

The Inflammasome: A Molecular Platform Triggering Activation of Inflammatory Caspases and Processing of proIL- β

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Summary

Generation of Interleukin (IL)-1 β via cleavage of its proform requires the activity of caspase-1 (and caspase-11 in mice), but the mechanism involved in the activation of the proinflammatory caspases remains elusive. Here we report the identification of a caspase-activating complex that we call the inflammasome. The inflammasome comprises caspase-1, caspase-5, Pycard/Asc, and NALP1, a Pyrin domain-containing protein sharing structural homology with NODs. Using a cell-free system, we show that proinflammatory caspase activation and proIL-1 β processing is lost upon prior immunodepletion of Pycard. Moreover, expression of a dominant-negative form of Pycard in differentiated THP-1 cells blocks proIL-1 β maturation and activation of inflammatory caspases induced by LPS *in vivo*. Thus, the inflammasome constitutes an important arm of the innate immunity.

Introduction

Interleukin-1 β (IL-1 β) is a proinflammatory cytokine produced by activated macrophages and monocytes. It functions in the generation of systemic and local responses to infection, injury, and immunological challenges and is the primary cause of chronic and acute inflammation (Dinarello, 1998). As an endogenous pyrogen, it is also a key player in the febrile response (Dinarello et al., 1999). IL-1 β is produced as an inactive cytoplasmic precursor (proIL-1 β , p35) that must be cleaved (at Asp116) to generate the mature active form (p17). The IL-1 β -converting enzyme, better known as caspase-1 (Alnemri et al., 1996; Cerretti et al., 1992; Thornberry et al., 1992) is required for this cleavage. Caspase-1 is the prototypic member of a family of inflammatory caspases (including human caspase-4 and -5, and mouse caspase-11 and -12) which all contain an N-terminal caspase recruitment domain (CARD). Various microbial agents such as lipopolysaccharide (LPS) are known to induce the activation of caspase-1, but the precise molecular mechanism by which this occurs is unknown.

Apaf-1, NOD, and related family members of the nucleotide binding-site (NBS) family play central roles in caspase activation and apoptosis as well as in the activation of the transcription factor NF- κ B (Inohara and Nunez, 2001). An important feature of these proteins is

that they possess several distinct protein/protein interaction domains which are used to assemble large multi-component complexes. Apaf-1, for example, contains an N-terminal CARD followed by a NBS/self-oligomerization domain and a C-terminal WD-40 repeat (Jaroszewski et al., 2000; Koonin and Aravind, 2000; van der Biezen and Jones, 1998) (Figure 1A). Via these domains, Apaf-1 assembles a complex (called the apoptosome) that triggers caspase-9 activation. Caspase-9 is inducibly recruited to Apaf-1 via CARD/CARD interactions, when cytochrome-c binds to the C-terminal WD-40 domain of Apaf-1 (Li et al., 1997). Apoptosome assembly brings several caspase-9 molecules into close proximity resulting in their crossactivation (Shi, 2002).

Recently, we and others have identified novel members of the NBS family: NALP1 (CARD7, NAC, DEFCAP) (Bertin and DiStefano, 2000; Chu et al., 2001; Hlaing et al., 2001; Martinon et al., 2001), NALP2 (NBS1) (Bertin and DiStefano, 2000; Martinon et al., 2001), and NALP3 (Cryopyrin, Pypaf1) (Aganna et al., 2002; Hoffman et al., 2001; Manji et al., 2002), which like Apaf-1/NOD are comprised of multiple protein/protein interaction domains. We have focused our attention on NALP1 which is widely expressed with highest expression levels found in PBLs, thymus, and spleen, suggesting a functional role for NALP1 in the immune system (Chu et al., 2001; Hlaing et al., 2001). Similar to the domain structure found in NOD family members (Inohara et al., 2002), the NBS domain of NALP1 is followed by leucine-rich repeats (LRRs); however, the N-terminal CARD motif is substituted by a CARD-like Pyrin domain (PYD), while a bona fide CARD domain is present at the C terminus (Figure 1A). The N-terminal PYD motif of NALP1 interacts with Pycard/Asc (Martinon et al., 2001), a bipartite adaptor protein containing an N-terminal PYD and a C-terminal CARD motif (Masumoto et al., 1999) (Figure 1A), thereby forming a putative protein complex armed with two distinct CARD motifs.

Results and Discussion

Pycard and NALP1 Bind and Activate Caspase-1 and Caspase-5

The CARD modules can mediate the interaction between CARD-containing caspases and their corresponding upstream activators, as exemplified by the interaction of caspase-9 and Apaf-1 (Hofmann, 1999), encouraging us to test whether the CARD-module of NALP1 also binds to a CARD-caspase (caspase-1, -2, -4, -5, and -9). A CARD-containing fragment of NALP1 strongly interacted with caspase-5, while a weak interaction was observed with caspase-2 and caspase-4, and no interaction with caspase-9 (Figures 1B and 1C). The caspase-5/NALP1 interaction induced processing of caspase-5, as reflected by the disappearance of the procaspase in cell extracts and the appearance of a 35 kDa cleavage product in coimmunoprecipitates (Figures 1C and 1F). Interestingly, only NALP1 mutants lacking the LRR, but not full-length NALP1, bind to both Pycard and caspase-5

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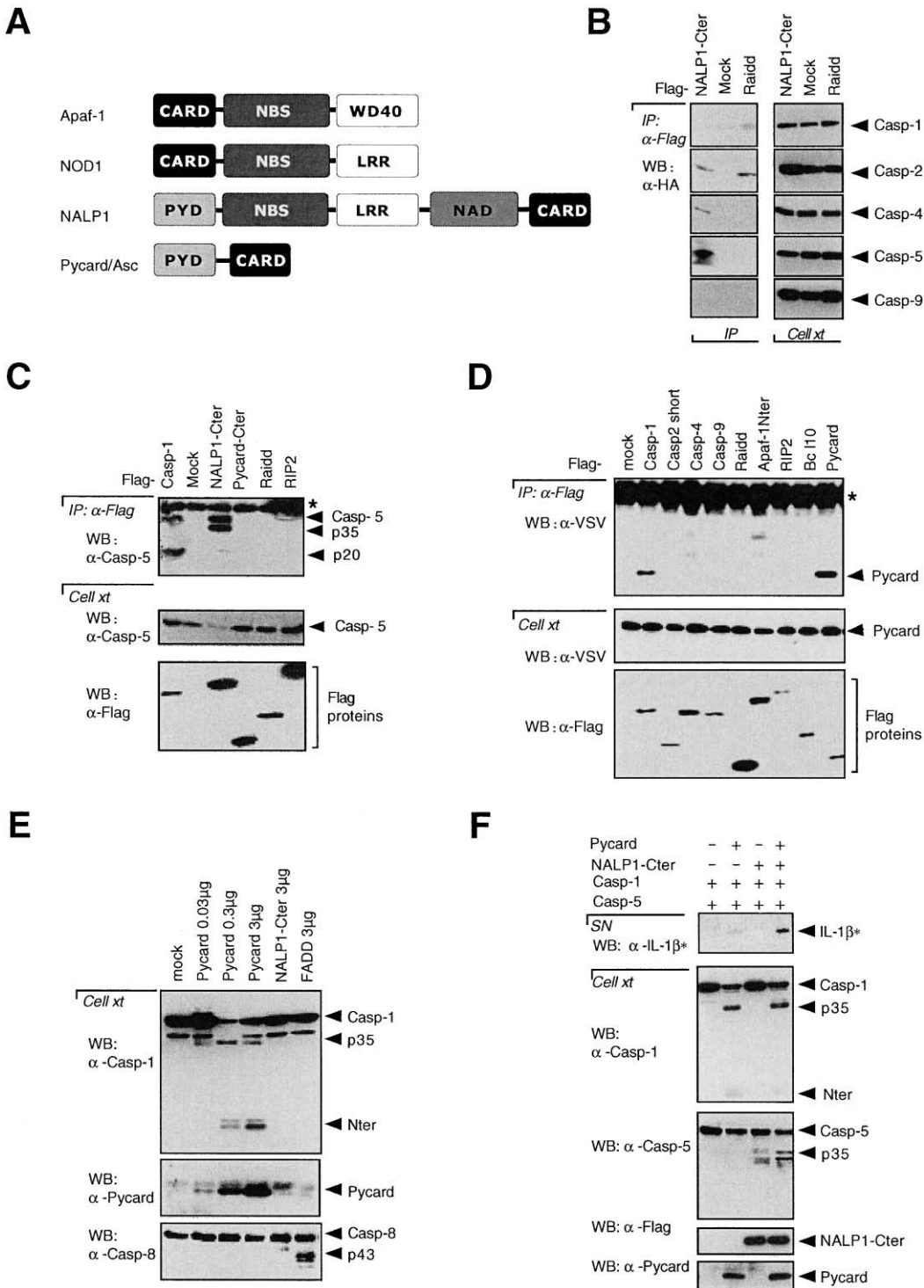


Figure 1. Pycard and NALP1 Bind and Activate Proinflammatory Caspases

(A) Domain structure of Pycard (Asc), NALP1, and the structurally related proteins Apaf-1 and NOD1. Caspase recruitment domain (CARD), Pyrin domain (PYD), leucine-rich repeats (LRR), nucleotide binding-site (NBS), WD domains, and a domain highly conserved in other NALP1-like proteins (NALP-associated domain, NAD) are indicated.

(B) Interaction of various CARD-caspases with NALP1-Cter. Flag-tagged proteins were immunoprecipitated from transfected 293T cells with a polyclonal anti-Flag antibody, and the associating HA-tagged caspases were detected via Western analysis with anti-HA antibodies.

(C) NALP1 interacts with caspase-5; caspase-5 was coexpressed with the indicated Flag-tagged expression constructs. Twenty-four hours after transfection, anti-Flag (M2) immunoprecipitates (IP) were analyzed for the presence of caspase-5, and cell extracts (xt) were immunoblotted with the indicated antibodies. p35, caspase-5 cleavage product containing the CARD domain, and the p20 caspase subunit are indicated. The asterisk indicates the IgG heavy chain.

and activate caspase-5 (data not shown). Removal of the LRR, therefore, constitutively activates NALP1, suggesting that the LRR may negatively regulate NALP1 activity in a manner similar to that proposed for the ligand binding domains of Ipaf and Apaf-1 (Adrain et al., 1999; Poyet et al., 2001). On the other hand, when we tested similar CARD-CARD interactions for Pycard we found that the CARD-module of Pycard strongly and specifically interacted with caspase-1 (Figure 1D), leading to caspase-1 processing/activation (Figure 1E). In a similar fashion, overexpression of FADD specifically induced activation of caspase-8 (Figure 1E). Thus, analogous to the adaptor protein FADD that links the death effector domain (DED) of caspase-8 to the death domain (DD) of Fas, Pycard may connect the CARD of caspase-1 to the PYD of NALP1.

Interestingly, both caspases recruited to the NALP1/Pycard complex, caspase-1 and caspase-5, were also capable of forming heterocomplexes when coexpressed in 293T cells (Figure 1C). However, under these conditions caspase-1 immunoprecipitates contained the full-length and the mature p20 fragment of caspase-5 in contrast to NALP1 immunoprecipitates which contained only partially cleaved caspase-5 (Figure 1C), suggesting that caspase-1 is necessary to achieve the complete maturation of caspase-5 (see below). A physical interaction between murine caspases-1 and -11 has previously been demonstrated (Wang et al., 1998), and genetic evidence supports a crucial role of both caspases in the processing of the proinflammatory cytokines IL-1 β and IL-18 (Kuida et al., 1995; Li et al., 1995; Wang et al., 1998). Although human caspase-5 and murine caspase-11 share only 54% identical amino acid residues, no other caspase in the human genome is more closely related to murine caspase-11, suggesting that human caspase-5 and murine caspase-11 are functional homologs. In support of this notion, the expression of both caspases is highly inducible by LPS and INF- γ (Lin et al., 2000; Wang et al., 1998). We therefore considered the possibility that caspase-5 might also be involved in IL-1 β processing. To test this, we expressed caspase-1 and caspase-5 in the presence or absence of their respective activator Pycard or the C-terminal segment of NALP1. IL-1 β processing was monitored with an antibody that recognizes only the mature form of IL-1 β . ProIL-1 β processing occurred most efficiently when both caspase-1 and caspase-5 were coactivated (Figure 1F). This suggests that in humans, like in mice, more than one proinflammatory caspase may be implicated in the generation of active IL-1 β .

Activation of Inflammatory Caspases in a THP-1 Cell-free System

To further investigate the role of NALP1 and Pycard in caspase activation, we developed a cell-free system

by adapting previously described systems (Ayala et al., 1994; Cerretti et al., 1992; Kostura et al., 1989; Miller et al., 1993; Thornberry et al., 1992; Yamin et al., 1996). In brief, inflammatory caspase activation and IL-1 β processing was specifically induced by mechanical disruption of the integrity of THP-1 cells and detected in the ensuing cytoplasmic fractions. The reason why cell rupture may trigger the activation of inflammatory caspases is discussed below. THP-1 cells were utilized as they resemble primary monocytes in that they constitutively express high levels of caspase-1, caspase-5, Pycard, and NALP1 (data not shown; Lin et al., 2000; Masumoto et al., 2001). Upon cell rupture, caspase-1 and caspase-5 were present in their proforms in the cell extracts. Subsequently, the precursors of caspase-1 and caspase-5 were rapidly cleaved into p35 kDa and mature fragments. This corresponds to the initial cleavage between the large and small caspase subunits, followed by the appearance of the N-terminal CARD fragment and p20 subunits (Figure 2A) as detected by Western analysis with specific anti-caspase-1 and anti-caspase-5 antibodies.

To follow concurrent processing of endogenous proIL-1 β (barely detectable in unstimulated or undifferentiated cells; see Figure 2B), proIL-1 β expression was induced either by a short treatment with LPS or by induction of THP-1 maturation into macrophage-like cells by treatment with PMA (Tsuchiya et al., 1982). Under these latter conditions, the rather remarkable extent of activation of the proinflammatory caspases was seen regardless of whether or not the cells had been previously stimulated with LPS for 1 hr (Figure 2B). Also, LPS treatment or PMA-specific differentiation did not by itself induce caspase activation which occurred only after cell rupture (Ayala et al., 1994) (Figure 2B). Further, indiscriminate caspase activation did not occur, as other caspases remained in their precursor forms. For example, caspase-9 was only processed when cytochrome-c was added to the cell extracts (Figures 2A and 2B). Activation of both caspase-1 and -5 correlated with the processing of proIL-1 β to its active p17 fragment. This was specifically inhibited by addition of the caspase-1 inhibitors z-VAD-fmk and YVAD-fmk, which block the activity of the mature caspase (Figure 2A) (Garcia-Calvo et al., 1998). When caspase activation was monitored in cytoplasmic extracts from HeLa cells, which contain detectable amounts of caspase-1 and caspase-5 but no Pycard or NALP1, activation of the inflammatory caspases was not detected, even though activation of caspase-9 could be induced by the addition of cytochrome-c to the same cell extracts (Figure 2B). This observation further confirms the specificity of the *in vitro* system and, importantly, indicates that molecules specific to monocytes/macrophages are involved in the activation of the inflammatory caspases.

(D) Pycard coimmunoprecipitates with caspase-1. Experiments were done as described in (C). The antibody used detects the N-terminal portion (CARD) of caspase-1. The asterisk indicates the IgG heavy chain.

(E) Overexpression of Pycard induces cleavage of caspase-1. 293T cells were transfected with 0.5 μ g of caspase-1 plasmid and the indicated amount of Pycard or FADD expression plasmids.

(F) Expression of both caspase-1 and caspase-5 is necessary for optimal cleavage of proIL-1 β . 293T cells were cotransfected with equal amounts of caspase-1 and/or caspase-5 and NALP1-Cter and/or Pycard. Empty vector was used to adjust the total amount of DNA in each transfection. Caspase-induced cleavage of proIL-1 β after Asp 116 was detected using an antibody specifically detecting the p17 cleavage product (IL-1 β^*). Cell extracts were immunoblotted to confirm expression of the transfected proteins.

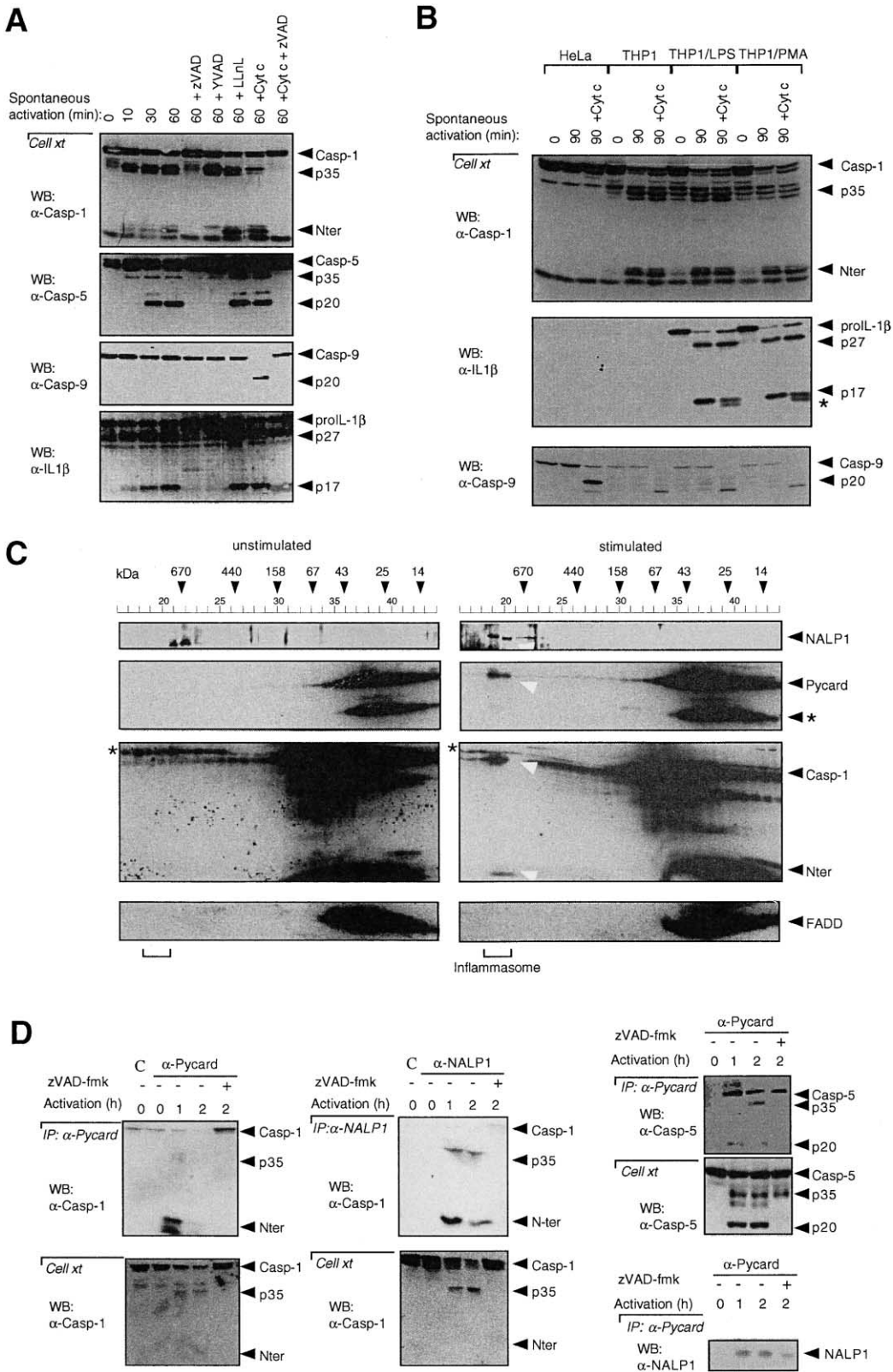


Figure 2. ProIL-1 β Processing in THP-1 Correlates with Parallel Activation of Caspase-1 and Caspase-5 and Inflammasome Formation
(A) The cytosolic fractions from LPS-prestimulated (1 hr) THP-1 cells were incubated at 30°C for the indicated periods of time, and activation of caspase-1, caspase-5, caspase-9, and proIL-1 β was monitored by Western analysis in the presence or absence of the caspase inhibitors zVAD-fmk (50 μ M), YVAD-fmk (50 μ M), and the proteasome inhibitor LLnL (50 μ M). Cytochrome-c (1 ng) was added to activate Apaf-1 and caspase-9. The monoclonal antibodies used to detect caspase-5 and caspase-9 both interact with the p20 caspase subunit.

Inducible Complex Formation between NALP1, Pycard, and Proinflammatory Caspases

Taken together, the above results suggested that assembly of NALP1, Pycard, caspase-1, and caspase-5 initiated cleavage of proIL-1 β . To further investigate this, stimulated and unstimulated cell extracts were analyzed by gel filtration in a manner similar to the approach used to characterize the Apaf-1 and caspase-9-containing apoptosome (Cain et al., 1999). When nonactivated cell extracts of THP-1 cells were separated by gel filtration, NALP1 had an apparent molecular mass of approximately 700 kDa, indicating that NALP1 (theoretical mass 156 kDa) is a component of a multiprotein complex even under resting conditions (Figure 2C). Triggering of caspase-1 activation further induced a shift of NALP1 toward a higher molecular weight fraction. Likewise, Pycard and caspase-1, which eluted at their predicted molecular masses of 30 and 60 kDa, respectively, in nonstimulated extracts, partially shifted into NALP1-containing fractions ($M_r > 700$ kDa). This points to the existence of an inducible high molecular weight complex containing NALP1, Pycard, and proinflammatory caspases, which we call the inflammasome.

To obtain more direct evidence for the existence of the inflammasome, caspase-1 was coimmunoprecipitated with either Pycard or NALP1 in THP-1 extracts in an activation-dependent manner (Figure 2D). In contrast to cell extracts, coimmunoprecipitated caspase-1 was mostly present in the processed form (only background levels of pro-caspase-1 were detected in the absence of z-VAD-fmk), indicating that caspase-1 is rapidly activated in the NALP1/Pycard/caspase-1 complex. Caspase-1 binding to NALP1/Pycard appeared to be transient, since at 2 hr poststimulation, less caspase-1 was detectable in the inflammasome (Figure 2D). Caspase-5 appears to be a component of this complex, since anti-Pycard antibodies coimmunoprecipitated caspase-5 and NALP1 in a stimulus-dependent manner (Figure 2D).

The Inflammasome Component Pycard Is Essential for the Activation of Caspase-1 and Caspase-5

To further demonstrate the importance of the inflammasome for caspase-1 and caspase-5 activation, we took advantage of the *in vitro* activating system described above. When caspase activation was monitored in lysates in the presence of two different anti-Pycard antibodies (a polyclonal [α -Pycard] or a monoclonal antibody [α -ASC]), activation of both caspase-1 and

caspase-5 was abrogated (Figures 3A and 3D). The inhibitory effect of anti-Pycard was dose dependent (Figure 3B) and specific, since no inhibition of caspase-9 cleavage was observed (Figure 3C). Further, denaturation of the anti-Pycard antibodies for 5 min at 95°C or addition of the specific blocking peptide (PE148) used as an antigen in generating anti-Pycard but not of an unrelated peptide (PE151) neutralized the antagonist activity of the anti-Pycard antibodies (Figure 3D). Although less apparent than with anti-Pycard antibodies, anti-NALP1 had a moderate inhibitory effect on the activation of caspase-1 and caspase-5 (Figure 3A), likely due to an inefficient blocking activity of the anti-NALP1 antibody and possibly also due to the redundancy of NALPs (see below). Further, when Pycard was immunodepleted from cytoplasmic extracts, caspase-1 and caspase-5 activation was completely inhibited (Figures 3E and 3F). Partial immunodepletion of NALP1 also diminished activation of caspase-1 and caspase-5. Finally, immunodepletion of Pycard from cells shortly primed with LPS resulted in a considerable reduction in proIL-1 β processing (Figure 3F).

A Dominant-Negative Version of Pycard Blocks LPS-Induced IL-1 β Maturation *In Vivo*

To analyze whether Pycard and NALP1 are crucial for IL-1 β maturation *in vivo*, we generated stable populations of cells expressing only the Pyrin motif of Pycard (DN Pycard) (Figure 4B). DN Pycard binds to NALP1 but not to caspase-1 (Martinon et al., 2001; data not shown) and is therefore predicted to act as a dominant-negative inhibitor of NALP1/Pycard-induced caspase-1 recruitment. Initially, we tried to detect proIL-1 β cleavage following stimulation of wild-type and DN Pycard-THP-1 cells with LPS. Although LPS stimulation of THP-1 induced the synthesis of the IL-1 β precursor in less than 2 hr, there was surprisingly barely detectable amounts of cleaved IL-1 β in cell extracts of wild-type cells. In contrast, a strong release of mature IL-1 β was observed when THP-1 cells were first differentiated with PMA (Tsuchiya et al., 1982) and then stimulated with LPS. The caspase-dependent IL-1 β maturation was confirmed by z-VAD-fmk pretreatment (Figure 4A, see also Figure 2A). Under these conditions, DN Pycard strongly blocked maturation of IL-1 β without, however, affecting LPS-induced proIL-1 β synthesis (Figure 4C). Initially, we like others (Ayala et al., 1994) were unable to detect the concomitant processing of caspases-1 and -5 in cell extracts of stimulated cells. Fortuitous analysis of the

(B) HeLa, THP-1, and THP-1 cells prestimulated for 1 hr with LPS or differentiated into macrophages with PMA were activated and analyzed as described in (A). The asterisk indicates an IL-1 β fragment generated during apoptosis.

(C) Formation of a complex containing NALP1, Pycard, caspase-1, and caspase-5 (inflammasome). THP-1 cell lysates prepared by mechanical disruption were either incubated at 30°C for 60 min to allow the spontaneous activation of proIL-1 β (see [A]) or left at 4°C to prevent activation. Both activated and nonactivated cell extracts were size fractionated on a Superdex S-200 column. The elution profiles of NALP1, Pycard, and caspase-1 are shown before and after activation. White arrows indicate the position of proteins shifted to a higher molecular weight complex (inflammasome, fractions 19 and 20). The elution profile of FADD was determined for control purposes. The asterisk in the Pycard blot points to its putative short form and in the caspase-1 blot to a nonspecific crossreactive band.

(D) NALP1, Pycard, caspase-1, and caspase-5 form a complex in a time-dependent manner. Extracts of THP-1 cells activated for various periods of time through incubation at 30°C were immunoprecipitated with α -Pycard, α -NALP1, or control antibodies (c) and probed for the presence of caspase-1, caspase-5, and NALP1, respectively.

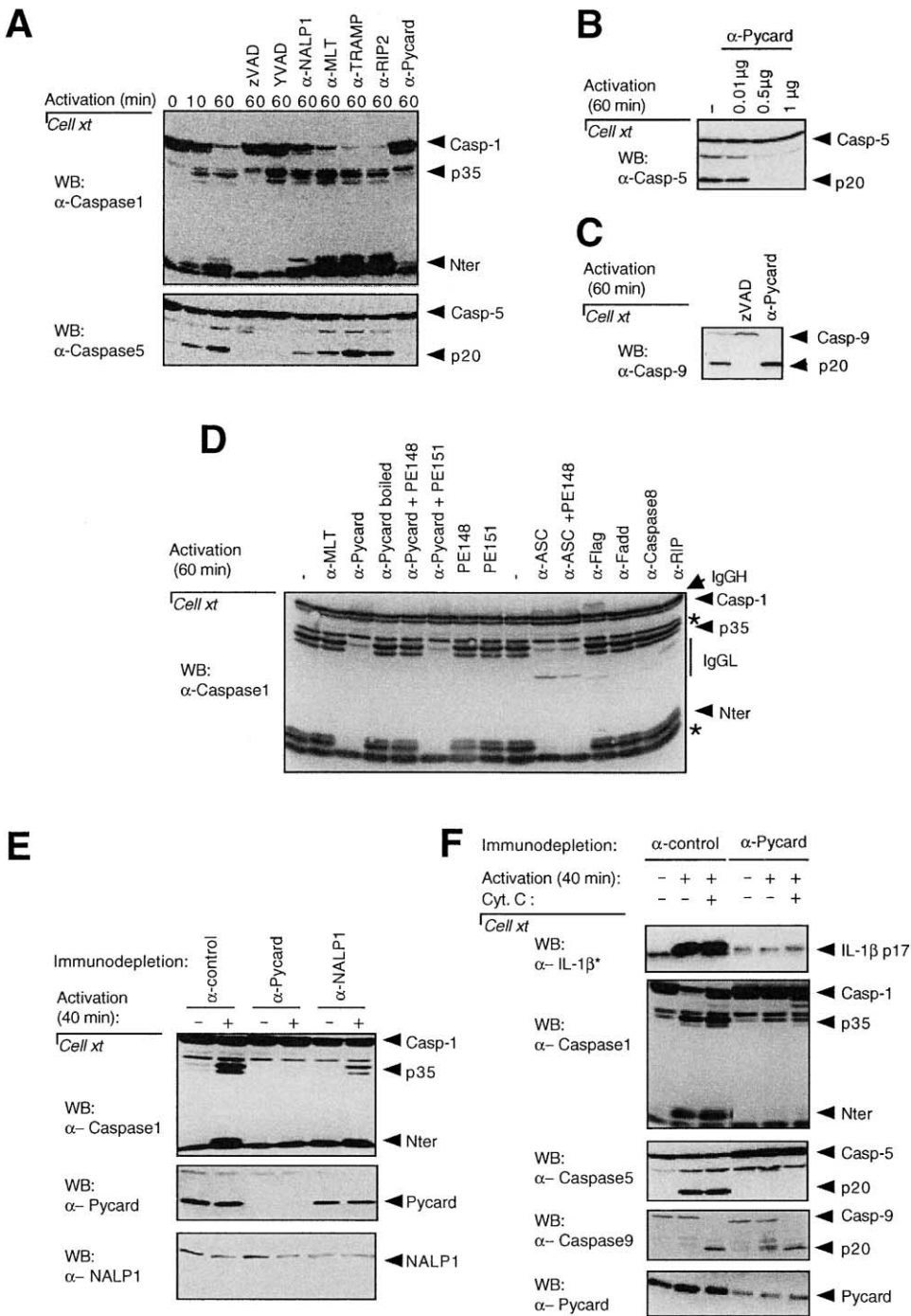


Figure 3. Pycard Is Essential for Caspase-1 and Caspase-5 Activation in THP-1 Cells

(A) THP-1 cell lysates were incubated at 30°C for various time periods in the presence or absence of antibodies to Pycard, NALP1, or various control antibodies (MLT paracaspase, TRAMP/DR3, and RIP2). Activation of the caspases was then followed as described in Figure 2B.

(B) Dose dependence of α-Pycard antibody mediated inhibition of caspase-5 activation.

(C) α-Pycard antibodies do not interfere with cytochrome-c-mediated caspase-9 activation (for experimental conditions, see Figure 2A).

(D) Boiled α-Pycard or α-Pycard preincubated with the specific peptide PE148 or a control peptide PE151 was tested for its ability to antagonize Pycard function. α-ASC, a monoclonal antibody against Pycard, or unrelated antibodies (α-Flag, α-FADD, α-caspase 8, or α-RIP) were also tested for their antagonist activity. The asterisk indicates nonspecific crossreactive bands.

(E) THP-1 cell extracts were preincubated on ice with protein-G-adsorbed antibodies to Pycard, NALP1, or control IgGs. After removal of the beads, immunodepletion of Pycard and NALP1 was analyzed by immunoblotting. Caspase-1 activation was then initiated by shifting the temperature from 0°C to 30°C.

(F) Pycard was immunodepleted, as described above, from extracts of THP-1 cells that had been stimulated with LPS for 1 hr as described in (E). IL-1β cleavage was analyzed with a specific antibody for the cleaved mature form of IL-1β (p17).

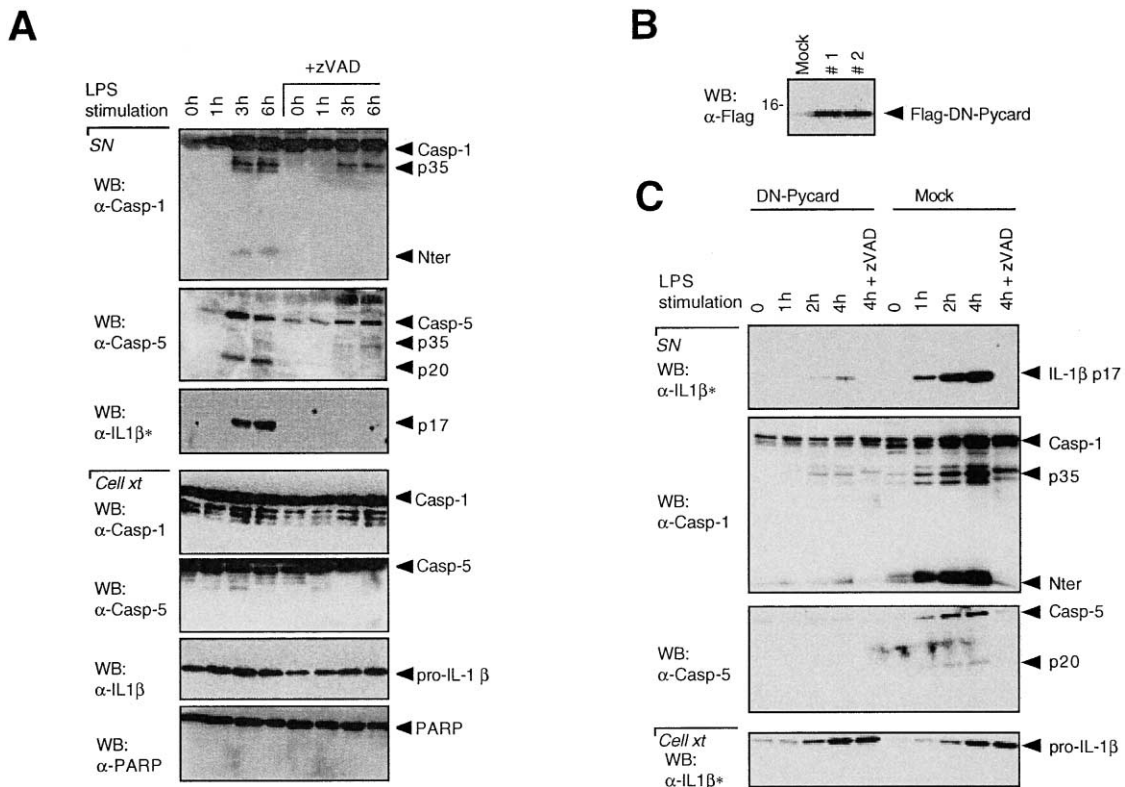


Figure 4. Inhibition of proIL-1 β Processing by Dominant-Negative (DN) Pycard

(A) Differentiated THP-1 cells were stimulated with LPS for the indicated periods of time in the presence or absence of z-VAD-fmk. Total cell extracts (Cell xt) or the culture media (SN) were analyzed by immunoblotting for caspase-1, -5, and IL-1 β . PARP was not cleaved (lower panel) under similar conditions.

(B) THP-1 cells were infected using a retroviral vector encoding Flag-tagged DN Pycard (aa 1–94). After selection with puromycin, stably transfected cell populations were assayed for the expression of Flag-tagged proteins by Western analysis (two distinct populations are shown).

(C) PMA differentiated THP-1 cells expressing DN Pycard were treated with LPS (10 μ g/ml) for the indicated time periods. Processing of proIL-1 β and caspases-1 and -5 was monitored in the cell culture media (SN). That DN Pycard expression has no effect on LPS-induced IL-1 β upregulation is shown by Western analysis of cell extracts in the lower panel.

culture media revealed that caspase-1 and caspase-5 were present in the supernatant and that the active forms of caspase-1 and caspase-5 were rapidly and specifically released into the supernatant following LPS stimulation of differentiated THP-1 cells (Figure 4A). In fact, the presence of the inflammatory caspases in the extracellular space was previously suggested by an electron microscopy study that observed active processed caspase-1 on the external surface of intact plasma membranes of monocytes (Singer et al., 1995). Further characterization of the role and the mechanism by which this externalization occurs is of great interest. The activation and release of the proinflammatory caspases was blocked in cells expressing the DN Pycard but not in wild-type and mock-transfected cells (Figure 4C). Therefore, the inflammasome component Pycard is a crucial regulator of proinflammatory caspase activation and IL-1 β maturation in vivo.

IL-1 activity is tightly regulated at multiple levels (Mantovani et al., 1998), one of which concerns the regulated cleavage of the inactive proIL-1 β to its active mature form by caspase-1. Reported activators of caspase-1 are the kinase RIP-2 (Humke et al., 2000; Thome et al.,

1998) and the NOD homolog Ipaf/CARD12 (Poyet et al., 2001). However, caspase-1 cleavage by these proteins was demonstrated only in overexpression systems, and the capacity of RIP-2 and Ipaf/CARD12-activated caspase-1 to process proIL-1 β has not been investigated. In this report we describe the identification of a protein complex called the inflammasome that links proIL-1 β to its upstream activator. This complex is composed of Pycard, NALP1, caspase-1, and likely caspase-5. Several lines of evidence suggest that Pycard is essential for the maturation of proinflammatory caspases: first, coexpression of Pycard and caspase-1 induces caspase-1 processing; second, interaction of Pycard and caspase-1 was detected at the endogenous level; third, in vitro, the specific removal of Pycard or its neutralization renders cytoplasmic extract defective in activating both caspase-1 and caspase-5; and finally, in vivo, activation of caspase-1 and caspase-5 is blocked by expression of a DN Pycard which binds NALP1 but cannot recruit caspase-1. In support of the importance of Pycard are also two recent reports demonstrating that forced oligomerization of Pycard leads to caspase-1 activation (Srinivasula et al., 2002) and that a novel NALP

(NALP12/Pypaf7) is also able to recruit Pycard (Wang et al., 2002).

The inflammasome complex is detectable at tangible quantities only in the *in vitro* system described here, which in essence recapitulates the one originally described for the characterization of the apoptosome (Li et al., 1997). In intact cells, the quantities of inflammasome complex must be minute or complex formation must be very transient, explaining why it was not possible to detect active proinflammatory caspases in cell extracts of stimulated monocytes. In this context it is noteworthy that in THP.1 cells, active caspases are found in the cell supernatant, raising the possibility that the whole inflammasome complex is secreted along with active IL-1.

In an analogous fashion to Apaf-1, we propose that NALP1 via its multiple domain structure plays a central role in the assembly of the inflammasome. Like the related Apaf-1 and NOD proteins, NALP1 self-associates (Chu et al., 2001), suggesting that the mechanisms of activation of caspase-1 and caspase-5 by NALP1 and of caspase-9 by Apaf-1 are similar. For Apaf-1 it has been shown that cytochrome-c triggers caspase-9 binding (Li et al., 1997), but how NALP1/Pycard activation occurs is currently unknown. It is tempting to speculate that the LRRs of NALP1 (like those found in the TLRs and NODs) may recognize pathogen-associated molecular patterns (PAMPS) (Girardin et al., 2002; Inohara et al., 2001; Janeway and Medzhitov, 2002) and/or endogenous nonforeign "alarm signals" (i.e., mammalian DNA and heat shock proteins) (Gallucci et al., 1999; Matzinger, 2002; Sauter et al., 2000; Vabulas et al., 2002) and thereby trigger inflammasome assembly. That disruption of the cellular integrity (likely resulting in the release of nonforeign alarm signals) is alone sufficient to activate IL-1 β processing supports the idea of the LRRs acting as a NALP regulatory unit. Interestingly, NALP1 is part of a large family of Pyrin domain-containing NBS-LRR proteins (>14 in humans). Since NALP2 and NALP3 also interact with Pycard (our unpublished data; Manji et al., 2002), different NALPs may have evolved to recognize a wide array of distinct pathogens and nonforeign signals.

There is also indirect evidence that NALP family members are involved in IL-1 β maturation. We and others have recently identified patients carrying mutations in the NBS (NACHT) domain of NALP3 with Muckle-Wells syndrome (Aganna et al., 2002; Hoffman et al., 2001). This autosomal-dominant syndrome is characterized by intermittent episodes of fever, often provoked by increased levels of inflammatory cytokines like IL-1 β . In view of the data presented in this report, it is tempting to propose that mutations in the NALP3 gene may lead to a deregulated activation of proinflammatory caspases resulting in spontaneous fever episodes.

Experimental Procedures

In Vitro Caspase-1/pro-IL-1 β Activation

THP-1 cells or HeLa were cultured in RPMI 1640/DMEM, respectively, supplemented with 10% heat inactivated fetal bovine serum (FCS), 50 μ M β -mercaptoethanol, and penicillin/streptomycin (100 μ g/ml of each). THP-1 cells were grown in suspension to a density of $\sim 1.5 \times 10^6$ cells/ml. Where indicated (i.e., Figures 2A and 2B),

THP-1 cells were prestimulated for 1 hr with LPS (1 μ g/ml) or treated with PMA as described in detail below (i.e., Figure 2B). Cytosolic extracts were prepared from stimulated or unstimulated THP-1 and HeLa cells as described previously (Liu et al., 1996). In brief, the cells were harvested and washed in cold phosphate-buffered saline. This was followed by swelling in 5 volumes of ice-cold hypotonic buffer W (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM MgCl₂, 1 mM Na EDTA, 1 mM Na EGTA, 0.1 mM PMSF, and the Roche protease inhibitor cocktail) and incubation on ice for 15 min. The cellular membrane integrity was then disrupted by passage 15 times through a G22 needle. Cell lysates were centrifuged, and the supernatants, following filtration (0.45 μ M), were used for this *in vitro* IL-1 β cleavage assay. Following a short incubation (30°C), caspase cleavage and IL-1 β processing were monitored by Western analysis with the specific antibodies indicated above. To test the requirements of Pycard and NALP1 in this system, antagonist and immunodepletion studies were carried out. For the antagonist experiments, 1 μ g of the indicated antibody was added to the prepared supernatants (about 500 μ g/reaction) prior to incubation. The activity of the anti-Pycard antibody was blocked by preincubation with its specific peptide (PE148). As a control, anti-Pycard was preincubated with the unrelated peptide (PE151).

For Pycard and NALP1 immunodepletion, THP-1 cell extracts were preincubated for 1 hr with the indicated antibodies, adsorbed to protein-G beads. After removal of the antibody/bead mixture, the extracts were processed as described above.

Additional experimental procedures can be found in the supplemental data at <http://www.molcell.org/cgi/content/full/10/2/417/DC1>.

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