Cryopyrin and pyrin activate caspase-1, but not NF-κB, via ASC oligomerization

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Abstract

Mutations in cryopyrin and pyrin proteins are responsible for several autoinflammatory disorders in humans, suggesting that these proteins play important roles in regulating inflammation. Using a HEK293 cell-based reconstitution system that stably expresses ASC and procaspase-1 we demonstrated that neither cryopyrin nor pyrin or their corresponding disease-associated mutants could significantly activate NF-κB in this system. However, both cryopyrin and two disease-associated cryopyrin mutants induced ASC oligomerization and ASC-dependent caspase-1 activation, with the disease-associated mutants being more potent than the wild-type (WT) cryopyrin, because of increased self-oligomerization. Contrary to the proposed anti-inflammatory activity of WT pyrin, our results demonstrated that pyrin, like cryopyrin, can also assemble an inflammasome complex with ASC and procaspase-1 leading to ASC oligomerization, caspase-1 activation and interleukin-1β processing. Thus, we propose that pyrin could function as a proinflammatory molecule.
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Abbreviations: CARD, caspase-associated recruitment domain; PYD, pyrin domain; DD, death domain; DED, death effector domain; NOD, nucleotide-binding oligomerization domain; LRR, leucine-rich repeat; NLR, NACHT-LRR; IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor; ASC, apoptosis-associated speck-like protein containing a CARD; PFS, periodic fever syndromes; FMF, familial Mediterranean fever; FCAS, familial cold autoinflammatory syndrome; FCU, familial cold urticaria; MWS, Muckle-Wells syndrome; NOMID, neonatal-onset multisystem inflammatory disease; CINCA, chronic infantile neurological cutaneous and articular syndrome; PSTPIP1, proline serine threonine phosphatase-interacting protein 1; PAPA, pyogenic arthritis, pyoderma gangrenosum, and acne syndrome; IKK, IκB kinase; PAMP, pathogen-associated molecular patterns; PGN, peptidoglycans; MDP, muramyl dipeptide; WT, wildtype; RT, reverse transcription; PCR, polymerase chain reaction; NACHT, domain present in NAIP, CIITA, HET-E and TP1

Introduction

The pyrin domain (PYD)-containing proteins pyrin (also known as marenostrin) and cryopyrin (also known as Pypaf1, Nalp3), have been implicated in the etiology of a number of inflammatory diseases in humans termed hereditary periodic fever syndromes (PFSs) (reviewed in Gumucio *et al.*,[1] Hull *et al.*,[2] McDermott and Aksentijevich,[3] Stehlik and Reed[4] and Tschopp[5]). Mutations in the *MEFV* gene which encodes pyrin are associated with familial Mediterranean fever (FMF),[6,7] while mutations in the *CIAS1* gene which encodes cryopyrin are responsible for three PFSs; familial cold autoinflammatory syndrome (FCAS)/familial cold urticaria (FCU), Muckle–Wells syndrome (MWS), and neonatal-onset multisystem inflammatory disease (NOMID)/chronic infantile neurological cutaneous and articular syndrome (CIN-CA).[8,9] These diseases are characterized by recurrent episodes of inflammation and fever, and because of the lack of an apparent stimulus and the involvement of autoantibodies and autoreactive T cells, they are defined as autoinflammatory syndromes (reviewed in Gumucio *et al.*,[1] Hull *et al.*,[2] McDermott and Aksentijevich,[3] and Stehlik and Reed[4]).

The human pyrin protein is a 781 amino-acid protein,[6,7] expressed predominantly in neutrophils, monocytes, and eosinophils.[10,11] It is composed of several identifiable conserved domains including an N-terminal PYD, which is a homotypic protein–protein interaction domain belonging to the six-helix bundle death domain (DD)-fold superfamily that includes DDs, death effector domains (DEDs), and caspase-associated recruitment domains (CARDs).[12–15] The PYD is followed by two central B-box zinc-finger and coiled coil domains and a C-terminal B30.2/rfp/SPRY domain. Most FMF-associated mutations are localized in B30.2/rfp/SPRY domain,[16] suggesting that this domain is involved in the regulation of the activity of pyrin. Pyrin associates through a PYD–PYD interaction with apoptosis-associated speck-like protein containing a CARD (ASC),[17,18] a 22 kDa adapter protein with an N-terminal PYD and a C-terminal CARD involved in the activation of caspase-1.[19–21] However, the physiological significance of the pyrin–ASC interaction for caspase-1 activation remains unclear because mice with total *MEFV* gene ablation are not yet available. Nevertheless, the importance of ASC for caspase-1 activation was recently revealed by gene knockout in mice, which showed an increased resistance to endotoxic shock of the *ASC*−/− mice and a severe defect in caspase-1 activation and interleukin (IL)-1β production in macrophages from these mice in

response to several pro-inflammatory molecules.[22] Pyrin has also been shown to interact with the proline serine threonine phosphatase-interacting protein 1 (PSTPIP1).[23] Interestingly, PSTPIP1 is mutated in the autoinflammatory pyogenic arthritis, pyoderma gangrenosum, and acne syndrome,[24] suggesting that pyrin and PSTPIP1 function in the same autoinflammatory pathway (reviewed in McDermott [25]).

Like pyrin, cryopyrin contains an N-terminal PYD that mediates its interaction with ASC. However, beyond the PYD there are no structural similarities between pyrin and cryopyrin. Cryopyrin belongs to the NACHT-LRR (NLR) protein family, which includes NOD1, NOD2, Ipaf and 14 other family members known as NALPs.[26,5] All family members contain a central nucleotide-binding oligomerization domain (NOD)/domain present in NAIP, CIITA, HET-E and TP1 (NACHT), and a C-terminal leucine-rich repeat (LRR) domain. The disease-associated cryopyrin mutations are located within or near the NOD/NACHT domain,[27] suggesting that these mutations might affect oligomerization of cryopyrin. Cryopyrin is primarily expressed in peripheral blood leukocytes.[28,29] Recent reports suggest that cryopyrin is involved in the regulation of NF-κB activation,[29–33] and this in turn regulates the expression of genes involved in the immune response and inflammation.[34] Cryopyrin also regulates IL-1β generation by assembling an inflammasome complex with ASC and caspase-1.[35] This activity of cryopyrin was recently shown to be regulated by bacterial peptidoglycan possibly through direct interactions with its LRR domain.[36]

In the present study, we investigated the NF-κB and caspase-1-activating abilities of WT and disease-associated pyrin and cryopyrin proteins using a stable 293T cell-based reconstitution system that expresses physiological amounts of procaspase-1 and the caspase-1 adaptor protein ASC. We demonstrated that expression of either pyrin or cryopyrin or their corresponding disease-associated mutants could induce ASC oligomerization and activation of caspase-1, but not NF-κB. The disease-associated mutants of cryopyrin were more potent than the WT protein in inducing caspase-1 activation because of increased self-oligomerization and interaction with ASC. Our data suggest that both pyrin and cryopyrin are capable of assembling independent inflammasome complexes with ASC and procaspase-1, and activating caspase-1 via ASC oligomerization.

Results

Cryopyrin and pyrin do not activate NF-κB in the presence of physiological amount of ASC

A number of recent studies provided conflicting results on the role of ASC in activation of NF-κB in response to proinflammatory cytokines such as lipopolysaccharide (LPS) and tumor necrosis factor (TNF)-α or expression of cryopyrin or pyrin proteins.[29–33] Since these studies were performed in transfected cells overexpressing ASC, the results of these studies may not reflect the normal physiological function of ASC. This is especially true in light of the results of the ASC knockout mice, which demonstrated that ASC does not appear to play a role in NF-κB activation in response to LPS or TNF-α.[22] However, since coexpression of ASC with the

disease-associated cryopyrin mutants, but not the WT cryopyrin, was reported to activate NF-κB,[31] it is possible that the disease-associated cryopyrin mutants, and perhaps also the disease-associated pyrin mutants, could abnormally recruit ASC to induce NF-κB activation. To test this possibility and to rule out any effect of ASC overexpression, we generated a stable HEK293T cell line expressing physiological amount of ASC (293-ASC cells) comparable to that present in human THP1 monocytes and periferal blood leukocytes (Figure 1a and Figure S1, Supplementary information). We investigated whether expression of WT cryopyrin or two cryopyrin mutants R260W and D303N, commonly found in patients with FCAS/FCU and NOMID/ CINCA, respectively, could induce NF-κB in 293-ASC cells by NF-κB reporter gene assay. Surprisingly, neither the WT nor the mutant cryopyrin proteins were able to induce NF-κB activation in these cells (Figure 1c), or in the parental 293T cells (Figure 1b), which lack ASC expression. On the contrary the WT and the mutant cryopyrin proteins reduced the basal NF-κB activity in both the parental 293T cells and the 293-ASC cells. Transfection of the 293T and the 293-ASC cells with TRAF6 induced potent NF-κB activation, ruling out the possibility that these cells contain a defect in NF-κB activation. Interestingly, coexpression of cryopyrin proteins with TRAF6 in 293T and the 293-ASC cells inhibited TRAF6-induced NF-κB activation (Figure 1b and c). Coexpression of cryopyrin proteins with Bcl10 also inhibited NF-κB activation by this protein (data not shown). Our data thus clearly demonstrate that the WT or disease-associated cryopyrin mutants do not induce NF-κB activation alone or in the presence of ASC, but rather they have a dampening effect on this pathway in this 293T system. Moreover, when ASC is expressed at normal levels in 293T cells, it does not collaborate with cryopyrin to induce NF-κB activation. This apparent discrepancy with the reported NF-κB-promoting activity of the disease-associated cryopyrin mutants in 293T cells[31] is most likely due to the use of transiently overexpressed ASC in the previous study. The transiently overexpressed ASC has been shown to interact with the IκB kinase (IKK) complex in 293T cells and suppresses its activity.[33] Cryopyrin interaction with ASC could displace ASC from the IKK complex, relieving the suppression of the IKK complex, and resulting in artificial NF-κB activation as reported previously.[33]

Coexpression of WT pyrin with ASC in HEK293T cells, was reported to activate NF-κB.[33] In contrast, a second study reported that coexpression of pyrin with ASC and cryopyrin in HEK293T cells inhibited cryopyrin–ASC-mediated NF-κB activation.[30] To investigate the role of pyrin in the NF-κB-signaling pathway we expressed the WT pyrin and two FMF-associated pyrin mutants M694V and V726A in the 293-ASC cells and the parental 293T cells. As shown in Figure 1d and e, expression of the different pyrin proteins did not induce significant NF-κB activity in either the parental or the 293-ASC cells. There was also little change in the NF-κB activity when pyrin was coexpressed with cryopyrin in these cells. However, unlike cryopyrin, expression of pyrin or the pyrin mutants with TRAF6 did not inhibit TRAF6-induced NF-κB activation indicating that the inhibitory effect observed with cryopyrin is specific to the cryopyrin protein. Taken together, our data indicate that neither cryopyrin nor pyrin activate the
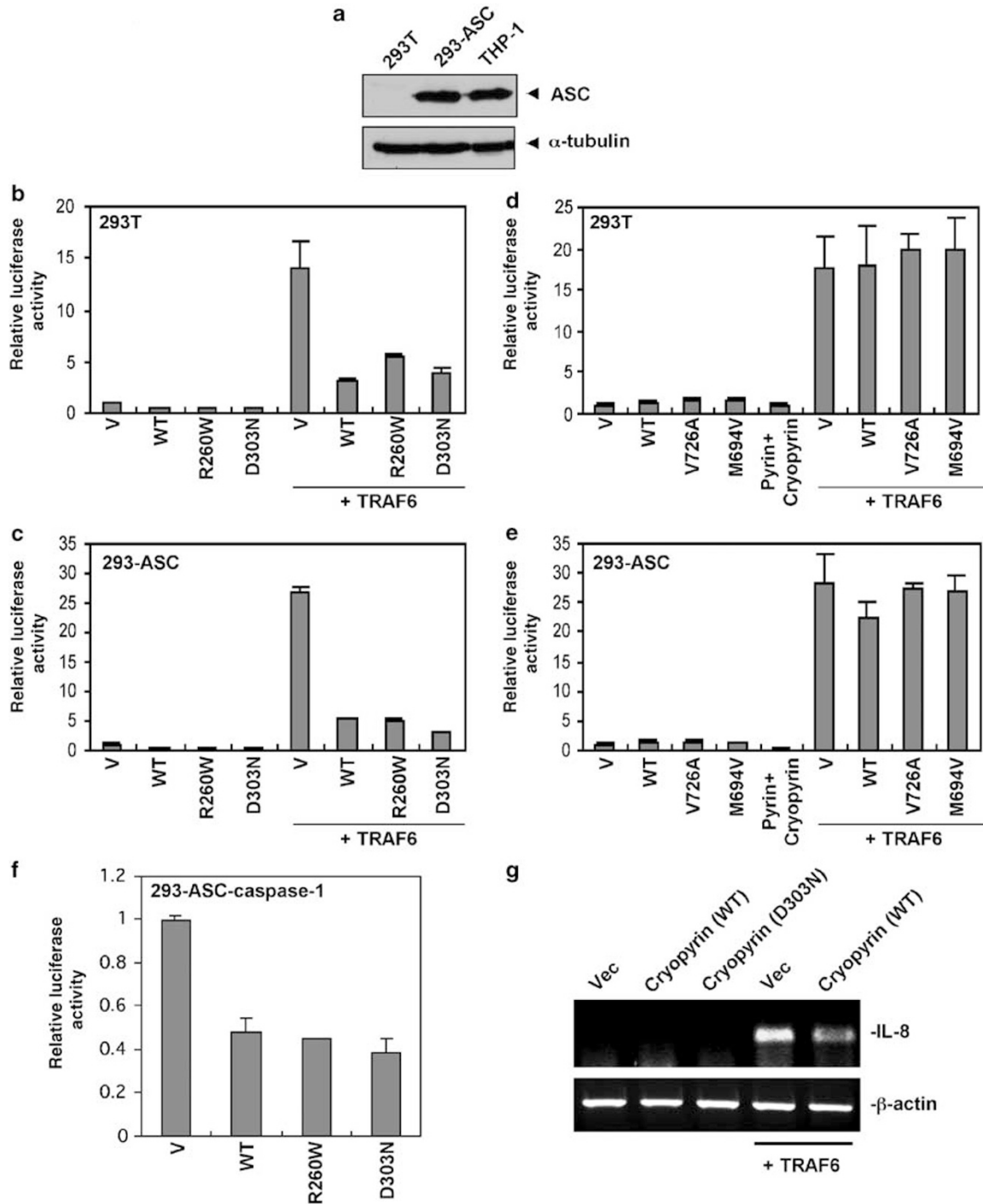
Figure 1 Cryopyrin and pyrin do not activate NF-κB. (**a**) Western blot analysis of total cell extracts from 293T, 293-ASC and THP1 cells with anti-ASC monoclonal antibody. Lower panel, shows the expression of α-tubulin in these cells. Note that the expression of ASC in 293-ASC cells is comparable to the expression of endogenous ASC in THP1 cells. (**b** and **c**) Effect of cryopyrin expression on NF-κB activity in 293T and 293-ASC cells. 293T (**b**) or 293-ASC (**c**) cells were transfected with empty vector (V) or expression constructs encoding wild-type (WT) or mutant R260W or D303N cryopyrins alone or together with TRAF6 as indicated. The NF-κB activity was assayed as described under Materials and Methods. (**d** and **e**) Effect of pyrin expression on NF-κB activity in 293T and 293-ASC cells. 293T (**d**) or 293-ASC (**e**) cells were transfected with empty vector (V) or expression constructs encoding wild-type (WT) or mutant V726A or M694A pyrins alone or together with TRAF6 as indicated. The NF-κB activity was assayed as in (**b**) and (**c**). (**f**) Effect of cryopyrin expression on NF-κB activity in 293-ASC-caspase-1 cells. 293-ASC-caspase-1 cells were transfected with empty vector (V) or expression constructs encoding wild-type (WT) or mutant R260W or D303N cryopyrins as indicated. The NF-κB activity was assayed as in (**b**) and (**c**). (**g**) Effect of cryopyrin and TRAF6 expression on IL-8 gene in 293-ASC-caspase-1 cells. 293-ASC-caspase-1 cells were transfected with empty vector (Vec, 1st lane), or expression constructs encoding wild-type (WT) cryopyrin (2nd lane), mutant D303N cryopyrin (3rd lane), TRAF6 (4th lane) or WT cryopyrin plus TRAF6 (5th lane) as indicated. The IL-8 gene expression was assayed by RT-PCR with specific human IL-8 gene oligonucleotide primers as described under Materials and Methods. β-actin was used as an internal control (lower panel)

NF-κB-signaling pathway when expressed in 293T cells containing physiological levels of ASC.

The cryopyrin disease-associated mutants exhibit increased ASC-dependent caspase-1 activation ability

Constitutive IL-1β secretion was observed in macrophages from patients with cryopyrin R260W mutation.[35] Since processing of pro-IL-1β is mediated by caspase-1 we investigated whether the cryopyrin disease-associated mutants have an enhanced ability to activate procaspase-1 in the absence of ligand stimulation. To do that, we generated stable HEK293T cell clones expressing either procaspase-1 alone (293-caspase-1) or procaspase-1 and ASC together (293-ASC-caspase-1). These cells were transfected with a constitutively active Ipaf as a positive control,[37] or with WT cryopyrin or the cryopyrin mutants R260W and D303N. Expression of Ipaf, which binds directly to procaspase-1 via CARD–CARD interactions independent of ASC,[37] induced autoprocessing of procaspase-1 in both 293-caspase-1 and 293-ASC-caspase-1 cells (Figure 2a). However, expression of WT cryopyrin or the cryopyrin mutants R260W and D303N resulted in autoprocessing of procaspase-1 in the 293-ASC-caspase-1 cells, but not in the 293-caspase-1 cells (Figure 2a, right panel), indicating that cryopyrin requires ASC to induce autoprocessing of procaspase-1. The processed caspase-1 was active as evidenced by its ability to cleave pro-IL-1β to generate mature IL-1β (Figure 2a, 2nd panel from top). Quantitative analysis of the activity of the WT compared to the activity of the cryopyrin mutants revealed that the activity of the cryopyrin mutants were significantly higher than that of the WT cryopyrin (Figure 2b). This was confirmed by expression of increasing amounts of the WT cryopyrin or the cryopyrin mutants in 293-ASC-caspase-1 cells (Figure 2c and d). This later experiment clearly showed that the cryopyrin mutants induced more, dose-dependent, caspase-1 activation compared to the WT cryopyrin, when the WT and the mutant cryopyrin proteins were expressed at equivalent levels. Among the two cryopyrin mutants, the D303N was the most active in inducing caspase-1 activation and pro-IL-1β processing (Figure 2c and d). Combined, these studies demonstrate that the disease-associated cryopyrin mutants have an enhanced ASC-dependent ability to activate more caspase-1 than the WT cryopyrin, a result consistent with the reported increased secretion of mature IL-1β in monocytes from patients harboring the cryopyrin R260W mutation.[35]

As with the 293-ASC cells (see Figure 1c and e), the stable expression of procaspase-1 alone (293-caspase-1 cells) or together with ASC (293-ASC-caspase-1) did not induce NF-κB activation in response to expression of WT or mutant cryopyrin, or pyrin proteins (Figure 1f and data not shown), confirming that cryopyrin and pyrin proteins do not induce NF-κB activation even in the presence of ASC and procaspase-1. Furthermore, additional experiments using an endogenous downstream NF-κB target gene (IL-8), confirmed the above results (Figure 1g). The results demonstrate clearly that expression of TRAF6 in the 293-ASC-caspase-1 cells leads to induction of the IL-8 gene (Figure 1g, 4th lane), whereas expression of TRAF6 in the 293-ASC-caspase-1 cells leads to induction of the IL-8 gene (Figure 1g, 4th lane), whereas expression of
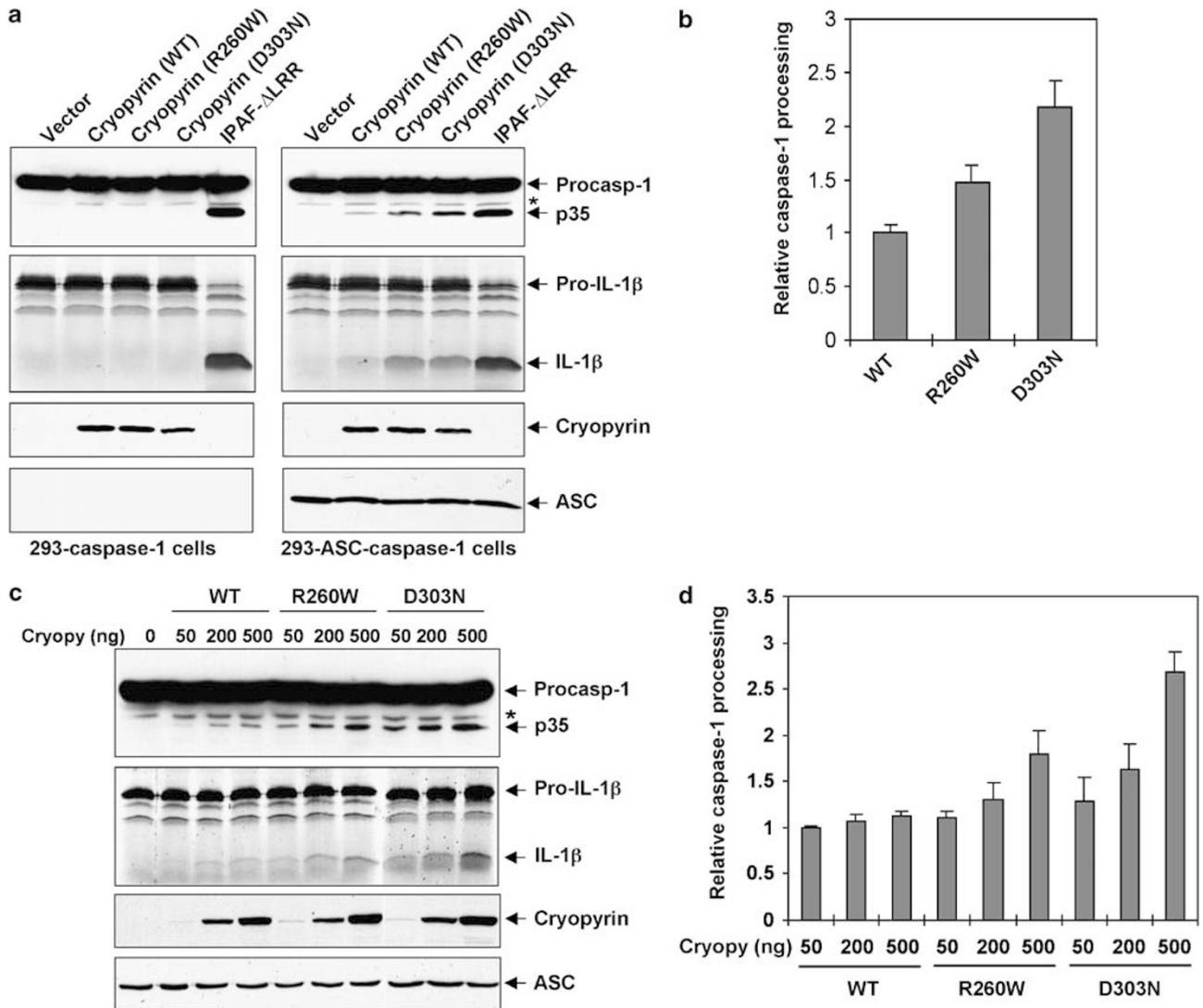
cryopyrin or the cryopyrin disease-associated D303N mutant in the same cells does not induce IL-8 (Figure 1g, 2nd and 3rd lanes). Similar results were obtained with the 293-ASC or 293-caspase-1 cells (data not shown). As with the NF-κB reporter gene assay (Figure 1b and c), coexpression of cryopyrin with TRAF6 in these cells also suppressed TRAF6-induced IL-8 gene expression (Figure 1g, 5th lane).

The cryopyrin disease-associated mutants exhibit increased oligomerization with ASC compared to WT cryopyrin

The molecular mechanism responsible for the constitutive activity of the disease-associated cryopyrin mutants is unclear. Since the majority of the disease-associated cryopyrin mutations are missense mutations located within the nucleotide and oligomerization domain (NOD), these mutations could cause conformational changes in this domain leading to increased oligomerization or aggregation of the mutant cryopyrin with ASC. Consequently, the oligomerized cryopyrin–ASC complex could serve as a platform to recruit and oligomerize procaspase-1, leading to its autoactivation. To test this hypothesis, we generated a stable 293T cell line expressing a GFP-tagged ASC to visualize the oligomerization of ASC in response to expression of the cryopyrin proteins by confocal microscopy. The ASC–GFP-expressing 293T cells were transfected with an empty plasmid or a plasmid encoding Flag-tagged WT cryopyrin or the cryopyrin mutants R260W and D303N. The transfected cells were stained with monoclonal anti-Nalp3 and rhodamine-red-conjugated anti-mouse IgG to visualize cryopyrin. In almost all the cells, the empty plasmid-transfected cells showed a homogeneous diffused ASC–GFP fluorescence over the entire cytoplasm and nucleus (Figure 3a, top panel, green channel). However, when the ASC–GFP cells were transfected with the cryopyrin disease-associated mutants (R260W or D303N) the entire ASC–GFP fluorescence colocalized with the cryopyrin mutants in bright perinuclear specks or aggregates in all the cells expressing the mutant cryopyrin proteins (Figure 3a, bottom panels). A similar but less dramatic aggregation of ASC–GFP and cryopyrin was seen in cells expressing the WT cryopyrin (Figure 3a, middle panel). A quantitative analysis of the percentage of cryopyrin-induced ASC–GFP aggregates in a large population of transfected cells revealed that the disease-associated mutant cryopyrin proteins induce significantly more specks/aggregates than the WT cryopyrin (Figure 3b). Interestingly, when the two mutants were compared with each other we found that the D303N mutant could induce significantly more ASC oligomerization than the R260W mutant (Figure 3b). These results suggest that the disease-associated mutations increase the tendency of cryopyrin to form oligomerized complexes with ASC, with the D303N mutant exhibiting the highest activity.

The cryopyrin disease-associated mutants are less soluble than the WT cryopyrin

To understand why the mutant cryopyrin proteins induce more ASC oligomerization than the WT cryopyrin, we expressed

Figure 2 Activation of caspase-1 by cryopyrin and the cryopyrin disease-associated mutants. (**a**) 293-caspase-1 cells (left panels) or 293-ASC-caspase-1 cells (right panels) were transfected with equal amounts (250 ng) of empty vector (vector), or the indicated cryopyrin proteins (2nd–4th lanes), or Ipaf-ΔLRR (5th lanes). After transfection cells were lysed, and the total cell lysates were Western blotted with anti-Flag antibody to detect caspase-1 (1st panels from top) and cryopyrin (3nd panels from top) or anti-ASC monoclonal antibody (4th panels from top). Cell lysates from the transfected cells were also incubated with ^{35}S-labeled pro-IL-1β and the reaction products were fractionated by SDS-PAGE and detected by autoradiography (2nd panels from top). The processed caspase-1 p35 subunit and the mature IL-1β are indicated. The asterisk indicates a nonspecific band. (**b**) Quantitative analysis of cryopyrin-induced caspase-1 processing. 293-ASC-caspase-1 cells were transfected with equal amounts (250 ng) of the indicated cryopyrin plasmids. After transfection the cell lysates were analyzed by Western blotting as in (**a**) and the amounts of caspase-1 p35 fragment were determined by densitometry. The relative caspase-1 processing was calculated as the amount of p35 fragment produced by each cryopyrin protein divided by the amount of p35 produced by WT cryopyrin. The values were normalized relative to the expression level of each cryopyrin protein. The values were calculated from at least three independent experiments. (**c**) 293-ASC-caspase-1 cells were transfected with increasing amounts of the indicated cryopyrin proteins. After transfection the cells were lysed and assayed as in (**a**). The asterisk indicates a nonspecific band. (**d**) Quantitative analysis of cryopyrin-induced caspase-1 processing. 293-ASC-caspase-1 cells were transfected with increasing amounts of the indicated cryopyrin proteins and analyzed as in (**c**). The relative caspase-1 processing was calculated as the amount of p35 fragment produced by each cryopyrin transfection divided by the amount of p35 produced by the lowest concentration of WT cryopyrin. The values were normalized relative to the expression level of each cryopyrin protein in each transfection. The values were calculated from at least three independent experiments

His6-tagged WT and cryopyrin mutants in Sf-9 cells with the baculovirus system. Cells were lysed in a native or a 6 M guandine-HCl-containing buffer and the soluble proteins were bound to Ni^{2+}-affinity resin. Western blot analysis of the Ni^{2+}-bound proteins revealed dramatically less mutant cryopyrins than WT cryopyrin bound to the Ni^{2+}-affinity resin when the cells were lysed in a native buffer (Figure 3c, upper panel). The least binding was observed with the

D303N mutant (Figure 3c, upper panel, third lane). However, there was no significant difference in the amounts of the mutant and WT cryopyrins bound to the Ni^{2+}-affinity resin when the cells were lysed in a 6 M guandine-HCl-containing buffer (Figure 3c, lower panel). These results demonstrate clearly that the mutant proteins are less soluble under native conditions than the WT protein due to increased self-oligomerization/aggregation, which could explain
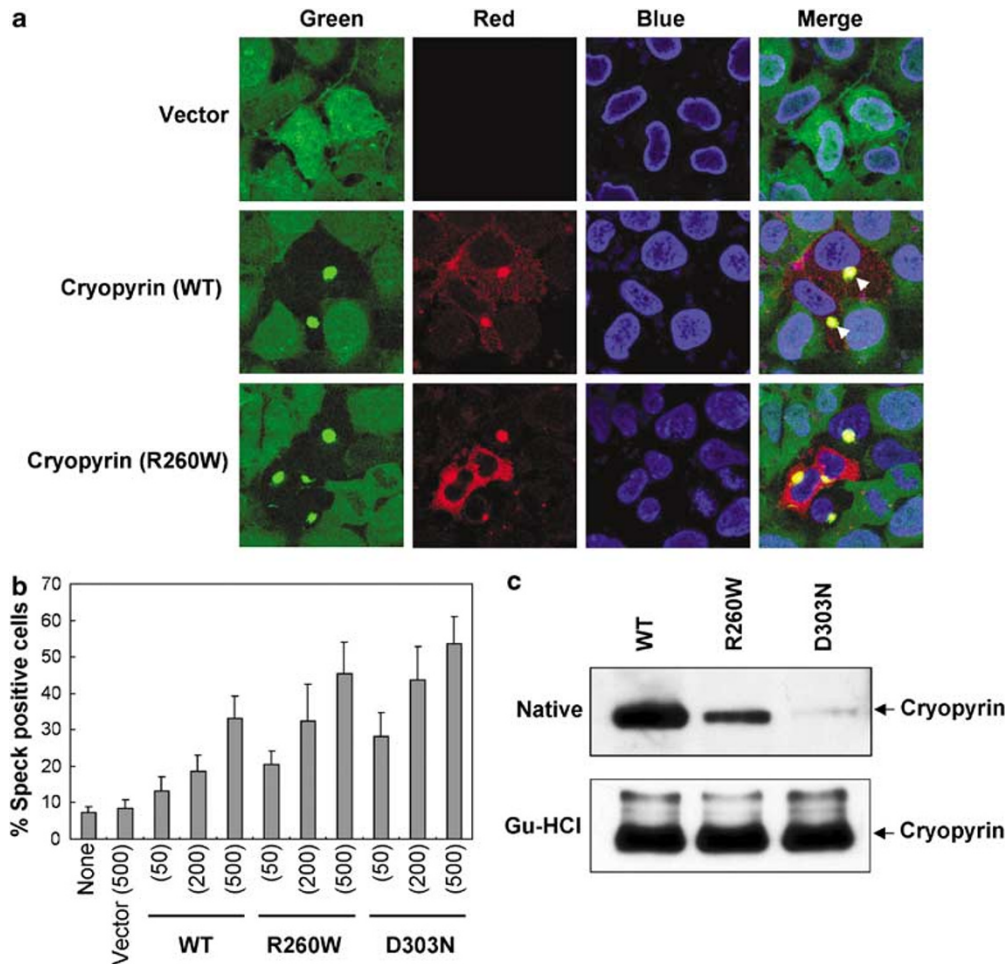
Figure 3 Cryopyrin induces oligomerization of ASC. (**a**) 293-ASC–GFP cells were transfected with empty vector (1st panels from top), or with the WT cryopyrin (2nd panels from top) or the R260W cryopyrin mutant (3rd panels from top). The cells were stained with anti-Nalp3/cryopyrin and rhodamine-red-conjugated secondary antibody (red channel) and nuclei were counterstained with DAPI (blue channel). The green channel represents the ASC–GFP fluorescence. The arrowheads indicate the perinuclear cryopyrin-ASC–GFP specks. Note that only cells expressing exogenous cryopyrin (red channel, cells in the center of the field) show cryopyrin–ASC specks. In the cells that do not express exogenous cryopyrin, ASC is seen in the entire cytoplasm (green channel). (**b**) 293-ASC–GFP cells were transfected with empty vector (vector) or the indicated amounts (ng) of cryopyrin expression plasmids. After transfection the percentages of cells containing cryopyrin–ASC–GFP specks were calculated as described under Materials and Methods. (**c**) The indicated His6-tagged WT and mutant cryopyrin proteins were expressed in Sf-9 cells and then bound to Ni^{2+}-affinity beads in a native (upper panel) or guanidine-HCl (lower panel) buffer as described under Materials and Methods. The bead-bound proteins were then fractionated by SDS-PAGE and immunoblotted with anticryopyrin/Nalp-3 antibody

their increased ability to induce more ASC oligomerization (Figure 3a and b). This is likely responsible for their higher caspase-1-activating ability in our cell-based reconstitution system (Figure 2a and b), and for the increased secretion of mature IL-1β in monocytes from patients harboring the cryopyrin R260W mutation.[35] Remarkably, the least soluble cryopyrin mutant, D303N, was also the most effective in inducing caspase-1 activation (compare Figure 3c with Figure 2b and d). This suggests that mutations that induce more self-oligomerization of cryopyrin may cause more caspase-1 activation, leading to a more severe disease phenotype in patients. Indeed, patients carrying the D303N mutation display a more severe disease phenotype compared to patients with the R260W mutation.[38] It remains to be determined whether this correlation applies to other cryopyrin mutations.

Pyrin induces ASC-dependent caspase-1 activation

Mutations in pyrin are associated with the autoinflammatory disease familial mediteranian fever (FMF), suggesting that pyrin is an important regulator of the inflammatory process. Pyrin has been shown to interact with ASC,[18] but whether this interaction leads to potentiation or inhibition of caspase-1 activation is still unclear. Based on evidence that a C-terminal truncation of pyrin in mice increased IL-1β processing while ectopic expression of WT pyrin in mouse RAW cells impaired IL-1β processing, a recent study proposed that the physiological function of pyrin is to inhibit the proteolytic activation of caspase-1 by sequestering ASC and preventing its interactions with caspase-1.[11] However, the findings that many pyrin mutations in the C-terminal B30.2/rfp/SPRY domain are

associated with FMF,[16] and that human pyrin is induced as an immediate-early gene by proinflammatory molecules[10] argue that the activity of pyrin could be proinflammatory. To test whether pyrin has an anti- or proinflammatory activity, we assayed the activity of WT pyrin, and two FMF-associated pyrin mutants, M694V and V726A, in our cell-based 293-ASC-caspase-1 reconstitution system. As shown in Figure 4a, expression of increasing amounts of WT pyrin or the FMF-associated pyrin mutants in 293-ASC-caspase-1 cells resulted in a dose-dependent autoprocessing of procaspase-1. In contrast, expression of these proteins in the 293–caspase-1 cells, which lacks ASC, did not induce autoprocessing of procaspase-1 (Figures 4b and 5a left panels), indicating that ASC is required for pyrin to recruit and induce autoprocessing of procaspase-1. Consistent with this result, expression of a pyrin mutant with two point mutations in the PYD (L16P/F24S), which are expected to prevent the interaction of pyrin with ASC (see Figure 6, bottom panels), also failed to induce autoprocessing of procaspase-1 (Figure 4a, 11th and 12th lanes). Surprisingly, unlike the disease-associated cryopyrin mutants, the two FMF-associated pyrin mutants did not show a significant increase in activity above that of the WT pyrin with

regard to caspase-1 activation (Figure 4c). In addition, there was no significant difference in the solubility between the mutant pyrin proteins and the WT pyrin in a native buffer (Figure 4d), indicating that these pyrin mutations do not increase pyrin oligomerization. One explanation for these results is that these FMF-associated pyrin mutations might have been acquired during evolution to confer enhanced responsiveness to certain pathogen-associated molecular patterns (PAMP).[16] In the absence of these PAMPs the difference between the activity of the WT and the mutant pyrin proteins might not be detectable.

Pyrin does not interfere with cryopyrin-induced caspase-1 activation

Recent observations suggested that pyrin negatively regulates the cryopyrin-signaling pathway by disrupting the cryopyrin–ASC interactions.[30] To determine the effect of pyrin on cryopyrin-induced caspase-1 activation, we cotransfected pyrin or the FMF-associated pyrin mutants together with cryopyrin into 293-caspase-1 or 293-ASC-caspase-1 cells. As shown in Figure 5a right panel, coexpression of pyrin or the
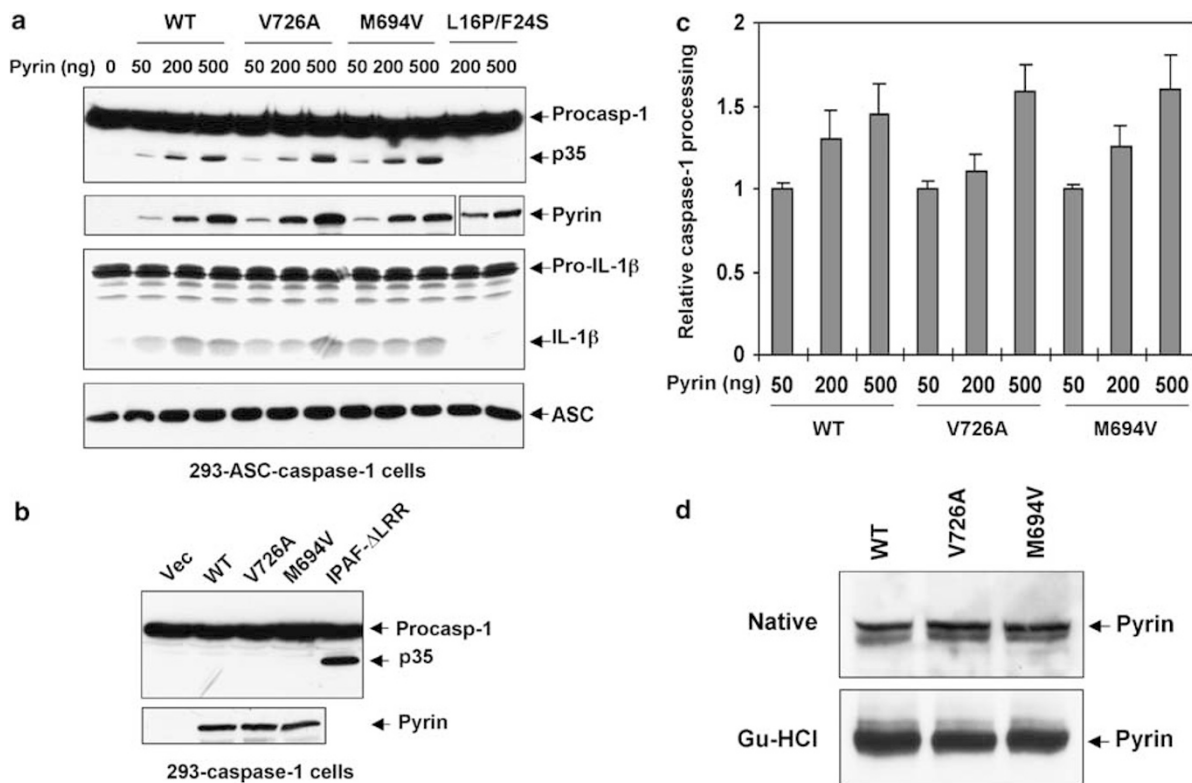

Figure 4 Activation of caspase-1 by pyrin. (**a**) 293-ASC-caspase-1 cells were transfected with increasing amounts of the indicated pyrin proteins. After transfection cells were lysed, and total cell lysates were Western blotted with anti-Flag antibody to detect caspase-1 (1st panel from top) and the pyrin mutant (2nd panel from top, 11th and 12th lanes), or anti-Myc antibody to detect WT, V726A and M694V pyrins (2nd panel from top, 2nd–10th lanes), or anti-ASC monoclonal antibody (4th panel from top). Cell lysates from the transfected cells were also incubated with ^{35}S-labeled pro-IL-1β and the reaction products were fractionated by SDS-PAGE and detected by autoradiography (3rd panel from top). The processed caspase-1 p35 subunit and the mature IL-1β are indicated. (**b**) 293-caspase-1 cells were transfected with equal amounts (500 ng) of empty vector (vec, 1st lane), or the indicated WT and mutant pyrin proteins (2nd–4th lanes), or Ipaf-ΔLRR (5th lane). After transfection cells were lysed, and the total cell lysates were fractionated by SDS-PAGE and detected by autoradiography as in (**a**). (**c**) Quantitative analysis of pyrin-induced caspase-1 processing. 293-ASC-caspase-1 cells were transfected with increasing amounts of the indicated pyrin proteins and analyzed as in (**a**). The relative caspase-1 processing was calculated as in Figure 2d legend. The values were calculated from at least three independent experiments. (**d**) The indicated His6-tagged WT and mutant pyrin proteins were expressed in Sf-9 cells and then bound to Ni^{2+}-affinity beads in a native (upper panel) or guanidine-HCl (lower panel) buffer as described under Materials and Methods. The bead-bound proteins were then fractionated by SDS-PAGE and immunoblotted with anti-His6 antibody

FMF-associated pyrin mutants together with cryopyrin in 293-ASC-caspase-1 cells did not inhibit processing of procaspase-1. On the contrary, there was a slight increase in procaspase-1 processing in the cells that express the two proteins (Figure 5b). These results indicate that the expression of the two proteins together in the same cell does not interfere with caspase-1 activation by either of the two proteins. No activation of caspase-1 by expression of the two proteins was observed in 293-caspase-1 cells, which lacks ASC (Figure 5a, left panels).

Collectively, our results indicate that pyrin, like cryopyrin, is a proinflammatory molecule that is capable of activating procaspase-1 in an ASC-dependent manner.

Pyrin induces ASC oligomerization

The ability of pyrin to induce ASC-dependent procaspase-1 activation suggests that pyrin, like cryopyrin, could induce ASC oligomerization. To visualize ASC oligomerization by
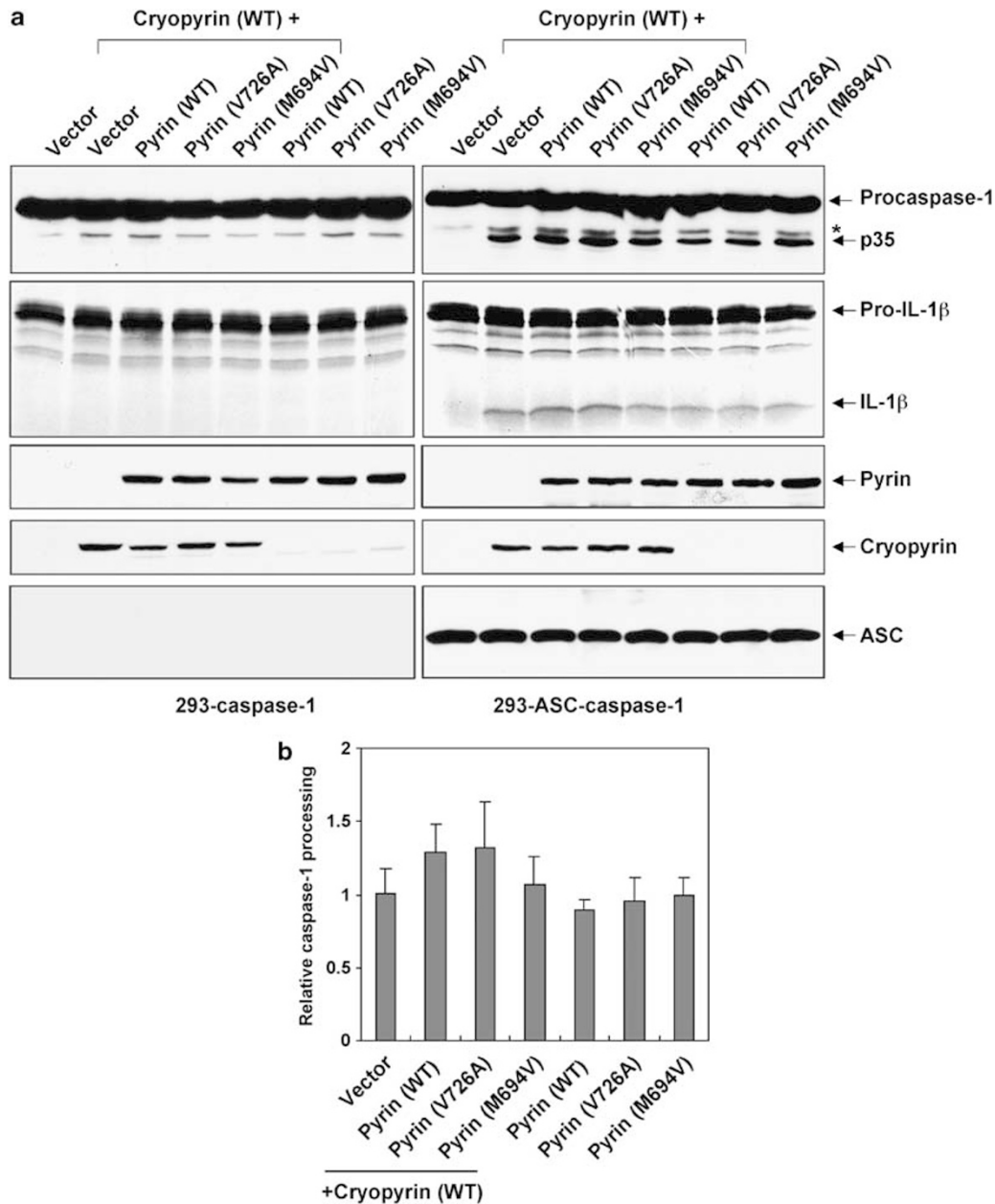
Figure 5 Pyrin does not interfere with cryopyrin induced caspase-1 activation. (**a**) 293-caspase-1 cells (left panels) or 293-ASC-caspase-1 cells (right panels) were transfected with the indicated pyrin constructs alone (750 ng each) (6th to 8th lanes), or together with WT cryopyrin (750 ng) (3rd–5th lanes). Cells transfected with empty vector alone (1st lane) or together with cryopyrin (2nd lane) were used as controls. After transfection the cells were lysed and assayed as in (**a**). The asterisk indicates a nonspecific band. (**b**) Quantitative analysis of pyrin plus cryopyrin-induced caspase-1 processing. 293-ASC-caspase-1 cells were transfected with the indicated pyrin constructs alone (5th–7th columns) or together with WT cryopyrin (2nd–4th columns) and analyzed as in (**a**). First column represents cells transfected with empty vector together with cryopyrin. The relative caspase-1 processing was calculated as the amount of p35 fragment produced by each transfection divided by the amount of p35 produced by WT cryopyrin plus vector (1st column). The values were calculated from at least three independent experiments

pyrin, we expressed WT pyrin or the pyrin mutants M694V and L16P/F24S in the 293-ASC-GFP cells. Expression of the WT pyrin or the pyrin mutant M694V in these cells resulted in complete oligomerization and colocalization of ASC-GFP with the WT pyrin in bright perinuclear specks (Figure 6). No ASC-GFP specks were observed when the cells were transfected with the PYD mutant L16P/F24S, indicating that this mutant does not interact with ASC. The lack of interaction with ASC is thus responsible for the inability of this mutant to induce procaspase-1 activation (see Figure 4a, 11th and 12th lanes). Together, these results indicate that the interaction of pyrin with ASC induces ASC oligomerization, a step that is essential to promote activation of procaspase-1.

Pyrin forms a ternary inflammasome complex with ASC and procaspase-1

The ability of pyrin to oligomerize ASC (Figure 6) and to induce ASC-dependent caspase-1 activation (Figures 4 and 5) suggests that pyrin is capable of assembling a functional inflammasome complex with ASC and procaspase-1. To test this hypothesis we generated C-terminal His6-tagged pyrin and cryopyrin proteins in the baculovirus system and immobilized them on Ni^{2+}-affinity beads. The immobilized proteins were incubated with extracts from the 293-caspase-1 or 293-ASC-caspase-1 cells. As shown in Figure 7a, pyrin, like cryopyrin proteins, was able to recruit

procaspase-1 only in the presence of ASC (2nd lane in each panel) but not in its absence (1st lane in each panel), indicating that the formation of a pyrin–ASC or cryopyrin–ASC complex is a prerequisite for recruitment of procaspase-1. We also noticed that the D303N cryopyrin mutant recruits more procaspase-1 and ASC compared with the WT cryopyrin, consistent with its increased caspase-1-activating ability.

To provide additional evidence that the pyrin–ASC complex could recruit procaspase-1, we immunoprecipitated a pyrin–ASC complex from pyrin-transfected 293-ASC cells, and incubated it with lysates from 293-caspase-1 cells (Figure 7b). As expected the pyrin–ASC complex (2nd lane), but not pyrin alone (3rd lane), was able to recruit procaspase-1. Taken together, our data indicate that pyrin, like cryopyrin, can assemble a ternary inflammasome complex with ASC and procaspase-1.

Discussion

Many cells of lymphoid origin express ASC, procaspase-1 and a large number of proinflammatory NLR proteins. The majority
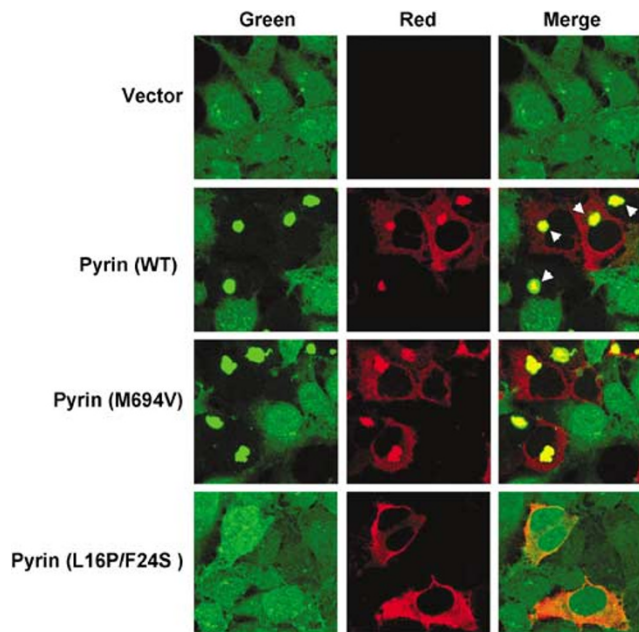
Figure 6 Pyrin induces ASC oligomerization. 293-ASC–GFP cells were transfected with empty vector (1st panel from top), or with the WT pyrin (2nd panel from top), the pyrin mutant M694V (3rd panel from top), or the pyrin mutant L16P/F24S (4th panel from top). The cells in the second and third panels were stained with anti-Myc and rhodamine-red-conjugated secondary antibody to visualize WT and M694V pyrin proteins (red channel). The cells in the fourth panels were stained with anti-Flag and rhodamine-red-conjugated anti-mouse IgG to visualize the pyrin L16P/F24S mutant (red channel). The green channel represents the ASC–GFP fluorescence. The arrowheads indicate the perinuclear pyrin-ASC–GFP specks. Note that the pyrin mutant L16P/F24S does not induce pyrin-ASC–GFP specks
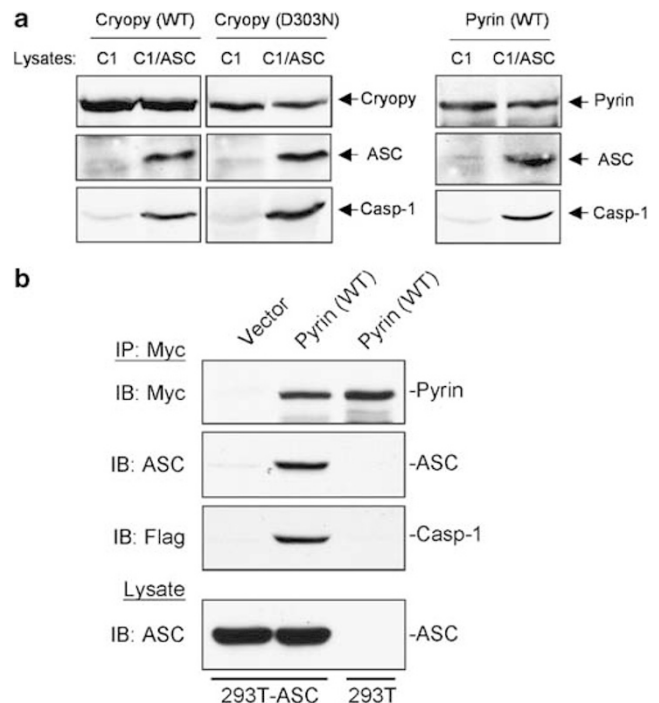

Figure 7 Pyrin recruits ASC and procaspase-1 to form an inflammasome complex. (a) His6-tagged WT pyrin (right panel) or cryopyrin proteins (WT and D303N mutant, left panels) were expressed in Sf-9 cells and bound to Ni^{2+}-affinity beads. The bead-bound proteins were then incubated with extracts prepared from 293-caspase-1 (C1) or 293-ASC-caspase-1 (C1/ASC) cells. After incubation the complexes were fractionated by SDS-PAGE and immunoblotted with the appropriate antibodies to detect cryopyrin, pyrin, ASC and procaspase-1. (b) 293-ASC or 293T cells were transfected with C-terminal Myc-tagged pyrin plasmid. 293-ASC cells were also transfected with an empty vector control. After transfection the cell lysates were immunoprecipitated with an anti-Myc antibody and protein G-sepharose beads. The beads were then incubated with lysates containing procaspase-1 prepared from 293-caspase-1 cells. The bead-bound proteins were then fractionated by SDS-PAGE and immunoblotted with the appropriate antibodies to detect pyrin, ASC and procaspase-1

of NLR proteins contain an N-terminal PYD followed by a NOD/NACHT domain and a regulatory C-terminal LRR domain, which is believed to function as a ligand-binding domain.[5,26,36] Many NLR proteins interact with ASC via PYD–PYD interactions, but their specific ligands and their exact role in inflammation is largely unknown.[5,26] One way to gain insights into the function of the individual NLRs is to reconstitute the NLR complexes in a cell line, such as 293T, that is deficient in ASC, procaspase-1 and proinflammatory NLRs. Here we have described a 293T cell-based reconstitution system that allows the determination of the pro- or anti-inflammatory potential of molecules that interact with ASC and procaspase-1. Unlike the previously described systems in which ASC and procaspase-1 are transiently expressed with different regulatory molecules, the new system utilizes 293T cells that stably express ASC and/or procaspase-1 at physiological levels thus eliminating any artificial results due to variations in ASC and procaspase-1 expression levels. Using this system we demonstrated that cryopyrin and pyrin or their corresponding disease-associated mutants do not induce ASC-dependent activation of NF-κB, thus making it less likely that these proteins regulate inflammation through NF-κB signaling. Rather, these proteins appear to exclusively induce ASC-dependent procaspase-1 activation through ASC oligomerization.

Our data support a model in which pyrin and cryopyrin regulate inflammation through assembly of two distinct and independent inflammasome complexes with ASC and pro-caspase-1 (Figure 8). What triggers the assembly of these inflammasome complexes is currently unknown. Recent findings that cryopyrin activity is regulated by bacterial peptidoglycans (PGN) independent of the LPS receptor TLR-4, raises the possibility that PGNs or its metabolized product muramyl dipeptide (MDP) interacts directly with the LRR of cryopyrin in the cytoplasm to regulate its activity.[36] These interactions could induce oligomerization of cryopyrin, leading to recruitment of ASC and procaspase-1 to this complex. As a result, procaspase-1 is activated by the induced proximity mechanism, and is then capable of processing pro-IL-1β into the mature IL-1β cytokine, which mounts the inflammatory response. Supporting this model crude LPS, which contains PGN could induce procaspase-1 activation independent of the TLR adaptors MyD88 and Trif,[39] suggesting that activation of caspase-1 could be mediated by direct interaction of LPS or PGN with cryopyrin or other related NLR family members in the cytoplasm.

Several missense cryopyrin disease-associated mutations have been described in patients with FCAS, MWS, and NOMID/CINCA.[8,9] Among these diseases NOMID/CINCA syndrome display the most severe phenotype with neonatal onset, chronic polymorphonuclear meningitis leading to progressive neurologic impairment, and recurrent flare-ups of joint inflammation.[38] The molecular mechanisms for the development of these autoinflammatory diseases are not
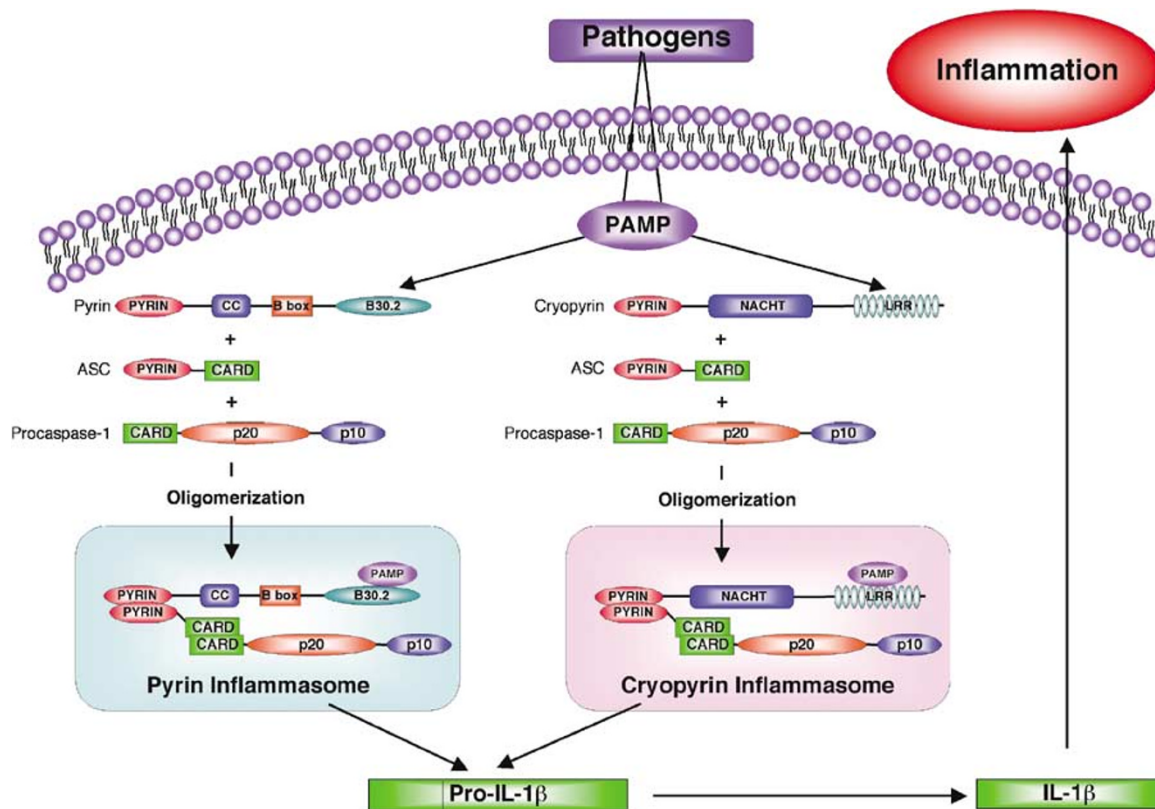
Figure 8 A hypothetical model illustrating the formation of the cryopyrin and pyrin inflammasomes in response to pathogen infection. Recognition of PAMPS by the LRR or B30.2 domains of cryopyrin or pyrin proteins leads to recruitment and oligomerization of ASC and procaspase-1 into two distinct inflammasome complexes. These complexes are then capable of processing pro-IL-1β into the mature IL-1β, which induces inflammation after its release from the infected cell

clear, but recent co-immunoprecipitation data showed that the cryopyrin disease-associated mutant proteins exhibit increased interaction with ASC.[31] However, we were unable to reproduce these results by co-immunoprecipitation because coexpression of the cryopyrin disease-associated mutants and ASC resulted in the formation of insoluble intracellular ASC–cryopyrin complexes/specks (Figure 3). Nevertheless, *in vitro* pull-down assays revealed that the D303N mutant does indeed interact more with ASC than with the WT protein (Figure 7a). In addition, using a 293T cell line that stably expresses a GFP-tagged ASC we were able to observe that the cryopyrin disease-associated mutants can interact with ASC and can also induce more ASC oligomerization/aggregation than the WT cryopyrin (Figure 3b). The reason for the increased ASC oligomerization by the mutant proteins is perhaps due to mutation-induced conformational changes in the oligomerization domain. A computer-generated model of the cryopyrin structure has suggested that the R260W and D303N mutations are located on one side of the protein, near the nucleotide-binding cleft, within a region possibly involved in oligomeric interactions, based on the known oligomeric structures of AAA + ATPases.[38] Therefore, these mutations may cause conformational changes leading to enhanced/increased oligomerization of the mutant cryopyrins. Indeed, analysis of the solubility of the mutant and the WT cryopyrins in a native buffer revealed that the mutant proteins are less soluble than the WT protein, which could explain their tendency to form more oligomers than the WT protein. Collectively, our data suggest that the cryopyrin disease-associated mutations may cause conformational changes in the oligomerization domain leading to self–self interaction and oligomerization. Thus, the cryopyrin disease-associated mutations may mimic activation induced by LPS or PGN (Figure 8).

Contrary to the current model in which it was suggested that pyrin inhibits caspase-1 activation by binding and sequestering ASC,[11] our data clearly demonstrate that pyrin forms a ternary complex with ASC and procaspase-1 and that formation of this complex leads to caspase-1 autoactivation. Indeed, pyrin was as effective as cryopyrin in inducing ASC-dependent caspase-1 activation, and coexpression of the two proteins in the same cell did not interfere with caspase-1 activation, thus ruling out the possibility that association of pyrin with ASC prevents ASC from binding to caspase-1. Considering that most FMF-mutations are located in the C-terminal B30.2/rfp/SPRY domain of pyrin,[16] which has been speculated to function in ligand binding,[40] or signal transduction,[41] it is likely that this domain regulates the activity of pyrin. In analogy with the C-terminal LRR domain of NLR family members, one could speculate that this domain represents a ligand-binding domain that senses PAMPs. Interaction of PAMPs with this domain induces oligomerization and binding of pyrin to ASC, which results in caspase-1 activation and IL-1β processing (Figure 8). The FMF-associated mutations might represent gain of function mutations that were acquired during evolution to sense a wider variety of PAMPs than the WT protein does. This would increase the immune response to a broad class of pathogens and confer a selective survival advantage to FMF carriers.

Consistent with this hypothesis human population studies revealed extremely high allele frequencies for several different pyrin mutations, leading to the conclusion that the mutant alleles confer a selective advantage.[42–44] It was also reported that some of the very same amino acids mutated in FMF carriers are often present as WT in primates and other species.[16]

The recently reported data that mice expressing a C-terminal truncated version of pyrin have a heightened sensitivity to endotoxic shock and an increased caspase-1 activation and IL-1β production in macrophages from these mice after stimulation,[11] is not inconsistent with our model. Several reports have shown that deleting the regulatory LRR domains of several members of the NLR family such as Ipaf, cryopyrin and Nalp1 results in generation of constitutively active molecules.[19,36,37] Considering that the C-terminal SPRY domain of pyrin is a regulatory domain then deleting it could result in a constitutively active pyrin. Alternatively, since macrophages from pyrin-truncation mice exhibit a defect in apoptosis compared to macrophages from WT mice in response to IL-4 and LPS treatment,[11] it is possible that accumulation of activated macrophages in the pyrin-truncation mice could be responsible for their reported heightened sensitivity to endotoxic shock.

Materials and Methods

Expression constructs

Mammalian expression constructs for WT and disease-associated mutant of cryopyrin (pcDNA-cryopyrin-Flag, pcDNA-cryopyrin-R260W-Flag, pcDNA-cryopyrin-D303N-Flag) and pyrin (pcDNA-pyrin-His/Myc, pcDNA-pyrin-M694V-His/Myc, pcDNA-pyrin-V726A-His/Myc) and expression constructs for Ipaf-ΔLRR, and Flag-procaspase-1 were described previously.[31,37,45] The expression plasmid for the pyrin mutant L16P/F24S was constructed in pcDNA3 with a C-terminal Flag tag. The expression plasmid for pro-IL-1β was generated by inserting a PCR generated pro-IL-1β cDNA in pcDNA3 (Invitrogen). pMSCV-ASC and pEGFP-N1-ASC were generated by inserting the cDNA of full-length human ASC in the pMSCV or pEGFP-N1 expression plasmids, respectively. The nucleotide sequences of all constructs were confirmed by automated sequencing.

Cell culture and stable cell lines

293T cells were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum, 200 μg/ml penicillin and 100 μg/ml streptomycin sulfate. Sf9 cells were grown in Sf-900 II SFM supplemented with 10% heat-inactivated fetal bovine serum and 10 μg/ml gentamycin. The N-terminal Flag-tagged human procaspase-1-expressing 293T cells (293-caspase-1 cells) were generated by stable transfection of 293T cells with a pcDNA3 expression vector containing the full-length human procaspase-1 cDNA (pcDNA-Flag-procaspase-1). Stable cell clones were selected using G418. The 293-ASC-caspase-1 cells or 293-ASC cells were generated from the 293-caspase-1 cells or 293T cells, respectively, by stable transfection with pMSCV–ASC expression plasmid. Stable cell clones were selected using puromycin. 293T cells expressing C-terminal GFP-tagged full-length ASC were generated by stable transfection with the expression plasmid pEGFP-N1-ASC and the empty pMSCV vector. Stable cell clones were selected using puromycin.

Expression of cryopyrin and pyrin proteins in the baculovirus system

The recombinant baculovirus transfer vectors for the WT and mutant cryopyrin and pyrin proteins were constructed in pVL-1393 with C-terminal His6 tags. The cryopyrin and pyrin transfer vectors were cotransfected with Sapphire™ baculovirus DNA (Orbigen) into Sf-9 cells to generate the recombinant baculoviruses according to the manufacturer's recommendation. To express cryopyrin or pyrin proteins in Sf-9 cells, 1×10^7 cells were infected with the respective recombinant baculoviruses at multiplicity of infection of 10. Cells were then seeded into T175 flasks and incubated for 48 h at 27°C. The expression of cryopyrin and pyrin proteins in Sf-9 cells was determined by Western blotting with anti-cryopyrin/Nalp3 antibody (Alexis) or anti-His6 antibody, respectively.

Cryopyrin and pyrin solubility assay

Sf-9 cells were infected with recombinant cryopyrin or pyrin baculoviruses for 48 h as described above. The infected cells were lysed by syringing ($20 \times$) in a native buffer (20 mM Hepes, pH 7.5, 1.5 mM $MgCl_2$, 100 mM NaCl, 1% Triton X100, 0.1 mM PMSF) or a denaturation buffer (50 mM Tris pH 7.5, 8.0 M guanidine-HCl) and centrifuged at $16\,000 \times g$ for 10 min. The cell lysates were then bound to Ni^{2+}-affinity beads and washed several times. The bead-bound proteins were then fractionated by SDS-PAGE and immunoblotted with the appropriate antibodies to detect cryopyrin and pyrin proteins.

Caspase-1 processing and IL-1β cleavage assay

Cells (2×10^5) were plated in six-well plates, and 16 h later, cells were transfected with the indicated plasmid using LipofectAMINE (Invitrogen). Cells were harvested 24 h after transfection, and lysed in a buffer containing 20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 2 μg/ml leupeptin, 2 mM DTT, 1 mM Na_3VO_4, 10 mM NaF by passing the cells several times through a 21 G needle. Lysates were then clarified by centrifugation at $16\,000 \times g$ for 10 min, and the supernatants were normalized for protein concentration. The normalized supernatants were then subjected to SDS-PAGE followed by immunoblotting with anti-Flag antibody to detect caspase-1 processing. The expression of the transfected pyrin-Myc or cryopyrin-Flag proteins was detected by Western blotting with the anti-Myc and anti-Flag antibodies, respectively. The expression of ASC was detected by Western blotting with the ASC monoclonal antibody.[17] For IL-1β cleavage assay, pro-IL-1β was *in vitro* translated in the presence of ^{35}S-methione using a TNT reticulocyte system (Promega), and incubated with the supernatant (50 μg) at 30°C for 90 min. The reactants were then analyzed by SDS-PAGE followed by autoradiography.

NF-κB reporter gene assays

Cells in 6-well plates were transfected with $5 \times$ κB-luciferase reporter plasmid (30 ng), pβ-gal (60 ng), cryopyrin or pyrin (0.4 μg). and TRAF6 (0.4 μg) as indicated. The total amount of DNA (0.8 μg) was kept constant by inclusion of empty vector DNAs. The luciferase activity was determined using a Dual-Luciferase Assay System (Promega). A LacZ-expressing plasmid was used for normalizing transfection efficiencies.

Induction of the endogenous NF-κB target gene IL-8 was determined 20 h after transfection of cells in six-well plates with the indicated constructs (500 ng) by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) using the IL-8 primers; 5′-primer, ATGACTTC CAAGCTGGCC and 3′-primer CTATGAATTCTCAGCCCTC. The amplification products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

Confocal microscopy

ASC–GFP expressing 293T cells grown on cover slips were transfected with the pyrin or cryopyrin expression plasmids. The transfections were carried out using Lipofectamine PLUS-reagent (Invitrogen) according to the manufacturer's instructions. All the plasmids for transfection were prepared by using Qiagen columns. After 24–48 h of transfection cells were stained with anti-Nalp3 antibody (Alexis) (for WT, R260W and D303N cryopyrins), anti-Flag antibody (for pyrin mutant L16P/F24S), or anti-Myc (for WT, M694V and V726A pyrins), and rhodamine-red-conjugated secondary antibodies, and nuclei were counterstained with DAPI. Cells were observed using a Zeiss LSM 510 Meta confocal microscope.

Speck quantitation assay

ASC–GFP expressing 293T cells grown in six-well plates were transfected with different amounts of the pyrin or cryopyrin expression plasmids as above. At 48 h after transfection the number of cells containing ASC–GFP specks were counted in randomly selected multiple fields. The percentage of ASC–GFP speck-positive cells was calculated as the number of specks divided by the total number of transfected cells. At least three independently transfected wells were analyzed per experiment.

Immunoprecipitation and pull-down assays

293-ASC or 293T cells were transfected with a C-terminal Myc-tagged pyrin plasmid (2 μg) in 100 mm dishes and 24 h after transfection the transfected cells were lysed by syringing ($20 \times$) in an IP buffer (20 mM Hepes, pH 7.4,. 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM PMSF, 2 μg/ml leupeptin, 1 mM Na_3VO_4, 5 mM NaF) and centrifuged at $16\,000 \times g$ for 10 min. The pyrin–ASC complex and pyrin alone were immunoprecipitated from the lysates with an anti-Myc antibody (Abcam) and immobilized on protein G-sepharose beads. The beads were then incubated with lysates containing procaspase-1 prepared from 293-caspase-1 cells in IP buffer for 3 h at 4°C. The bead-bound proteins were then fractionated by SDS-PAGE and immunoblotted with the appropriate antibodies to detect pyrin, ASC and procaspase-1.

In vitro pull-down assays were performed with recombinant C-terminal His6-tagged cryopyrin and pyrin proteins expressed in the baculovirus system. Cryopyrin and pyrin proteins were isolated from Sf-9 cell lysates by Ni^{2+}-affinity purification on Ni^{2+}-affinity beads. The bead-bound proteins were then incubated for 2 h at 4°C with extracts prepared from 293-caspase-1 or 293-ASC-caspase-1 cells. After incubation the complexes were fractionated by SDS-PAGE and immunoblotted with the appropriate antibodies to detect cryopyrin, pyrin, ASC and procaspase-1.

Acknowledgements

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Supplementary information accompanies the paper on the Cell Death and Differentiation web site (http://www.nature.com/cdd)