that some of the "sensitizing" IAPs exert a stabilizing activity on other IAPs. All these data suggested that IAPs can influence the stability of each other.

Among the "sensitizing" IAPs, the most evident observation was the regulation of IAP-2 stability by XIAP. To understand the possible mechanisms, we have established the permanent silencing of XIAP by lentiviral transfer of shRNA (shXIAP) into HeLa cells. Chlamydial infection induced the up-regulation of cIAP-2 mRNA levels in vector control and shXIAP-transduced cells (unpublished data). To check if the loss of XIAP disrupts the formation of the IAP-IAP complex, we performed gel filtration experiments with the cytosol of XIAP-silenced cells. Immnunoblot analysis revealed that cIAP-1 and most of the cIAP-2 were no longer detected in the 400-kDa fractions of XIAP-silenced cells (Figure 4). In addition, a portion of cIAP-2 was consistently present in the high-molecular-weight fraction of around 1 to 1.5 MDa, suggesting that cIAP-2 is recruited to a different complex in the absence of XIAP (Figure 4). As cIAP-2 has an inherent destructive capability by autoubiquitination and proteasome-mediated degradation [27], we checked if the loss of the infection-induced up-regulated fraction of cIAP-2 can be rescued by pretreatment of these cells with proteasome inhibitors. As expected, infection of the control (empty vector-carrying) cells leads to an increase in cIAP-2 protein levels. Consistent with the previous observations, we could not detect the up-regulated fraction of cIAP-2 in the infected cells expressing shXIAP (Figure S7A). When the cells were treated with proteasome inhibitors, the up-regulated fraction of cIAP-2 was indeed rescued in the infected XIAP-silenced cells (Figure S7A). Since proteins are degraded not only in the proteasomes but also in the lysosomes [28], we checked if the turnover of cIAP-2 protein levels in these cells is also influenced by the degradation by lysosomes. Surprisingly, pretreatment of cells with the lysosomal H^+-ATPase inhibitor Bafilomycin also inhibited the degradation of cIAP-2 in the control and XIAP-silenced cells in a similar fashion (Figure S7A), suggesting that the degradation of cIAP-2 in these cells is mediated by both proteasomes and lysosmes. To investigate whether cIAP-2 overexpressed by transfection of a eukaryotic expression vector behaves similarly to cIAP-2 up-regulated during infection, cIAP-2 was transfected into XIAP-silenced cells and treated with proteasome and lysosome inhibitors. The inhibitors failed to prominently increase the amounts of cIAP-2 in transfected control and XIAP-silenced cells (Figure

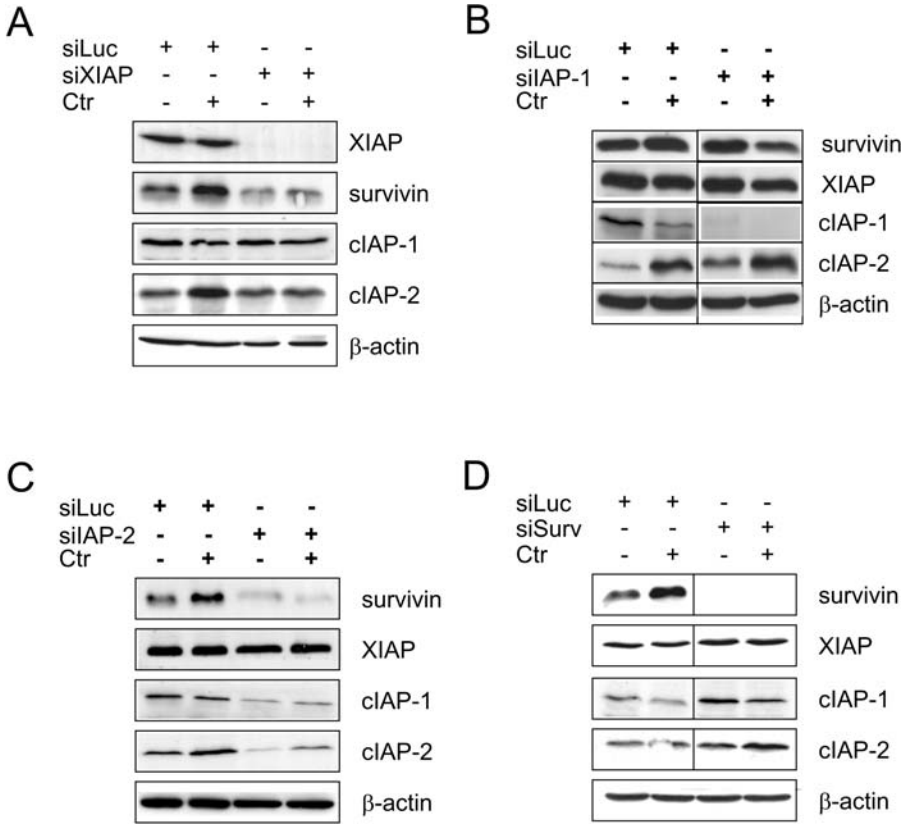

Figure 5. IAPs Cross-Regulate Each Other

siRNAs directed against (A) luciferase or XIAP, (B) cIAP-1, (C) cIAP-2, or (D) *survivin* were transfected in HeLa cells, and the protein levels of all IAPs were determined in infected (+) or uninfected (–) samples by immunoblot analysis as indicated.

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S7B). Interestingly, cIAP-2 transfected control cells contained reduced amounts of XIAP, which was partially rescued by proteasomal inhibitor MG132 (Figure S7B). These data showed that, different than in infection-induced up-regulation, overexpression of transfected cIAP-2 could not be rescued by either proteasomal or lysosomal inhibitors but rather caused the proteasomal degradation of endogenous XIAP.

Discussion

Many obligate intracellular bacteria have been shown to actively inhibit apoptosis in their host. However, functional data on the molecular mechanisms underlying the apoptosis block in infected cells are rare. We show here that the activity and stability of IAPs are regulated by chlamydial infection causing resistance to TNF/CHX-induced apoptosis. We demonstrate here the existence of large heteromeric native IAP complexes involved in the control of caspase activation.

HeLa cells infected with Ctr resisted TNF/CHX-induced apoptosis in an IAP-dependent fashion. A possible explanation for the marked apoptosis resistance observed in infected cells was therefore the infection-induced up-regulation of cIAP-2 specifically blocking the activation of effector caspase-3. Although cIAP-1 and cIAP-2 are also part of the TNF-receptor complex potentially modulating the activity of caspase-8 [29,30], a direct inhibition of caspase-8 activity by IAPs has not been demonstrated yet. We found the levels of active caspase-8 slightly reduced in infected cells. The caspase-8 substrate BID was, however, cleaved as efficiently as in noninfected cells, suggesting the effector caspases as major targets for apoptosis inhibition by IAPs. We have detected increased amounts of coprecipitating XIAP in the cIAP-2 immunoprecipitates from the infected cells. Since XIAP has been found to tether mature caspase-3 to the apoptosome and release of caspase-3 required the XIAP-antagonizing protein Smac [31], the IAP complex formed in cells infected with Ctr may thus favor the association of IAPs and recruitment of caspase-3 in a way refractory to activation. Consistent with this, neither cytochrome *c* nor Smac is released from the mitochondria of infected cells (unpublished data), although BID is similarly processed in the infected and control cells treated with TNF/CHX, suggesting that chlamydial infection employs multiple strategies to resist host cell apoptosis.

Mammalian IAPs have been reported to form homodimers and heterodimers, which raises the possibility that they can cross-regulate each other. It was shown that cIAP-1$^{-/-}$ cells have increased levels of cIAP-2 protein consistent with the down-regulation of cIAP-2 by cIAP-1 [32]. Silke et al. [33] demonstrated a direct interaction of XIAP with overexpressed cIAP-1 via their RING domains to modulate the stability of XIAP in melanoma cells. In addition, it was shown that in response to cell death stimuli, survivin associated with XIAP via the BIR domain to promote the stability of XIAP [34]. Apoptosis resistance of infected cells required cIAP-2 up-regulation but also XIAP and cIAP-1, as depletion of the

latter two sensitizes the cells to apoptosis despite cIAP-2 up-regulation. The most likely explanation was therefore that all these IAPs are organized in a functional complex. We could indeed confirm the direct association of cIAP-1, cIAP-2, and XIAP in these cells by immunoprecipitation and gel filtration experiments, suggesting that they function in unison to modulate the activation of caspases in vivo. We found no effect of survivin on apoptosis of infected cells, although survivin-depleted cells underwent mitotic catastrophe and formed giant cells (unpublished data) as previously described [35], suggesting that the depletion by RNAi was effective. Consistent with this, although survivin is stabilized in the infected cells, loss of survivin did not influence the resistance of *Chlamydia*-infected cells to cell death (Figure 3C).

By applying gel filtration assays,we could demonstrate the existence of a high-molecular-weight heteromeric complex in the range of 400 kDa containing cIAP-1, cIAP-2, and XIAP. However, we have also detected IAPs in other high-molecular-fractions, suggesting the existence of several IAP-containing complexes. In support of our sensitization data, we could not detect survivin in the IAP-containing high-molecular-weight fractions.

Since depletion of XIAP by siRNAs affected the stability of cIAP-2 (Figure 5A), we asked whether loss of XIAP led to disintegration of IAP complexes. Gel filtration experiments with XIAP-silenced cells revealed that this is indeed the case as cIAP-1 was detected only in the monomeric fractions. However, apart from the monomeric fractions and trace amounts in the 400-kDa fractions, a significant portion of cIAP-2 was also found in a high-molecular-weight fraction of around 1 MDa, which may reflect the recruitment to a different complex possibly involved in the fate of cIAP-2 under these conditions. These results are in contrast to what was reported from XIAP knockout MEFs which contained larger amounts of cIAP-1 and cIAP-2 than wild-type MEFs [36]. Besides species-specific differences, it may also reflect differences between the knockdown and knockout conditions.

If IAPs are organized into stable complexes, what is their role during apoptosis? Several studies have demonstrated that all the IAPs can bind to caspases directly to inhibit cell death. However, we found a direct role only for cIAP-1, cIAP-2, and XIAP in maintaining apoptosis resistance under these conditions. Silencing any one of the IAPs removes the blockade in the processing of caspase-3 to the active p17 fragment in response to TNF/CHX in infected cells (Figure 3B and unpublished data). Therefore, it is tempting to speculate that a heteromeric IAP-IAP complex is more potent to block caspases than individual IAPs in vivo.

As we have detected IAPs in heteromeric complexes, we addressed the question if Smac directly associates with these complexes during apoptosis. Interestingly, gel filtration experiments with apoptotic cells revealed the presence of Smac and active caspase-3 in all the IAP-containing high-molecular-weight fractions, irrespective of the apoptosis inducer, suggesting that Smac indeed binds and inactivates IAP-IAP complexes in vivo to induce effector caspase activation and cell death.

The role of ubiquitination in sorting the proteins for proteasomes and lysosomes is well established. While poly-ubiquitination of substrates frequently leads to the degradation of proteins by the proteasome, monoubiquitination or multimonoubiquitination (one ubiquitin on several lysine residues within the substrates) could target the substrate for a different subcellular compartment like the lysosomes [28]. Here we revealed that cIAP-2 levels in the infected cells are regulated by both lysosomes and proteasomes. Treatment of the cells with proteasome and, surprisingly, with lysosomal inhibitors could rescue cIAP-2 in control and XIAP-silenced cells suggesting different ubiquitin modifications in cIAP-2. However, when we overexpressed cIAP-2 by transfection of expression constructs, we could not rescue XIAP, in the presence of neither MG132 nor Bafilomycin (Figure S7B). Consistent with results described by Silke et al. (2005), overexpression of cIAP-2 by transfection caused the degradation of endogenous XIAP (Figure S7B). Because we did not observe degradation of XIAP in infected cells with high cIAP-2 levels, it suggests that *Chlamydia* infection interferes with this process, or that NF-κB targets other factors, which influence relative stabilities of IAPs. Loss of cIAP-1 has also been described to result in the increase of cIAP-2 but not XIAP [32]. While we did not observe these effects in our siRNA experiments, it appears that a general theme of posttranscriptional regulation by IAPs among themselves is emerging.

We are currently investigating the effect of overexpressed IAPs on the native IAP complexes demonstrated here. Taken together, our data of a constitutive "IAPosome," where IAPs positively affect the stability of other IAPs important for their apoptosis-inhibiting activity, are a new aspect of how these proteins function. Considering the already visible levels of complexity, it will be an intriguing task to unravel the functional interplay of these fascinating apoptosis regulators. Moreover, cIAP-2 is not only involved in antiapoptosis induced by Ctr in epithelial cells but is also up-regulated in monocytes infected with *C. pneumoniae* [37] and plays an important role in the apoptosis resistance of epithelial cells infected with *C. pneumoniae* [38]. Pathogenic bacteria and viruses known to stimulate antiapoptotic activities in their host cells induce the up-regulation of cIAP-2 [39–41], suggesting that the mechanism proposed here has general relevance for the host–pathogen interaction.

Materials and Methods

Cell culture and growth of Ctr. HeLa, HEp-2, HecIB, and Jurkat cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (GIBCO-BRL, San Diego, California, United States) and penicillin (100 U/ml)/streptomycin (100 μg/ml) (GIBCO-BRL) at 37 °C in 5.0% CO_2. *C. trachomatis* LGV serovar L2 was routinely propagated in the human laryngeal epithelial HEp-2 cells (ATCC [American Type Culture Collection, Manassas, Virginia, United States] CCL23) grown in cell growth medium (CGM), which consists of RPMI medium supplemented with 2 mM glutamine, 25 mM HEPES, 10% (vol/vol) FBS, and 10 μg of gentamicin/ml. Stock organisms were prepared essentially as described previously [42,43] and stored at −75 °C. Fresh stocks were used for each experiment.

Transfection of siRNAs and subsequent infection. To inhibit expression of genes with siRNAs, 50,000 cells/well were seeded in a 12-well plate at least 20 h prior to transfection. siRNAs designed for the inhibition of the genes under investigation and for Lamin A/C (siLam) or luciferase (siLuc) as negative control were transfected using the Transmessenger transfection kit or the RNAiFect kit (Qiagen, Valencia, California, United States). One day post transfection, the nearly confluent cells were trypsinized and seeded out in 12-well plates with glass coverslips or in six-well plates. The cells were then infected with Ctr at an MOI of 5 at 36 to 40 h post transfection, and 24 h later, the samples were analyzed by Western blot or induced to apoptosis. The following siRNAs were used in this study. siLuc:

AACUUACGCUGAGUACUUCGA, siIAP-1: AACAUAGUAGCUUGUUCAGUG, siIAP-1 [2] CUAGGAGACAGUCCUAUUCAA, siIAP-2: AAUUGGGAACCGAAGGAUAAU, siIAP-2 [2]: TTCAAGAUACACAGUUUCUAA, siXIAP: AAGUGCUUUCACUGUGGAGGA, siXIAP [2]: AACGAGAGAUUUGGAAAGATA, siSurv: AAGCAUUCGUCCGGUUGCGCU, siSurv [2]: UGCACCACUUCCAGGGUUUAU, siLamin A/C: AA CUGGACUUCCAGAAGAACA.

Permanent silencing of XIAP by shRNAs. HeLa cells stably expressing the XIAP shRNAs were generated according to http://www.tronolab.com. Briefly, 293T cells were transfected by the calcium phosphate method with the target vectors pLVTH-M–shXIAP and viral packaging vectors. After 48 h, lentiviral supernatants were filtered through a 0.45-μm filter and concentrated by ultracentrifugation at 106,750 × *g*, and infections of HeLa cells were performed in the presence of polybrene (Sigma, St. Louis, Missouri, United States). At 8 h postinfection, medium was exchanged, and after 48 h, GFP-positive cells were enriched by FACS (FACSDiva; Becton-Dickinson, Palo Alto, California, United States). The knockdown efficiency of the shRNA was quantified by immunoblotting and qRT-PCR analysis. Single-cell clones of shXIAP-expressing cells were isolated by FACS analysis, and the efficiency of the knockdown was quantified by qRT-PCR (see Protocol S1). The clone with the best knockdown efficiency was then expanded and used for various experiments. HeLa cells transduced with the empty vector were used as a control.

SDS-PAGE and Western blot. For SDS-PAGE, cells were lysed in single detergent buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 1 μg/ml aprotinin, 0.5 μg/ml leupeptin, 1 mM Pefabloc [Pentapharm Ltd.], 10 μM pepstatin) for 15 to 20 min on ice and sonicated twice for 15 s. Lysates were cleared by centrifugation for 10 min at 13,000 rpm. Sample buffer (3% 2-mercaptoenthanol, 20% glycerin, 0,05% bromphenol blue, and 3% SDS) was added to these lysates and boiled at 90 °C for 2 min before loading onto the gels. However, in most of the experiments, cells were directly lysed with the sample buffer. The lysed cells were collected and loaded onto SDS-PAGE after heating the lysates to 90 °C for 5 min. After separation, the proteins were transferred to PVDF membranes. For immunoblot analysis, membranes were blocked with 3% BSA in TBS with 0.5% Tween-20 for 2 h and incubated with antibodies directed against β-actin (Sigma), active anti-caspase-3 antibody (Cell Signaling Technology, Beverly, Massachusetts, United States), caspase-9 (Upstate, Millipore, Billerica, Massachusetts, United States), caspase-10 (Santa Cruz Biotechnology, Santa Cruz, California, United States), cIAP-1 mouse monoclonal antibody (PharMingen, San Diego, California, United States), cIAP-2 mouse monoclonal antibody (PharMingen), anti-XIAP mouse monoclonal anibody, *survivin* (Santa Cruz Biotechnology), or Lamin A/C (Chemicon, Temecula, California, United States). Antigen–antibody complexes were detected by horseradish peroxidase–coupled antibodies (PharMingen) followed by enhanced chemiluminescence (New England Biolabs, Beverly, Massachusetts, United States). To check for the presence of several IAPs in the immunoprecipitation experiments, the True Blot system from eBioscience (San Diego, California, United States) was used to avoid cross-reaction of the antibody heavy and light chains with the secondary antibody.

The rescue experiments with proteasomal and lysosomal inhibitors were performed as follows. Control and XIAP-silenced cells were infected at an MOI of 3 and treated with 5 nM Bafilomycin (Calbiochem, San Diego, California, United States) or 10 μM MG132 (Calbiochem) for 5 to 6 h. The cells were lysed and immunoblot analyses were performed. In case of transfection experiments, 1 μg of cIAP-2 was transfected into control and XIAP-silenced cells using LipofectAMINE (Invitrogen, Carlsbad, California, United States) following the manufacturer's instructions.

Immunoprecipitation. To check for the organization of IAPs in complexes, we established a single-step immunoprecipitation protocol. Infected and noninfected HeLa cells grown in 10-cm tissue culture dishes were treated with TNF/CHX as mentioned above. Cells were lysed with phosphate lysis buffer (100 mM NaCl, 10 mM $NaHPO_4$, 2 mM EGTA, 1 mM EDTA, 10 mM NaF, 10 mM $Na_2P_2O_7$, 0.5% sodium deoxycholate, 0.05% SDS, 5% glycerol, and 1% NP-40) with complete protease inhibitor cocktails on ice. The cells were collected by scraping the cells from the surface using a plastic rubber policeman and then sonicated for a few seconds to lyse the cells. All subsequent steps were performed in the cold room to avoid the nonspecific degradation of the host cell proteins by chlamydial proteases at room temperature. The immunoprecipitations were performed using antibodies against mouse monoclonal antibodies against cIAP-2, XIAP, and cIAP-1 (PharMingen) or *survivin* (Santa Cruz Biotechnology) overnight at 4 °C in an overhead rotator. Antigen–antibody complexes were then collected by protein A/G slurry for 2 h and washed with IP buffer before adding sample buffer. Samples were subjected to Western blot analysis using the True Blot system (eBioscience) following the manufacturer's instructions.

Subcellular fractionation. HeLa cells were cultured in 150-cm^2 flasks and harvested using a rubber policeman. Apoptosis was induced with TNF/CHX as described in Protocol S1, and the apoptotic cells were enriched by repeated washing with PBS. The cells were collected by centrifugation for 5 min at 340 × *g* at 4 °C. The pellet was washed with PBS and then resuspended in MB buffer (400 mM Sucrose, 50 mM Tris, 1 mM EGTA, 5 mM β-mercaptoethanol, 10 mM KH_2PO_4 [pH 7.6]). The suspension was kept on ice for 20 min, and the cells were then disrupted with a Dounce homogenizer. The nuclei and cell debris were removed by centrifugation at 1,075 × *g* for 2 min. The supernatant was centrifuged again at 10,000 rpm for 10 min to remove the mitochondria. The resulting supernatant was used as the cytosolic fraction to perform gel filtration experiments.

Gel filtration chromatography. The mitochondria-free cytosol fraction was applied to a Superdex 200 HR 26/60 column (Amersham Pharmacia, Little Chalfont, United Kingdom) at a flow rate of 1 ml/min. The column was equilibrated with PBS, and 5-ml fractions were collected starting at 80 ml (column void volume is 100 ml). The fractions were mixed with an equal volume of 25% trichloroacetic acid and kept on ice for 30 min. Precipitated proteins were collected at 3,775 × *g* for 30 min. The pellet was washed twice with acetone and dissolved in SDS sample buffer for analysis by SDS-PAGE and immunoblotting.

Supporting Information

Figure S1. Caspase-8 Is Active in Infected Cells

Active caspase-8 was monitored in living HeLa cells infected with Ctr and treated with TNFα/CHX (TNF) as described in Protocol S1 (Detection of active caspases). Shown are immunofluorescence and phase contrast images of representative fields. Note the increase in fluorescing cells irrespective of an intact cell layer in the infected treated sample.

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Figure S2. siRNAs Reduce the mRNA and Protein Levels of IAPs

(A) siRNAs directed against different IAPs strongly reduce the respective mRNA as was measured by qRT-PCR (see Protocol S1).
(B) Lysates of siRNA transfected and control cells either infected (+) or not (–) were analyzed by immunblotting for the amount of the respective IAP protein as indicated. Equal loading was controlled by staining with anti–β-actin antibody.

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Figure S3. IAPs Interact with Each Other

(A) Endogenous cIAP-1 was immunoprecipitated from control and apoptotic cells as mentioned in Materials and Methods. The coprecipitating cIAP-2 and XIAP were checked by immunoblot analysis.
(B) *survivin* is not present in the cIAP-2 and XIAP IPs. The cIAP-2 and XIAP IPs were probed for *survivin* by immunoblotting. The whole-cell lysate (WCL) was loaded as a control.

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Figure S4. IAPs Are Organized in Heteromeric Complexes in HecIB Cells

The IAP complexes were isolated by gel filtration and detected by immunoblotting as described in Materials and Methods.

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Figure S5. IAPs Are Organized in Heteromeric Complexes in Jurkat T Cells

(A) The IAP complexes were isolated by gel filtration and detected by immunoblotting as described in Materials and Methods.
(B) The cytosol from Jurkat T cells induced to apoptosis with CD95 was isolated by subcellular fractionation and gel filtration was performed as mentioned in Materials and Methods. The proteins were separated by SDS-PAGE, and the immunoblot analyses were performed as before.

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Figure S6. No Cross-Silencing of IAP Genes by Transfection of siRNAs

siRNAs directed against different IAPs and siLuc were transfected and mRNA levels of *cIAP-1* (A), *cIAP-2* (B), *XIAP* (C), and *survivin* (D) were measured by qRT-PCR in control (–) and infected (+) cells.

Shown are the results of four independent experiments and error bars represent ±SD of the mean.

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Figure S7. Degradation of cIAP-2 Is Mediated by Proteasomes and Lysosomes

(A) Control and shXIAP-expressing cells were infected with Ctr at an MOI of 3 for 16 h and treated with either 5 nM Bafilomycin and/or 10 μM MG132 for 6 h. The cells were lysed as mentioned in Materials and Methods, and the levels of XIAP and cIAP-2 were monitored by immunoblot analysis. Densitometric analysis revealed an increase of 30% of cIAP-2 levels in the infected control compared to the infected XIAP-depleted cells.

(B) Control and XIAP-silenced cells were transfected with 1 μg of pcDNA3–cIAP-2 construct and treated with 10 μM of MG132 for 6 h before lysis. Levels of cIAP-2 and XIAP were monitored by immunoblot analysis.

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Protocol S1. Supplemental Information

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