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# Active Caspase-1 Is a Regulator of Unconventional Protein Secretion

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## SUMMARY

Mammalian cells export most proteins by the endoplasmic reticulum/Golgi-dependent pathway. However, some proteins are secreted via unconventional, poorly understood mechanisms. The latter include the proinflammatory cytokines interleukin(IL)-1β, IL-18, and IL-33, which require activation by caspase-1 for biological activity. Caspase-1 itself is activated by innate immune complexes, the inflammasomes. Here we show that secretion of the leaderless proteins proIL-1α, caspase-1, and fibroblast growth factor (FGF)-2 depends on caspase-1 activity. Although proIL-1α and FGF-2 are not substrates of the protease, we demonstrated their physical interaction. Secretome analysis using iTRAQ proteomics revealed caspase-1-mediated secretion of other leaderless proteins with known or unknown extracellular functions. Strikingly, many of these proteins are involved in inflammation, cytoprotection, or tissue repair. These results provide evidence for an important role of caspase-1 in unconventional protein secretion. By this mechanism, stress-induced activation of caspase-1 directly links inflammation to cytoprotection, cell survival, and regenerative processes.

## INTRODUCTION

Caspase-1 is a cysteine protease originally cloned as IL-1β-converting enzyme (Cerretti et al., 1992; Thornberry et al., 1992). It is an essential regulator of inflammatory responses through its capacity to process and activate proIL-1β, proIL-18, and proIL-33 (Ogura et al., 2006). IL-1β and IL-18 are potent proinflammatory cytokines, and IL-33 promotes responses mediated by type 2 helper T cells (Dinarello, 2002; Ogura et al., 2006). Caspase-1 knockout mice cannot activate proIL-1β and proIL-18, and they are resistant to endotoxic shock (Kuida et al., 1995; Li et al., 1995; Los et al., 1999). Therefore, caspase-1 is considered as an "inflammatory" caspase. In contrast to mice lacking the apoptotic caspases-3, -8, or -9, caspase-1-deficient mice show no developmental defects, and cells isolated from these mice are sensitive to most apoptotic inducers (Kuida et al., 1995; Li et al., 1995; Los et al., 1999).

Caspase-1 is initially expressed as an inactive precursor. Similar to the activation of the proapoptotic caspases-9 and -8 by the apoptosome or the death-inducing signaling complex, respectively, inflammasome complexes that are required for activation of caspase-1 have been identified (Martinon et al., 2002; Martinon and Tschopp, 2004). Oligomeric NOD-like receptors such as NALP1, NALP3, or IPAF act as stress sensors for inflammasome assembly and subsequent activation of caspase-1, which in turn is responsible for activation of proIL-1β and secretion of the mature cytokine (Mariathasan and Monack, 2007; Ogura et al., 2006). We recently demonstrated that the inflammasome is also responsible for UV-induced secretion of IL-1β from human keratinocytes (Feldmeyer et al., 2007), suggesting that proIL-1β maturation by the inflammasome is not restricted to professional immune cells.

ProIL-1β, proIL-18, and -33 lack a signal peptide, and they are secreted by an unconventional, endoplasmic reticulum (ER)/Golgi-independent pathway, which is poorly characterized (Qu et al., 2007; Ogura et al., 2006; Donaldson et al., 1998; Dinarello, 2002; Carta et al., 2006; Andrei et al., 2004). Several other leaderless but secreted proteins have been identified, which may leave the cell by a similar pathway as IL-1β (Cleves, 1997; Nickel, 2005; Rubartelli, 2005). Some of them, e.g., the proangiogenic growth factor FGF-2 (basic FGF), clearly fulfill an extracellular function, whereas such a function is less obvious for annexins 1 and 2, galectins, or EBBP/TRIM16 (Rubartelli, 2005; Nickel, 2005; Feldmeyer et al., 2007; Cleves, 1997). Interestingly, caspase-1 and other inflammasome proteins are also released from activated macrophages or UV-irradiated keratinocytes, although all these proteins lack a signal peptide (Martinon et al., 2002; Martinon and Tschopp, 2004; Mariathasan et al., 2004; Feldmeyer et al., 2007). Secretion occurs quickly after activation of the inflammasome, which is thought to prevent apoptosis (Mariathasan et al., 2004; Martinon et al., 2002; Martinon and Tschopp, 2004). The correlation between inflammasome activation and secretion also raises the questions of whether inflammasome activation and unconventional protein secretion are linked and whether the inflammasome and caspase-1 in particular are components of the secretion machinery (Ogura et al., 2006).

ProIL-1α, the functional analog of proIL-1β, is also secreted independently of the ER/Golgi system (Dinarello, 1998). However, it is not a substrate of caspase-1 and does not require

processing for receptor binding (Dinarello, 1998). Surprisingly, activated macrophages from caspase-1- or NALP3-deficient mice are not only impaired in IL-1β secretion but also release less IL-1α (Kuida et al., 1995; Li et al., 1995; Sutterwala et al., 2006). Although it is not known whether this is a direct effect of caspase-1 expression or activity, it raises the possibility that caspase-1 and the inflammasome are generally involved in secretion of leaderless proteins.

Here we demonstrate that caspase-1 activation by the inflammasome is directly linked to IL-1α secretion from activated macrophages and UV-irradiated keratinocytes. In addition, secretion of FGF-2 also depends on caspase-1 expression and activity. Both proteins bind to caspase-1, suggesting a role of the protease as a carrier in an ER/Golgi-independent protein secretion pathway. Interestingly, secretion of caspase-1 itself requires enzymatic activity, and caspase-1 inhibition therefore prevents secretion of its binding proteins. Finally, using a proteomics approach, we identified known as well as unknown leaderless proteins, which are secreted in a caspase-1-dependent manner.

## RESULTS

### Secretion of proIL-1α Requires Caspase-1 Activity

To confirm the reduced secretion of IL-1α from caspase-1-deficient macrophages (Kuida et al., 1995; Li et al., 1995) and to determine the underlying mechanism, we isolated peritoneal macrophages from these mice and their wild-type littermates and activated them with lipopolysaccharide (LPS) and ATP. We measured the percentage of released IL-1α and -β by ELISA. As a control for unspecific lysis, we analyzed the ratio between the activity of the cytoplasmic enzyme lactate dehydrogenase (LDH) in the supernatant and the total activity (cell lysate plus supernatant [Figure 1A]). As expected, we found significantly reduced levels of IL-1α in the supernatant of caspase-1-deficient cells, although the reduction in IL-1β secretion was more pronounced (Figure 1A). Activated wild-type macrophages treated with a specific caspase-1 inhibitor (ac-YVAD-cmk) or a pan-caspase inhibitor (z-VAD-fmk) showed a similar reduction in IL-1α and -β secretion to caspase-1 knockout macrophages (Figure 1A), demonstrating that the enzymatic activity of caspase-1 is required. In contrast to previous studies (Kuida et al., 1995; Li et al., 1995), we still detected secretion of low amounts of IL-1β by caspase-1-deficient cells. This result may be in part due to a minor crossreactivity of our ELISA with the pro-form of the cytokine or it may indicate that (pro)IL-1β secretion is predominantly but not fully dependent on caspase-1 activity in macrophages.

Keratinocytes secrete proIL-1α and -β upon UVB irradiation, and we recently showed that maturation of proIL-1β is inflammasome dependent (Feldmeyer et al., 2007). In contrast to macrophages, keratinocytes do not process proIL-1α. This allows a quantitative determination of its secretion, irrespective of differences in the affinity of the ELISA to the pro- or mature forms. siRNA-mediated reduction of caspase-1 levels strongly decreased the secretion of IL-1β as well as of proIL-1α upon irradiation with a physiological dose of 50 mJ/cm$^2$ UVB (Figure 1B). As a positive control we reduced the expression of EBBP/TRIM16, which is an enhancer of IL-1β secretion (Munding et al., 2006). These results revealed that secretion of both types of IL-1 by UVB-irradiated keratinocytes correlates with expression of caspase-1 and EBBP.

Furthermore, treatment of keratinocytes with caspase-1 inhibitors strongly inhibited maturation and secretion of proIL-1β and also reduced secretion of proIL-1α to the same extent (Figure 1C). Therefore, efficient secretion of proIL-1α by keratinocytes also depends on caspase-1 activity and not only on expression of the protease.

To exclude the possibility that proIL-1α secretion is a result of caspase-1-mediated release of IL-1β or other factors, we incubated keratinocytes with conditioned medium of UVB-irradiated keratinocytes. This treatment did not enhance proIL-1α secretion but rather reduced it (Figure 1D).

To determine the specificity of the effect for IL-1 we analyzed the release of various other cytokines from UVB-irradiated keratinocytes using a multiplex cytokine assay. Whereas secretion of IL-1β was clearly reduced by caspase-1 siRNA, secretion of cytokines, which harbor a signal peptide, was not affected (data not shown).

Caspase-1 is activated by inflammasome complexes in macrophages and keratinocytes (Ogura et al., 2006; Feldmeyer et al., 2007). Since macrophages from mice lacking the inflammasome protein NALP3 secrete lower amounts of IL-1α than macrophages from wild-type littermates (Sutterwala et al., 2006), we analyzed the influence of several inflammasome proteins on the UVB-induced proIL-1α secretion from keratinocytes by siRNA-mediated knockdown (Figure 1E). Interestingly, knockdown of Asc and NALP3, which are required for proIL-1β maturation, also affected proIL-1α secretion. These results suggest that a common mechanism involving caspase-1 and inflammasome components underlies the secretion of both IL-1 variants.

### Secretion of IL-1 and Caspase-1 Depends on Enzymatic Activity of Caspase-1

Upon assembly of inflammasome complexes caspase-1 is activated, resulting in the formation of an $\alpha_2\beta_2$ heterotetramer, which consists of two p20 (containing the active site) and two p10 subunits (Dinarello, 1998; Ogura et al., 2006). Since proIL-1α is not a substrate of the active caspase-1 heterotetramer, it is unclear why secretion of IL-1α is reduced by caspase-1 inhibitors (Figures 1A and 1C). Either the active heterotetramer has secretion-promoting activity or this function can be fulfilled by the heterotetramer, irrespective of its activity. Caspase-1 inhibitors prevent tetramerization of caspase-1 and could therefore block IL-1α secretion by this mechanism. To distinguish between these possibilities, we performed experiments with recombinant proteins expressed in COS-1 cells, which allowed us to determine the influence of active or inactive $\alpha_2\beta_2$ heterotetramers with a mutated active site cysteine. First, we compared the effect of wild-type and inactive procaspase-1 on maturation of proIL-1β and secretion of proIL-1α and IL-1β (Figure 2A). As expected, COS-1 cells overexpressing caspase-1 with a mutated active site cysteine (C285A, C285S) secreted less proIL-1α, mature IL-1β, and interestingly also proIL-1β. This was not due to unspecific release after cell lysis, as we found the same amount of β-actin and similar LDH activities in the supernatants of all transfected cells (Figure 2A). Second, transfection of cells with expression vectors encoding the individual p20 and p10 subunits of

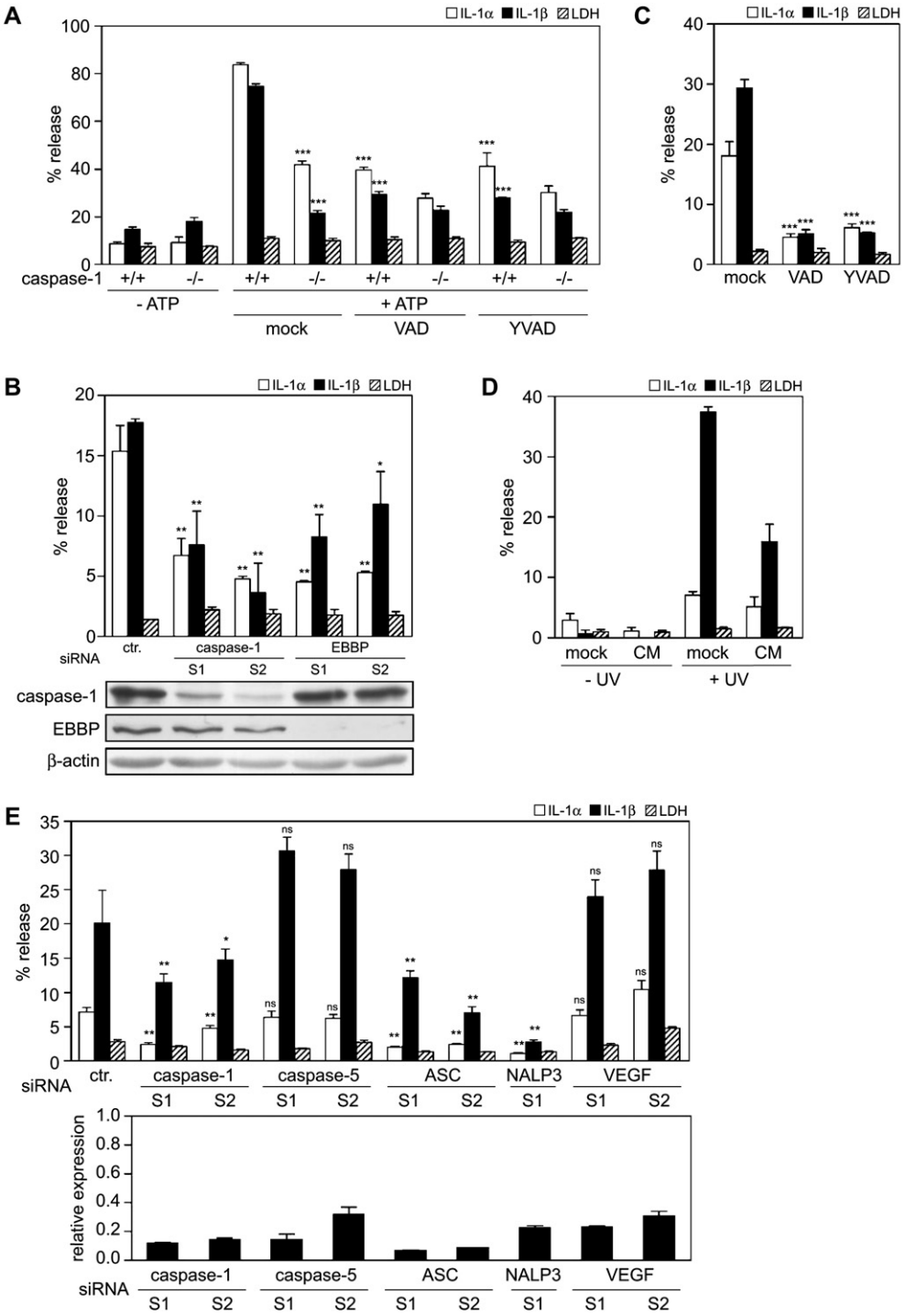



**Figure 1. Secretion of proIL-1α Requires Caspase-1 Activity**

(A) Peritoneal macrophages from caspase-1-deficient (−/−) or wild-type (+/+) mice were stimulated with LPS (1 μg/ml; 16 hr) followed by ATP (5 mM; 30 min) or mock treated. Before ATP stimulation, cells were treated with a pan-caspase inhibitor (VAD; 10 μM) or a caspase-1 inhibitor (YVAD; 10 μM) or mock treated. The percentages of IL-1α and -β release were determined by ELISA measurements of cytokine levels in supernatant and cell lysates. LDH activity served as a control for cell lysis.

(B and E) Human primary keratinocytes were transfected with random (ctr.) or specific siRNAs (S1, S2) against caspase-1, EBBP (B), or the indicated inflammasome proteins (E). Forty-eight hours after transfection, cell lysates were analyzed by western blot analysis with the indicated antibodies (B) or 24 hr after transfection by real-time RT-PCR (E, for knockdown efficiencies at the protein level see similar experiment in Feldmeyer et al., 2007). Alternatively, they were irradiated 24 or 48 hr after transfection with 50 mJ/cm$^2$ and incubated for 4 hr. The percentages of released IL-1α and -β and cell lysis were determined as in (A).

caspase-1 resulted in formation of an active enzyme as reflected by processing of proIL-1β, and secretion of IL-1β and proIL-1α (Figure 2B). This activity was absent when p10 or p20 were transfected alone. Upon transfection of the inactive p20(C285A) subunit instead of the wild-type subunit, maturation of proIL-1β was hardly detectable. Most importantly, the concentration of IL-1α found in the supernatant under these conditions was also much lower. These results demonstrate that enzymatically active full-length caspase-1 or active heterotetramer are required for efficient secretion of proIL-1α and IL-1β.

Since secretion of caspase-1 itself required an intact active site (Figure 2A), we determined if this is also the case for the $\alpha_2\beta_2$ tetramer. For this purpose we transfected cells with Myc-tagged p20. Processing of proIL-1β and secretion of IL-1β and proIL-1α demonstrated the formation of a functional heterotetramer (Figure 2C). Similar to procaspase-1 (Figure 2A), only active p20 was secreted but not the inactive mutants. Furthermore, secretion of p20 was only detected in the presence of the p10 subunit, demonstrating that only the tetramer but not the isolated p20 subunit is secreted (Figure 2D). However, secretion of enzymatically inactive procaspase-1 was restored in the presence of wild-type caspase-1 (Figure 2E). As expected, only caspase-1 induced IL-1β secretion under these conditions, but not caspases-5 or -9 (Figure S1 available online). To further address the role of caspase-1 activity for secretion we performed an siRNA-mediated knockdown of caspase-1 in keratinocytes and restored the levels of caspase-1 by transfection with plasmids encoding either wild-type or active site-mutated caspase-1. Upon UV irradiation, only the wild-type protein restored secretion but not the mutated protein (Figure S2). Similarly, YVAD treatment of keratinocytes before irradiation reduced the secretion of procaspase-1 and particularly of activated caspase-1 (Figure 2F).

We conclude from these experiments that procaspase-1 and the $\alpha_2\beta_2$ heterotetramer require enzymatic activity to promote secretion of IL-1 and their own secretion.

## Caspase-1 Binds to proIL-1α

ASC is an example of an unconventionally secreted protein, which is not a substrate of caspase-1 but a binding partner (Mariathasan et al., 2004). To determine if proIL-1α binds to caspase-1, we immunoprecipitated IL-1α from lysates of UVB-irradiated or control keratinocytes. Indeed, caspase-1 was present in the precipitate of both samples. Interestingly, the amount of caspase-1 bound to IL-1α was clearly enhanced 30 min after irradiation (Figure 3A). Therefore, UV irradiation enhances direct or indirect association of both proteins and concomitant secretion of IL-1α. Binding was confirmed by coimmunoprecipitation of proteins overexpressed in COS-1 cells (Figure 3B), indicating a direct interaction. ProIL-1α bound to the p20 subunit of caspase-1 but not to the CARD, which is an adaptor domain for homotypic interactions (Figure 3B). Interaction between proIL-1α and the p20 subunit was independent of the active site cysteine (Figure 3C).

## Caspase-1 Is a Binding Partner of FGF-2 and Enhances Its Secretion

The effect of caspase-1 on secretion of both IL-1 variants raises the possibility of a more general role of caspase-1 in unconventional secretion. To address this question we determined if caspase-1 affects release of FGF-2. The latter is a secreted growth factor, although it lacks a signal peptide (Nickel, 2005). Interestingly, FGF-2 exhibits a weak homology to IL-1 and shares the same β strand fold (Dinarello, 1998). We used primary skin-derived fibroblasts to study FGF-2 secretion because expression of this growth factor is very low in macrophages and keratinocytes (data not shown). Irradiation of human fibroblasts with a physiological dose of 5 mJ/cm$^2$ UVA strongly increased FGF-2 secretion (data not shown). Interestingly, knockdown of caspase-1 in fibroblasts significantly reduced FGF-2 secretion compared to cells transfected with scrambled siRNA or siRNA corresponding to the unrelated VEGF-A (Figure 4A). The dependence of FGF-2 secretion on caspase-1 expression was confirmed with fibroblasts from caspase-1 knockout mice and wild-type littermates (Figure 4B). Furthermore, treatment of human fibroblasts with caspase-1 inhibitors significantly reduced the amount of secreted FGF-2 (Figure 4C), demonstrating that efficient FGF-2 secretion requires not only caspase-1 expression but also caspase-1 activity. However, UV-induced secretion of FGF-2 was not fully dependent on caspase-1 since low amounts were still secreted in the absence of this proteinase.

We next examined a possible interaction between caspase-1 and FGF-2 in UVA-irradiated human fibroblasts by coimmunoprecipitation (Figure 4D). Indeed, interaction of the endogenous proteins was observed in these cells. As shown for the interaction with IL-1α, caspase-1 interacted with FGF-2 through the p20 subunit (Figure 4E). These experiments revealed that caspase-1 is also important for secretion of FGF-2, and that this effect is most likely mediated via physical interaction.

As FGF-2 plays an important role in vascularization during cutaneus wound repair (Broadley et al., 1989), we analyzed this process in 5 day wounds of caspase-1 knockout mice and wild-type littermates. Interestingly, wound angiogenesis was significantly reduced in caspase-1 knockout mice, suggesting that the caspase-1-mediated release of FGF-2 may be relevant in vivo under stress conditions (Figure S3).

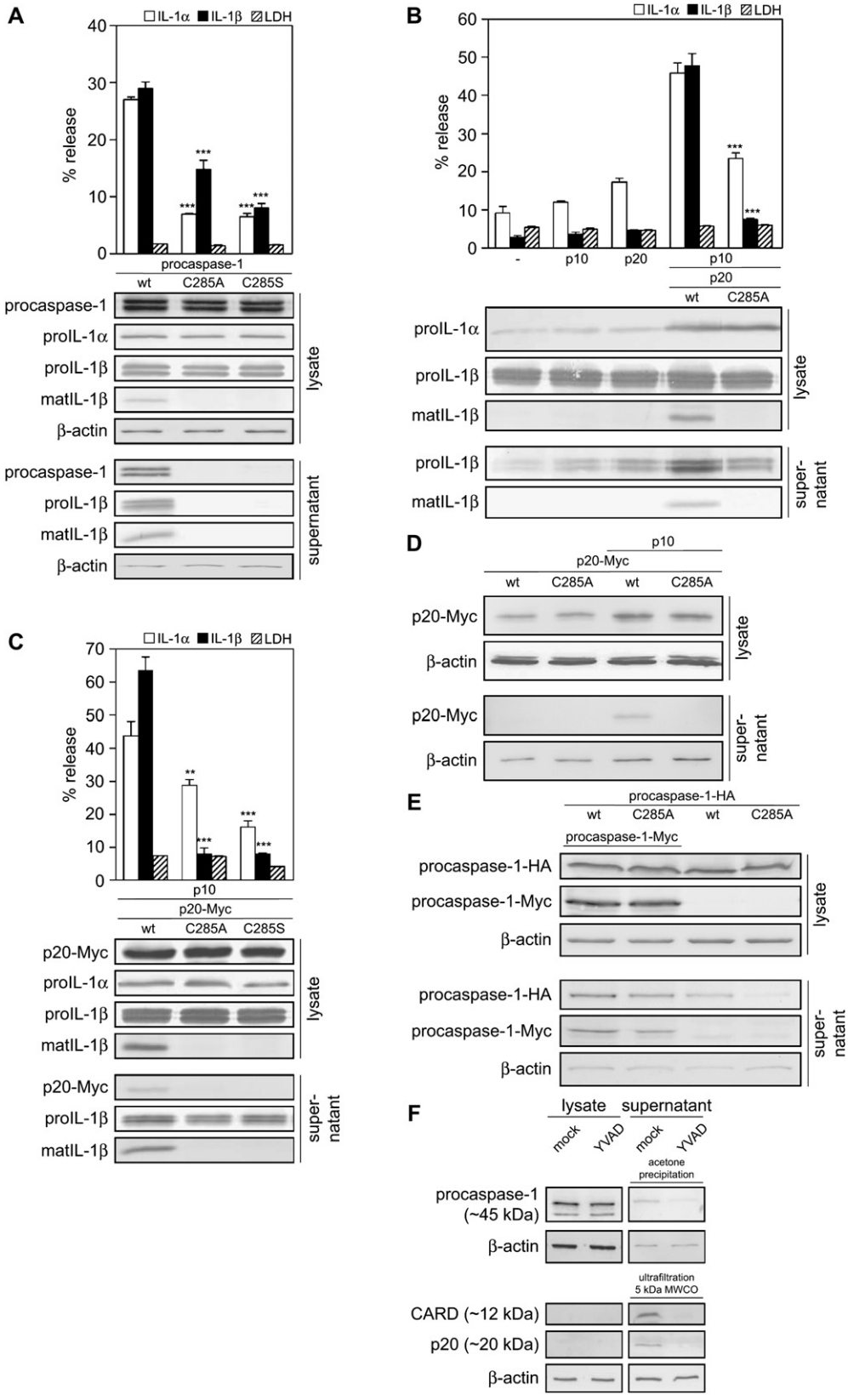
## iTRAQ Analysis Identifies Proteins that Are Secreted in a Caspase-1-Dependent Manner

Finally, we used an unbiased proteomics approach to determine a possible role of caspase-1 as a general regulator of unconventional protein secretion. The mass-spectrometry-based iTRAQ method (Ross et al., 2004) allows quantitative comparison of

(C) Human keratinocytes were treated with caspase inhibitors as in (A) and subsequently UVB-irradiated as in (B).

(D) Human keratinocytes were either incubated with conditioned medium (CM) or control medium and UVB-irradiated as in (B) or mock treated. CM was from UVB-irradiated keratinocytes incubated for 4 hr and contained 73 ± 2 pg/ml IL-1α and 251 ± 22 pg/ml IL-1β. These values were subtracted accordingly before calculating the IL-1 release of the cells grown in CM.

Bars represent mean ± standard deviation (SD) of three independent experiments. In (A) and (C), p values were calculated by Student's t test against mock-treated cells. In (B) and (E), p values were calculated by ANOVA with post Dunnett's test against control cells.


A
IL-1α IL-1β LDH
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matIL-1β
β-actin
supernatant
B
IL-1α IL-1β LDH
% release
- p10 p20 p10 p20
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IL-1α IL-1β LDH
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β-actin
lysate
procaspase-1-HA
procaspase-1-Myc
β-actin
supernatant
F
lysate supernatant
mock YVAD mock YVAD
acetone precipitation
procaspase-1 (~45 kDa)
β-actin
ultrafiltration 5 kDa MWCO
CARD (~12 kDa)
p20 (~20 kDa)
β-actin

protein abundance by comparing MS-peak intensities of different labels. We used keratinocytes for this experiment, as they are less sensitive to cell lysis and concomitant unspecific protein release than macrophages or fibroblasts. Using this approach we identified proteins that are secreted from keratinocytes within 4 hr after UVB irradiation in the presence or absence of YVAD.

Proteins from the culture supernatants were concentrated, digested, labeled with distinct iTRAQ labels, and mixed (ctr. iTRAQ-116; YVAD iTRAQ-114). After two-dimensional LC-fractionation, peptides were identified and quantified by tandem mass spectrometry.

In total, 961 proteins were identified (see Supplemental Data). The iTRAQ 116/114 ratios of the identified proteins ranged from 0.420 (secretion inhibited by caspase-1) to 1.889 (secretion promoted by caspase-1). IL-1 receptor antagonist (IL-1Ra) was the protein with the lowest iTRAQ ratio (1.220) for which we could verify a caspase-1-dependent secretion by western blot analysis or multiplex cytokine array (Figure 5A and data not shown). Therefore, we set the threshold to 1.220. Seventy-seven proteins remained, which are likely to be secreted in a caspase-1-dependent manner. The function of at least 26 of these proteins is known (Table 1). Interestingly, seven of them are known to be secreted via an ER/Golgi-independent pathway. They include annexin A2 (Danielsen et al., 2003), peroxiredoxin-1 (Chang et al., 2006), thymosin β-10 (Huang et al., 2006a), cystatin-A (Heidtmann et al., 1997), HMGI-C/HMGA2 (Donaldson et al., 1998), as well as macrophage migration inhibitory factor (MIF) and galectin-3 (Rubartelli, 2005; Nickel, 2005). Eight other proteins harbor a signal peptide. Four proteins, contactin-1, MMP14/(MT1-MMP), jagged-1, and desmoglein-3, are transmembrane proteins, suggesting that their detection in the supernatant is due to shedding.

To verify the results of the screen we analyzed the secretion of several identified proteins in independent experiments. For this purpose we performed western blot analyses of lysates and supernatants from UV-irradiated cells after YVAD treatment (Figure 5A) or after caspase-1 knockdown (Figure 5B). We verified caspase-1-dependent secretion for uncleaved Bid, Aip-1/Wdr-1, annexin A2, and peroxiredoxin-1. Secretion of the leaderless protein thioredoxin-1 (Rubartelli, 2005) was also dependent on caspase-1, although its iTRAQ ratio suggested a caspase-1-independent secretion. In addition, caspase-1-independent release or secretion was verified for β-actin, gelsolin, and FGF-BP.

Annexin A2 has been reported to be associated with exosomes (Fevrier and Raposo, 2004). Therefore, we analyzed the possibility that annexin A2, caspase-1, and other unconventionally secreted proteins are associated with vesicles in the supernatant. However, ultracentrifugation of supernatant from UV-irradiated keratinocytes (4 hr after irradiation) revealed that the investigated proteins were not in vesicles but were in the soluble fraction (Figure S4 and data not shown).

Finally, we determined whether the observed influence of caspase-1 on secretion of various proteins is restricted to keratinocytes. For this purpose we analyzed cell lysate and supernatant of activated THP-1 macrophages in the presence or absence of YVAD (Figure 5C). The caspase-1-dependent secretion of the investigated proteins strongly correlated with the results obtained with keratinocytes.

In summary, these experiments demonstrate a general role of stress-activated caspase-1 and the inflammasome in unconventional protein secretion.

## DISCUSSION

Caspase-1 has a well-established function in inflammation via the activation of proinflammatory cytokines (Dinarello, 1998). In addition, it has been reported to mediate either cell death (Cookson and Brennan, 2001) or survival (Gurcel et al., 2006) upon infection by certain pathogens. Here we found that activation of caspase-1 by inflammasome complexes is directly linked to unconventional secretion of a variety of leaderless proteins, most likely via a direct or indirect physical interaction.

Unconventional secretion has been a mystery since its discovery but can be clearly distinguished from the classical secretion pathway. Nevertheless, a generally accepted mechanism of leaderless secretion is still missing, although several models exist (Andrei et al., 2004; Carta et al., 2006; MacKenzie et al., 2001; Nickel, 2005; Qu et al., 2007; Rubartelli, 2005). It seems to be a fast process, making it difficult to track proteins during their release. In addition, analysis of unconventional secretion has been hampered by the fact that secretion was studied in different cell types, making a comparison of the results very difficult. Furthermore, it is usually activated by cellular stress (e.g., heat shock for FGF-2 release), which also results in cell lysis and in unspecific release that masks regulated secretion.

In this study we demonstrate a role of enzymatically active caspase-1 in secretion of leaderless proteins: (1) We confirmed that activated macrophages from caspase-1 knockout mice secrete lower levels of IL-1α, which is not a substrate of caspase-1 (Figure 1A). Dependency of proIL-1α secretion on caspase-1 was also observed in UV-irradiated human keratinocytes (Figures 1B–1E), which represent an even better model because

**Figure 2. Secretion of IL-1 and Caspase-1 Depends on Enzymatic Activity of Caspase-1**

COS-1 cells were transiently transfected with expression plasmids coding for the indicated proteins (A–E). Lysates and supernatants were collected 12 hr after transfection. Expression and secretion were determined by western blot analysis with the indicated antibodies. When tagged proteins were expressed, antibodies against these epitopes were used. Cells were transfected with vectors encoding proIL-1α and -β and wild-type (wt) or an active site mutated (C285A, C285S) procaspase-1 (A), the p10 subunit and the wt or C285A/C285S p20 subunit of caspase-1 (B), or the Myc-tagged wt or C285A/C285S p20 subunit (C). (A–C) The percentages of IL-1α and -β release were determined by ELISA measurements of cytokine levels in the supernatants and cell lysates. LDH activity served as control for cell lysis. Bars represent mean ± SD of three independent experiments; p values were calculated by ANOVA with post Dunnett's test against wt transfected cells. (D) Cells were transfected with expression vectors coding for p10 and Myc-tagged wt or C285A p20 subunits as indicated. (E) Cells were transfected with vectors encoding Myc-tagged wt procaspase-1 and HA-tagged wt or C285A procaspase-1. (F) Human keratinocytes were treated with YVAD or mock treated and irradiated with 50 mJ/cm$^2$ UVB. Four hours post-irradiation, cell lysate and supernatant were collected and analyzed by western blot. Proteins from the supernatant were either acetone precipitated (concentration factor = 3 in comparison to the amount of lysate) or concentrated by ultrafiltration (concentration factor = 65 in comparison to the amount of lysate). The antibodies used are indicated.

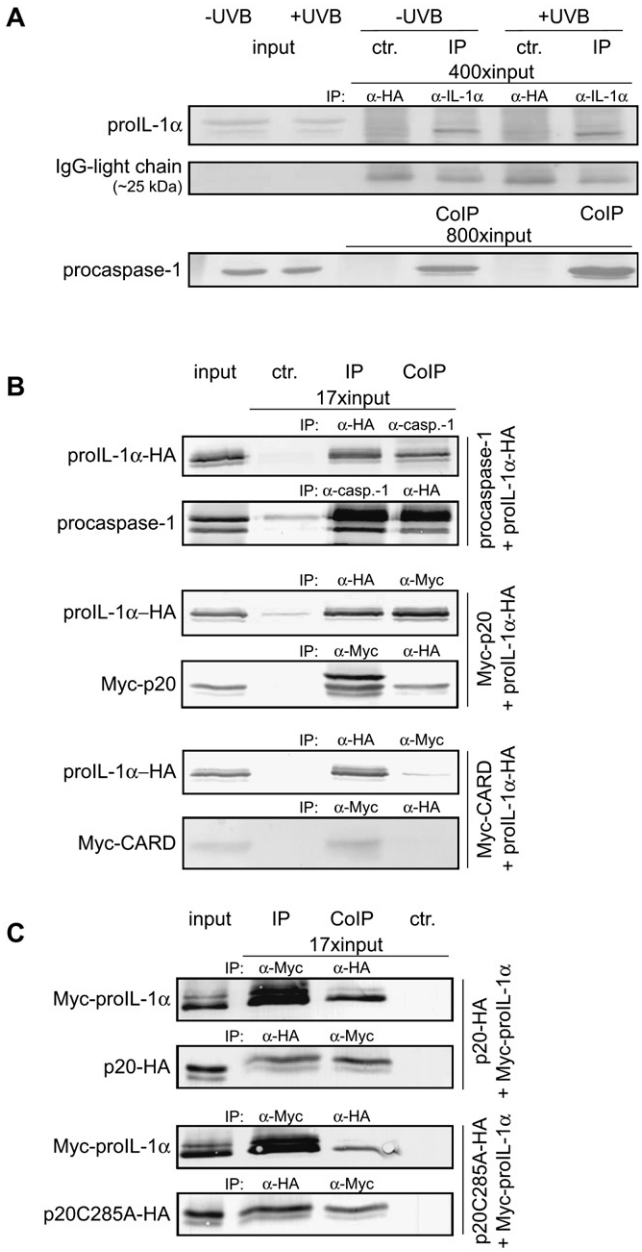


**Figure 3. Caspase-1 Binds to proIL-1α**

Coimmunoprecipitation (CoIP) of proIL-1α and procaspase-1 or subdomains of this protein from cell lysates is shown. Antibodies used for IP or western blot analysis are indicated. When tagged proteins were expressed, antibodies against these epitopes were used.

(A) Endogenous proIL-1α was precipitated from lysate of human primary keratinocytes, which had been irradiated with 50 mJ/cm$^2$ UVB 30 min before lysis or not irradiated. Incubation with antibodies against the unrelated HA-epitope was performed as a negative control (ctr.).

(B) COS-1 cells were transiently cotransfected with expression plasmids encoding the indicated proteins. Where indicated, HA- or Myc-tagged versions of the proteins were expressed. Incubation with protein A sepharose alone served as control (ctr.).

(C) As in (B) but with wild-type p20 compared to enzymatically inactive p20 (C285A).

proIL-1α is not processed in these cells. We further demonstrated that activation of caspase-1 and therefore secretion of proIL-1α is mediated by the inflammasome (Figure 1E); (2) We found that proIL-1α physically interacts with caspase-1 (Figure 3), suggesting that caspase-1 binding is a prerequisite for unconventional secretion and/or that caspase-1 acts as a transport vehicle in this pathway. This interaction is enhanced by UV irradiation but is independent of the active site cysteine; (3) We showed that the active site cysteine in procaspase-1 and in the activated $\alpha_2\beta_2$ tetramer is required for secretion of proIL-1α and of pro- and mature caspase-1 itself (Figure 2). However, it is not clear whether active caspase-1 is the carrier in the transport process or whether it activates a secretion machinery via its activity; (4) Secretion of FGF-2, which is released independently of a signal peptide, was also dependent on UV-induced caspase-1 activity in fibroblasts (Figures 4A and 4B), and FGF-2 also interacted with caspase-1 in these cells (Figures 4D and 4E); and (5) iTRAQ proteomics identified additional proteins, which are secreted upon activation of caspase-1. Because the vast majority of identified proteins were found at similar concentrations in the supernatant of caspase-1 inhibitor-treated as well as mock-treated cells (see Supplemental Data), these proteins can be considered as controls for unspecific release due to cell lysis (e.g., LDH) or to be secreted via the classical or another caspase-1-independent pathway (e.g., gelsolin). Most importantly, we identified several proteins that are secreted independently of the ER/Golgi pathway. Caspase-1 may regulate the secretion of some proteins indirectly, e.g., by enhancing their expression, since only the supernatant was analyzed in the screen. At least expression of IL-1Ra and RNase 7 is enhanced by IL-1 (Mee et al., 2005; Harder and Schroder, 2002) and, therefore, indirectly by caspase-1 activity. However, this possibility could be ruled out for Bid, Aip-1/Wdr-1, annexin A2, and peroxiredoxin-1 by western blot analysis of the lysates (Figure 5). Caspase-1 secretion may also play a direct or indirect role in shedding because we identified different amounts of transmembrane proteins in caspase-1 inhibitor- and mock-treated cells (contactin-1, MMP14/MT-MMP, desmoglein-3, jagged-1). Interestingly, amino-terminal fragments of desmoglein-3, which cover the iTRAQ peptide identified by MS/MS analysis, are present in human serum and are the targets of autoantibodies in Pemphigus Vulgaris (Lanza et al., 2006). Therefore, the stress-induced caspase-1 activation may result in higher levels of antigen in patients suffering from this autoimmune disorder.

Thioredoxin-1 is also secreted without a signal peptide (Rubartelli, 2005). Nevertheless, the average iTRAQ ratio of all detected thioredoxin-1 peptides suggests a caspase-1-independent secretion. We noticed, however, that the standard deviation between detected iTRAQ peptides was rather high in this case. By western blotting we found that thioredoxin-1 secretion was in fact strongly dependent on caspase-1 expression and activity. The reason for this discrepancy between iTRAQ and the western blot results may be due to posttranslational modifications of thioredoxin-1 (Haendeler, 2006), which may give rise to variants that are differentially detected by MS and by our antibody.

Although caspase-1 enhances secretion of various proteins, low amounts of these proteins seem to be secreted in a caspase-1-independent manner. This is especially true for proIL-1α

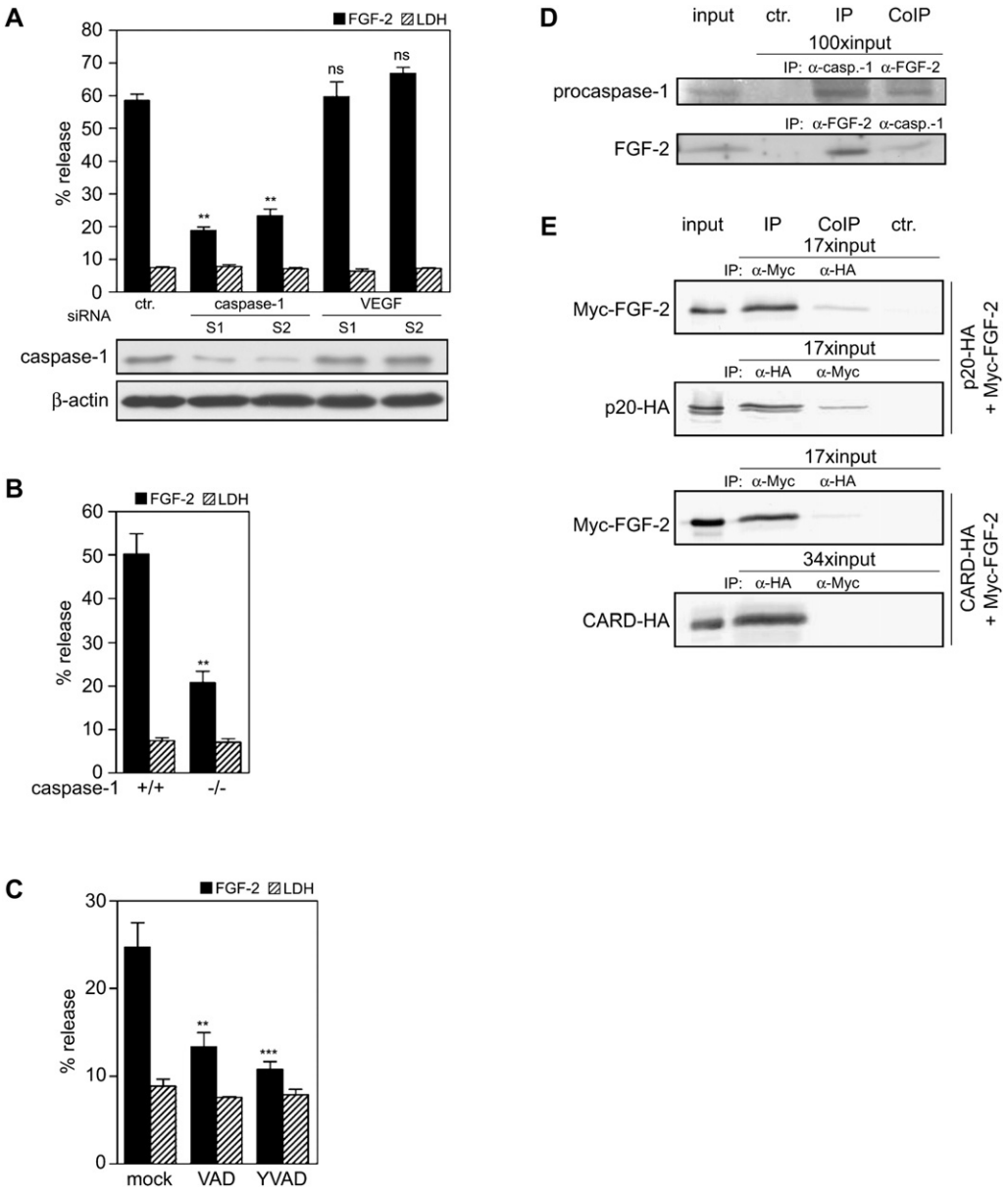


**Figure 4. Caspase-1 Binds to FGF-2 and Promotes Its Secretion**

(A–C) The percentage of FGF-2 release was determined by ELISA measurements of the growth factor in supernatants and cell lysates. LDH activity served as control for cell lysis. Bars represent mean ± SD of three independent experiments. (A) Human primary fibroblasts were transfected with random (ctr.) or specific siRNAs (S1, S2) against caspase-1 or VEGF. Forty-eight hours later, cells were either harvested for western blot analysis with the indicated antibodies or UVA-irradiated with 5 mJ/cm$^2$ and incubated for 6 hr. p values were calculated by ANOVA with post Dunnett's test against control cells. (B) Caspase-1-deficient murine fibroblasts (−/−) and wild-type (+/+) cells were UVB-irradiated with 40 mJ/cm$^2$ UVB or mock treated. Cells and supernatants were harvested 6 hr later. p values were calculated by Student's t test against stimulated wild-type cells. (C) As in (A) but treated with caspase-1 inhibitors. p values were calculated by Student's t test against mock-treated cells.

(D and E) CoIP of FGF-2 and procaspase-1 or its subdomains is shown. Antibodies used for IP or western blot analysis are indicated. When tagged proteins were expressed, antibodies against these epitopes were used. Treatment with protein A sepharose alone served as a negative control (ctr.). (D) CoIP of endogenous FGF-2 and procaspase-1 from lysates of human primary fibroblasts. (E) COS-1 cells were transiently cotransfected with expression plasmids coding for the indicated HA- or Myc-tagged proteins; lysates were used for CoIP.

(e.g., Figure 1A) and FGF-2 (Figures 4A and 4B). Importantly, however, the relative amount of these proteins found in the supernatant is significantly higher than the corresponding LDH activity. This argues against unspecific release of these proteins due to cell lysis and for a partial caspase-1-independent secretion of these leaderless proteins. This may also explain why the phenotype of mice lacking FGF-2 differs from that of caspase-1 knockout mice under homeostatic conditions. However, we

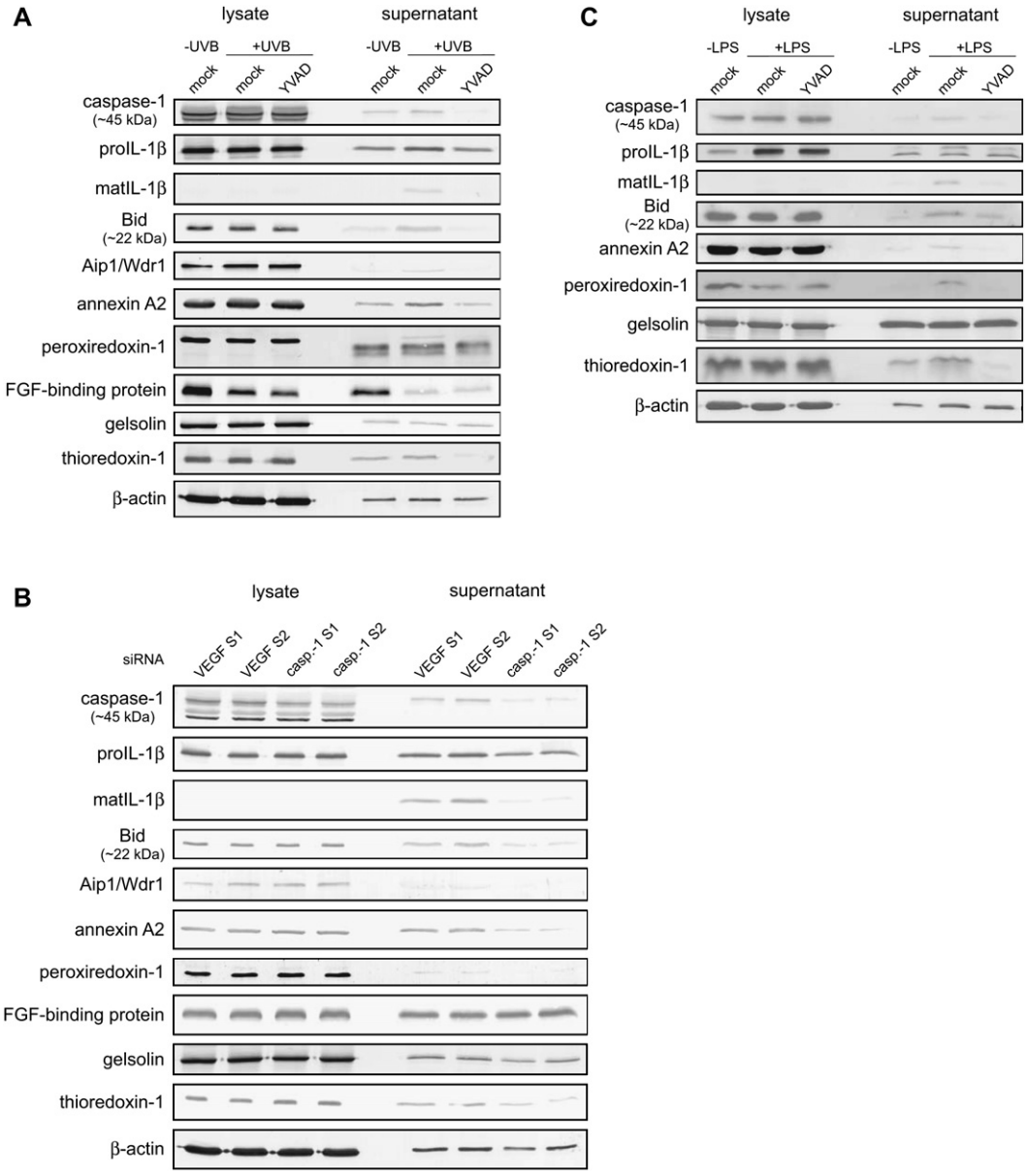


**Figure 5. Identification of Proteins Secreted in a Caspase-1-Dependent Manner**

Western blot analysis of selected secreted proteins using cell lysate and supernatant 4 hr after irradiation with 50 mJ/cm$^2$ UVB (A and B) or 6 hr after LPS stimulation (C). (A) Human keratinocytes were treated with YVAD or mock treated. (B) Human keratinocytes were transfected with specific siRNAs (S1, S2) against caspase-1 or VEGF as a control. (C) THP-1 cells were differentiated with TPA (20 ng/ml; 72 hr). Cells were either YVAD or mock treated and stimulated with LPS (1 μg/ml) or solvent.

found reduced wound angiogenesis in caspase-1 knockout mice, which correlates with the known role of FGF-2 in vascularization of skin wounds (Broadley et al., 1989). This suggests that caspase-1-mediated release of FGF-2 may be particularly important under stress conditions.

Interestingly, many of the proteins, which are secreted in a caspase-1-dependent manner, are involved in inflammation, cytoprotection, and/or tissue repair. Therefore, activation of caspase-1 by various environmental challenges, e.g., UV irradiation, infection, or wounding, seems to be coupled to the release of proteins, which helps to restore the cell and tissue homeostasis. This hypothesis is consistent with the predominant expression of caspase-1 by inflammatory and epithelial cells (Lin et al., 2000), which are particularly involved in cellular defense mechanisms. Proteins such as IL-1, MIF, and thioredoxin are critical extracellular regulators of inflammation (Rubartelli, 2005; Schwertassek et al., 2007). FGF-2 plays an important role in tissue repair and angiogenesis, processes that are required during and after acute or chronic inflammation (Nickel, 2005; Rubartelli, 2005). Peroxiredoxin-1 and thioredoxin are

**Table 1. Function and Properties of Selected Proteins Identified by iTRAQ**

| Protein | Function | Subcellular Localization | SP | Secretion | iTRAQ 116/114 | iTRAQ STDEV | iTRAQ Peptides | Verification (Western Blot) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Keratinocytes (UVB Irradiated) | | Macrophages (THP-1; LPS/ATP stim.) |
| | | | | | | | | Inhibitor (YVAD) | siRNA | Inhibitor (YVAD) |
| Bid (P55957) | apoptosis, cytochrome *C* release | CP, mt membrane | – | unknown | 1.889 | – | 1 | ↓↓ | ↓ | ↓↓ |
| Aip1/Wdr1 (O75083) | cell motility, actin disassembly | CP | – | unknown | 1.813 | 1.345 | 3 | ↓↓ | ↓ | – |
| Coronin-3 (Q9ULV4) | actin dynamics, signal transduction | CP | – | unknown | 1.448 | – | 1 | – | – | – |
| Annexin A2 (P07355) | $Ca^{2+}$-dependent phospholipid binding, membrane dynamics | membrane-associated basement membrane, EC | – | nonclassical [1–3] | 1.408 | 0.405 | 48 | ↓ | ↓ | ↓↓ |
| Glutathione S-transferase theta 2 (P30712) | detoxification | CP | – | unknown | 1.402 | – | 1 | – | – | – |
| Contactin-1 (Q12860) | GPI-anchor, cell adhesion, Notch ligand | cell membrane (EC) | + | unknown | 1.379 | – | 1 | – | – | – |
| MMP14 (P50281) | activates progelatinase, angiogenesis, tumor invasion | single-pass type I membrane protein | + | unknown | 1.365 | – | 1 | – | – | – |
| Macrophage migration inhibitory factor (P14174) | inflammation, cell motility, macrophage regulation at sites of inflammation | CP, EC | – | nonclassical [4, 5] | 1.347 | 0.488 | 3 | – | – | – |
| Calnexin (P27824) | ER chaperone | ER membrane, single-pass type I membrane protein | + | unknown | 1.341 | – | 1 | – | – | |
| Fli-1 (Q01543) | transcription factor | nucleus | – | unknown | 1.335 | – | 1 | – | – | – |
| Desmoglein-3 (P32926) | component of desmosomes, cell-cell contact | single-pass type I membrane protein | + | unknown | 1.332 | 0.001 | 2 | – | – | – |
| ARPC1 (O15143) | actin dynamics | CP | – | unknown | 1.330 | – | 1 | – | – | – |

(*Continued on next page*)

**Table 1.** *Continued*

| Protein | Function | Subcellular Localization | SP | Secretion | iTRAQ 116/114 | iTRAQ STDEV | iTRAQ Peptides | Verification (Western Blot) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Keratinocytes (UVB Irradiated) | | Macrophages (THP-1; LPS/ATP stim.) |
| | | | | | | | | Inhibitor (YVAD) | siRNA | Inhibitor (YVAD) |
| Kallikrein 10 (O43240) | protease | EC | + | classical | 1.289 | – | 2 | – | – | – |
| PAI-1 (P05121) | protease inhibitor | EC | + | classical | 1.283 | 0.090 | 6 | – | – | – |
| Peroxire-doxin-1 (Q06830) | redox regulation, interacts with the thioredoxin system | CP, EC | – | nonclassical [6] | 1.265 | 0.190 | 13 | ↓ | ↓ | ↓↓ |
| Laminin β3 (Q13751) | mediates cell-ECM contacts | EC | + | classical | 1.261 | 0.083 | 7 | – | – | – |
| Thymosin β-10 (P63313) | cytoskeletal organization | CP, EC | – | nonclassical [7] | 1.255 | 0.221 | 5 | – | – | – |
| Cystatin-A (P01040) | cathepsin inhibitor | CP, EC | – | nonclassical [8] | 1.251 | – | 1 | – | – | – |
| Jagged-1 (P78504) | notch signaling | single-pass type I membrane protein | + | unknown | 1.247 | 0.028 | 2 | – | – | – |
| Galectin-3-binding protein (Q08380) | mediates integrin-mediated cell adhesion | EC | + | classical | 1.241 | 0.205 | 4 | – | – | – |
| LEI (P30740) | regulates various proteases | CP | – | unknown | 1.240 | 0.137 | 7 | – | – | – |
| RNase 7 (Q9H1E1) | antimicrobial, induced by IL-1β | EC | + | classical | 1.235 | – | 1 | – | – | – |
| Laminin α3 (Q6VU68) | ECM component | EC | + | classical | 1.233 | 0.227 | 20 | – | – | – |
| HMGA2 (P52926) | chromatin regulation | nucleus, EC | – | nonclassical reported for other HMG proteins [9] | 1.231 | – | 1 | – | – | – |
| Galectin-3 (P17931) | Lectin, which binds IgE, stimulates endothelial cell migration | nucleus, EC | – | nonclassical [5, 10] | 1.220 | 0.137 | 8 | – | – | – |
| IL-1Ra (P18510) | inhibits IL-1R type I | CP, EC | + | classical | 1.220 | 0.178 | 3 | – | – | – |
| FGF-binding protein (Q14512) | tissue repair, angiogenesis, enhances FGF signaling | ES | + | classical | 1.208 | 0.032 | 2 | → | → | – |
| Gelsolin (P06396) | cell motiliy | CP, EC | + | classical | 1.077 | 0.154 | 5 | → | → | → |
| LDH (P00338) | metabolism, lysis marker | CP | – | no | 1.071 | 0.255 | 6 | – | – | – |

**Table 1. *Continued***

| Protein | Function | Subcellular Localization | SP | Secretion | iTRAQ 116/114 | iTRAQ STDEV | iTRAQ Peptides | Verification (Western Blot) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Keratinocytes (UVB Irradiated) | | Macrophages (THP-1; LPS/ATP stim.) |
| | | | | | | | | Inhibitor (YVAD) | siRNA | Inhibitor (YVAD) |
| Thioredoxin-1 (P10599) | redox regulation | CP, EC | – | nonclassical [8, 11–12] | 0.957 | 0.472 | 13 | ↓↓ | ↓ | ↓↓ |
| Galectin-1 (P09382) | apoptosis, differentiation | CP, EC | – | nonclassical [5, 10, 13] | 0.939 | 0.240 | 12 | – | – | – |
| EPLIN (Q59FE8) | actin dynamics | CP | – | unknown | 0.742 | 0.144 | 10 | – | – | – |
| GAPDH (P04406) | metabolism | CP | – | unknown | 0.699 | 0.139 | 30 | – | – | – |
| Lamin-A/C (P02545) | nuclear lamina | nuclear lamina | – | unknown | 0.551 | 0.113 | 17 | – | – | – |

Selection of representative proteins identified by an iTRAQ secretome screen. Proteins with a known biological function as well as those for which secretion is described in the literature are listed. The complete list of proteins with iTRAQ quantification can be downloaded (see Supplemental Data). The verification by western blot corresponds to data in Figure 5. Abbreviations and symbols: CP, cytoplasm; EC, extracellular; mt, mitochondria; SP, (ER) signal peptide; ↓, reduced; ↓↓, strongly reduced; →, secretion unaltered. Selected References: [1] Danielsen et al., 2003; [2] Faure et al., 2002; [3] Zhao et al., 2003; [4] Lolis and Bucala, 2003; [5] Nickel, 2003; [6] Chang et al., 2006; [7] Huang et al., 2006b; [8] Di Quinzio et al., 2007; [9] Nickel, 2005; [10] Hughes, 1999; [11] Nakamura et al., 2006; [12] Rubartelli et al., 1995; [13] Cooper and Barondes, 1990.

cytoprotective enzymes through their ability to detoxify reactive oxygen species (Nakamura et al., 2006; Rubartelli, 2005). In contrast, an extracellular function of caspase-1, Bid, and Aip-1/Wdr-1 has not been described yet. It may well be that release of these proteins is a means to reduce their intracellular concentration. It has been suggested that secretion of active caspase-1 prevents cell death (Martinon et al., 2002; Martinon and Tschopp, 2004). This could also be the case for Bid. Aip-1/Wdr-1 regulates cell migration via cofilin-mediated actin depolymerization (Li et al., 2007). It can interact with caspase-11, which in turn binds to caspase-1 (Wang et al., 1998). Therefore, this link between Aip-1/Wdr-1 and caspase-1 suggests that a caspase-1-mediated reduction in Aip-1/Wdr-1 via secretion inhibits cell migration (Li et al., 2007). For annexin A2 and thymosin β-10 an extracellular function has as yet not been described. However, it has been demonstrated that these proteins regulate exocytosis (Lorusso et al., 2006; Muallem et al., 1995). Therefore, the observed secretion of these proteins may indicate a function in the secretion process itself. It remains to be determined if some of these proteins are direct caspase-1 substrates, which regulate unconventional secretion, or if additional caspase-1 substrates are involved in this pathway. Recently, several caspase-1 substrates have been identified, of which some are involved in the regulation of the cytoskeleton and its dynamics (Shao et al., 2007). In addition, some of the unconventionally secreted proteins that we identified in our iTRAQ screens (Supplemental Data and data not shown) may also be caspase-1-binding proteins and substrates, and this should be tested in the future. Possible candidates are involved in the dynamics of microtubuli (e.g., EB1) or of the actin cytoskeleton (ADF, Aip-1, cofilin, ERM proteins). Others are involved in exocytosis and membrane protein mobility (synaptotagmin, annexins) or in the regulation of membrane composition (phospholipases and their regulators, e.g., annexin A1). In support of this hypothesis, phospholipases play a prominent role in lysosome-mediated secretion of IL-1β (Andrei et al., 2004). All these proteins may be involved in vesicular trafficking and may thus be components of a complex machinery that mediates unconventional secretion. The characterization of the latter will be a major task for the future.

In summary, our results demonstrate a role of caspase-1 in ER/Golgi-independent protein secretion, one of the most poorly characterized processes in cell biology. In particular, they strongly suggest that stress-induced activation of the inflammasome and thereby of caspase-1 has unexpected consequences: It not only induces inflammation via activation and secretion of proinflammatory cytokines, but it also regulates the extracellular levels of proteins involved in tissue repair and cytoprotection. Therefore, it directly links inflammation to protective and regenerative processes.

## EXPERIMENTAL PROCEDURES

### Plasmid or siRNA Transfection of Cells

COS-1 cells were transiently transfected with expression plasmids using Lipofectamine 2000 (Invitrogen, Basel, Switzerland). Cells were grown to 80% confluency in 6-well plates. Immediately before transfection with 8 μg of plasmid DNA, growth medium was replaced by OptiMEM (Invitrogen).

Human keratinocytes were transfected with siRNA in 12-well plates using Profectin65 (Atugen, Berlin, Germany) as described (Feldmeyer et al., 2007) or INTERFERin (Polyplus, Illkirch, France). Human fibroblasts were grown to semiconfluency and transfected with siRNAs (final concentration: 100 nM) in 12-well plates using INTERFERin.

### Stimulation of Protein Secretion

Murine macrophages ($2 \times 10^5$ cells/cm$^2$) were grown in 24-well plates and stimulated with 1 μg/ml LPS for 16 hr and subsequently with 5 mM ATP for 30 min. Medium was changed, and conditioned medium was harvested after 3 hr. Cells from four animals per genotype were pooled.

THP-1 cells (2 × $10^5$ cells/$cm^2$) were differentiated with 20 ng/ml TPA for 3 days. Medium was changed and 10 μM caspase-1 inhibitor or solvent (DMSO) was added. Thirty minutes later, cells were treated with 1 μg/ml LPS. Cells and supernatant were harvested after 4 hr.

Human keratinocytes were either transfected with siRNA or treated with 10 μM caspase inhibitors or solvent (DMSO) only. Forty-eight hours after transfection the medium was changed if not otherwise indicated and cells were irradiated with UVB (50 mJ/$cm^2$). For treatment with inhibitors the medium was changed before addition of inhibitor; cells were irradiated 45 min later. Secreted proteins were collected after 4 hr.

Murine and human fibroblasts were grown to confluency in 12-well plates in medium lacking FCS but supplemented with 1 U/ml heparin. Cells were then irradiated (40 mJ/$cm^2$ UVB for murine cells, 5 mJ/$cm^2$ UVA for human cells) in medium lacking phenol red, and conditioned medium was harvested 6 hr later. Irradiation was performed 48 hr after siRNA transfection or 30 min after addition of inhibitor. For experimental replicates, explant cultures from different animals were used.

COS-1 cells were left untreated, but medium was replaced 24 hr posttransfection, and secreted proteins were collected 12 hr later.

### Measurement of Protein Release from Stimulated Cells

After stimulation of cells, the supernatant was removed and adherent plus pelleted cells (1500 × g for 10 min) from the supernatant were lysed in 2% Triton X-100 in PBS. For immunoblotting, the supernatant was acetone precipitated. For ELISA, multiplex cytokine, and LDH measurements, the lysate was diluted with culture medium to the initial culture volume. The percent of release was individually calculated for each well according to the formula: $\%\,release = \frac{SN}{SN+Lys}$, where SN is the amount in the supernatant and Lys the amount in the lysate.

Each experiment was performed with triplicate dishes. ELISA and LDH measurements were performed in duplicate. ELISA kits against human and murine IL-1α, IL-1β, and FGF-2 were from R&D systems (Minneapolis, MN, USA). LDH activity assay was from Promega (Madison, WI, USA). Multiplex cytokine measurements were performed with the Bioplex multiplex cytokine measurement system (Bio-Rad, Hercules, CA, USA).

### iTRAQ Labeling

Human keratinocytes were grown in 14 cm dishes to 70% confluency. After three PBS washes, medium was replaced with Hanks' balanced salt solution (H 6648, Sigma) supplemented with 0.09 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Cells were either treated with 10 nM caspase inhibitors or solvent only and UVB-irradiated as described above. After centrifugation the supernatant was cleared by filtration (0.45 μm pore size). Supernatant of 10 dishes was concentrated to a volume of 1 ml with Amicon ultrafiltration units (5 kDa MWCO, Millipore, Billerica, MA, USA). Protein concentrations were determined by Bradford assay and by optical density at 280 nm.

Both samples were adjusted to the same protein concentration, and 150 μg of each was precipitated with acetone. Trypsin digestion and labeling with iTRAQ reagents were performed according to the supplier's instructions (Applied Biosystems, Foster City, CA, USA). The inhibitor-treated sample was labeled with the 114-dalton label, the DMSO treated sample with the 116-dalton label, respectively.

### Statistical Analysis

Statistical analysis was performed using the Prism Software (GraphPad Software, San Diego, CA, USA). Where multiple groups were compared to one control group, one-way ANOVA with posterior Dunnett's correction was performed. Different treatments were compared to control treatment by Student's t test. All tests were one-tailed; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

## SUPPLEMENTAL DATA

Supplemental Data include Experimental Procedures, four figures, and one table and can be found with this article online at http://www.cell.com/cgi/content/full/132/5/818/DC1/.

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