Cryopyrin-induced Interleukin 1β Secretion in Monocytic Cells

ENHANCED ACTIVITY OF DISEASE-ASSOCIATED MUTANTS AND REQUIREMENT FOR ASC

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Several autoinflammatory disorders are associated with missense mutations within the nucleotide-binding oligomerization domain of cryopyrin. The mechanism by which cryopyrin mutations cause inflammatory disease remains elusive. To understand the molecular bases of these diseases, we generated constructs to express three common cryopyrin disease-associated mutations, R260W, D303N, and E637G, and compared their activity with that of the wild-type protein. All cryopyrin mutant proteins tested were found to induce potent NF-xB activity when compared with the wild-type protein. This activation was dependent on the expression of ASC, an adaptor protein previously suggested to mediate cryopyrin signaling. When the disease-associated mutants were expressed in monocytic THP-1 cells (which express endogenous ASC), each induced spontaneous IL-1β secretion, whereas wild-type protein did not. In the absence of stimuli, wild-type cryopyrin was unable to bind to ASC, whereas the three mutant co-immunoprecipitated with ASC, suggesting a mechanism involved in the constitutive activation of mutant proteins. The induction of cryopyrin activity by enforced oligomerization in THP-1 cells resulted in ASC binding and the secretion of IL-1β, an effect that was abolished by the inhibition of ASC expression with small interfering RNAs. Thus, cryopyrin-mediated IL-1β secretion requires ASC in monocytic cells. Further, these results indicate that cryopyrin disease-associated mutants are constitutively active and able to induce NF-xB activation and IL-1β secretion at least in part by an increased ability to interact with ASC.

Human cryopyrin (also termed PYPAF1/NALP3/CATERPILLER 1.1†) is a protein encoded by the CIAS1 gene on chromosome 1q44. Missense mutations in cryopyrin are responsible for several autoinflammatory disorders, including familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and neonatal-onset multisystem inflammatory disease (NOMID). The NOD contains several distinct motifs including nucleotide-binding and Mg2+-binding sites, referred to as Walker A and B motifs, respectively. Many of the disease-associated mutants are clustered near the predicted Mg2+-binding site (1, 7). Notably, similar missense mutations have been found in the NOD of Nod2 in patients with Blau syndrome, another autosomal-dominant autoinflammatory syndrome (8, 9). Interestingly, the R260W cryopyrin mutation (identified in both FCAS and MWS) and the R334W mutation (in Blau syndrome) involve amino acid residues at analogous sequence positions, suggesting a common molecular mechanism for the development of autoinflammatory disease (8, 9).

The signaling pathways activated through cryopyrin have just begun to be identified. Previous studies have shown that the PD of cryopyrin interacts with ASC, a PD/CARD-containing adaptor molecule that has been suggested to mediate cryopyrin signaling (5, 10). Oligomerization through the NOD has been suggested to be critical to interaction with downstream molecules and the function of several NOD family members including Apaf-1, Nod1, and Nod2 (3, 11). Similarly, oligomerization of cryopyrin PD in the presence of ASC has been shown to induce NF-xB activation and apoptosis (10). Other studies have reported that overexpression of ASC promotes caspase-1 activation through a CARD-CARD interaction, an event that can result in the processing of pro-IL-1β to mature IL-1β (12, 13).

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amount expressed in the cells (14–16). Thus, the role and requirement of ASC for cryopyrin-mediated IL-1β production remain unclear.

Several lines of evidence suggest that IL-1β is an important mediator of inflammation in patients with cryopyrin mutations (17–19). IL-1β was found to be up-regulated in unstimulated monocytes from patients with NOMID syndrome (17). More significantly, clinical studies involving nonrelated MWS patients harboring the cryopyrin disease-associated R260W variant had a rapid clinical and serologic response following injection with an IL-1 receptor antagonist (18, 19). In the current studies, we compared the functional activity of wild-type and disease-associated cryopyrin mutants to understand the molecular basis of FCAS, MWS, and NOMID diseases. We found that cryopyrin mutants act as constitutively active proteins, providing a mechanism whereby cryopyrin mutations lead to dominant autoinflammatory disease. In addition, we provide evidence that ASC is required for IL-1β secretion induced through cryopyrin activation in monocytic THP-1 cells.

EXPERIMENTAL PROCEDURES

Expression Plasmids—Wild-type and LRR deleted mutants were described previously by Dowds et al. (10). The QuikChange XL site-directed mutagenesis kit (Stratagene) was used to generate pcDNA3-cryopyrin R260W-Flag (nucleotide C778T), pcDNA3-cryopyrin D303N-Flag (nucleotide G907A), and pcDNA3-cryopyrin E627G-Flag (nucleotide A1880G) as described by the manufacturer’s protocol. The mutagenic oligonucleotide primers (Invitrogen) used with this protocol are as follows: R260W, 5′-TTCTATATCCACTGTTGGGAGGTGAGC-3′; D303N, 5′-CTCTATATCCAGGTGGTGGATGCCTTG-3′; and E627G, 5′-GCCCAAGCCGACTGGTTGTCTACTGTTGTT-3′. The retroviral constructs were generated by PCR from templates described previously in Ref. 10 and subcloned into pMSCV-puro vector (BD Biosciences) and the manufacturer’s recommended protocol. ELISAs were developed for 5 min using tetramethylbenzidine substrate (Bio FX, Owings Mills, MD) and then stopped with 2 N H2SO4. Within 30 min, the absorbance was measured with a microplate reader (Bio-Tek Instruments, Winooski, VT).

RESULTS AND DISCUSSION

NF-κB Assay—1 × 10^6 HEK293T cells were co-transfected with a construct of interest in the presence of 33 ng of pEF1BOS-β-gal and 2.2 ng of pBsXVI-luc reporter. NF-κB luciferase reporter activity was measured 24 h post-transfection, and the values were normalized to β-galactosidase from triplicate cultures. The results are given as the mean ± S.D.

IL-1β ELISA—Assays were performed using matched Ab pairs (BD Biosciences) and the manufacturer’s recommended protocol. ELISAs were developed for 5 min using tetramethylbenzidine substrate (Bio FX, Owings Mills, MD) and then stopped with 2 N H2SO4. Within 30 min, the assay plates were read at 450 nm with a correction at 570 nm and analyzed with KC Jr. software (Bio-Tek Instruments, Winooski, VT).

Immunoprecipitation and Detection of Proteins—1 × 10^6 THP-1 stably expressing cryopyrinPD-Fpk3-Myc or Fpk3-Myc were incubated in the presence or absence of 200 nM AP1510 (Ariad Pharmaceuticals) or 1 ng/ml lipopolysaccharide from Escherichia coli 055B5 (Sigma) was used to treat the THP-1 cells. The retroviral constructs were generated by PCR from templates described previously in Ref. 10 and subcloned into pMSCV-puro vector (BD Biosciences) and the manufacturer’s recommended protocol. ELISAs were developed for 5 min using tetramethylbenzidine substrate (Bio FX, Owings Mills, MD) and then stopped with 2 N H2SO4. Within 30 min, the assay plates were read at 450 nm with a correction at 570 nm and analyzed with KC Jr. software (Bio-Tek Instruments, Winooski, VT).

Cryopyrin Disease-associated Mutants Activate NF-κB in the Presence of ASC—We engineered full-length cryopyrin expres-
The Cryopyrin Disease-associated Mutants Exhibit Increased Functional Activity

**Expression of Cryopyrin Disease-associated Mutants Induces Secretion of IL-1β in THP-1 Cells**—In addition to NF-κB activation, cryopyrin has been suggested to regulate a signaling pathway leading to the production of IL-1β (5, 15). Therefore, we compared the ability of disease-associated mutants and wild-type cryopyrin to induce secretion of IL-1β in human monocytic THP-1 cells. THP-1 cells express endogenous ASC; therefore, we transfected only the disease-causing mutants R260W, D303N, and E627G or wild-type cryopyrin and measured IL-1β production in the culture supernatants 24 h post-transfection. All three disease-associated mutants, as well as a mutant cryopyrin lacking the LRR that exhibits enhanced activity (5, 10), induced secretion of IL-1β, whereas wild-type cryopyrin did not (Fig. 2A). Consistent with these results, THP-1 cells stably transfected with the disease-associated R260W mutant spontaneously produced IL-1β, whereas cells transfected with wild-type cryopyrin or vector alone did not.

**Interaction of ASC with Cryopyrin Disease-associated Mutants.** HEK293T cells were cotransfected with 2 μg of pcDNA3, pcDNA3-cryopyrin-Flag (WT), pcDNA3-cryopyrinR260W-Flag (R260W), pcDNA3-cryopyrinD303N-Flag (D303N), or pcDNA3-cryopyrinE627G-Flag (E627G) in the presence of pCMV-β-gal and supernatants were assayed for IL-1β using ELISA 24 h post-transfection. Values are normalized to β-galactosidase from triplicate cultures. The results are given as the mean ± S.D. B, supernatants from 1 × 10^6 THP-1 stably transfected with pcDNA3, pcDNA3-cryopyrin-Flag (WT), pcDNA3-cryopyrinR260W-Flag (R260W) were assayed for IL-1β using ELISA. The results are given as the mean ± S.D. of triplicate cultures.

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*2 T. A. Dows, unpublished data.*
failed to bind to ASC (Fig. 3). In contrast, the cryopyrin mutants R260W, D303N, E627G, or ΔLRR readily co-immunoprecipitated (IP) with polyclonal myc Ab (top panel), and total lysates (bottom panel) from stable cells were immunoblotted with monoclonal ASC Ab. The same blots were reprobed with monoclonal tubulin Ab as a loading control. B, stable cryopyrinPD-Fpk3 or Fpk3 vector THP-1 cell lines were co-transfected with 0, 15, 30, or 60 pmol of siRNA ASC + AP1510 or cryopyrinPD-Fpk3 with 60 pmol siRNA GFP + AP1510 were immunoblotted with ASC Ab. The same blots were reprobed with monoclonal tubulin Ab as a loading control. B, stable cryopyrinPD-Fpk3 or Fpk3 vector THP-1 cell lines were transfected with ASC siRNA or GFP siRNA as indicated in the presence (+) or absence (−) of Fpk3 ligand AP1510. Following treatment, secreted IL-1β was measured by ELISA. The results are given as the mean ± S.D. of triplicate cultures.

Fig. 5. Elimination of ASC prevents cryopyrin-mediated IL-1β secretion. A, total lysates from cryopyrinPD-Fpk3 or Fpk3 vector THP-1 cell lines transfected with 0, 15, 30, or 60 pmol of siRNA ASC + AP1510 or cryopyrinPD-Fpk3 with 60 pmol siRNA GFP + AP1510 were immunoblotted with ASC Ab. The same blots were reprobed with monoclonal tubulin Ab as a loading control. B, stable cryopyrinPD-Fpk3 or Fpk3 vector THP-1 cell lines were co-transfected with ASC siRNA or GFP siRNA as indicated in the presence (+) or absence (−) of AP1510. Following treatment, secreted IL-1β was measured by ELISA. The results are given as the mean ± S.D. of triplicate cultures.
ciated cryopyrin mutants suggested that the interaction of cryopyrin with ASC is important for signaling. However, the role of ASC in cryopyrin-mediated IL-1β secretion has not been determined in monocytes that express endogenous ASC. To examine this, we developed a THP-1 cell line that stably expressed a chimeric protein composed of the effector PD of cryopyrin fused to three tandem Fkbp domains (cryopyrinPD-Fpk3) that can be oligomerized by the cell-permeable ligand AP1510 (Fig. 4A) (10). Using this inducible system of cryopyrin activation, we found that cryopyrinPD-Fpk3 co-immunoprecipitated with endogenous ASC only in the presence of AP1510, suggesting that oligomerization of cryopyrin PD is required for endogenous ASC binding (Fig. 4B). Moreover, this interaction correlated with the ability of these stable THP-1 cells to secrete IL-1β after incubation with the dimerizer AP1510 (Fig. 4C). In contrast, THP-1 cells expressing Fpk3 alone did not bind to endogenous ASC or secrete IL-1β in the presence or absence of AP1510 (Fig. 4, B and C).

ASC Plays a Critical Role in Cryopyrin-mediated IL-1β Secretion—To establish a direct role for ASC in cryopyrin-mediated IL-1β secretion, we used siRNAs to inhibit the expression of endogenous ASC in cryopyrinPD-Fpk3 or Fpk3 THP-1 stable cells and measured the IL-1β secretion in the presence or absence of AP1510 (Fig. 5, A and B). We tested four siRNAs to target different ASC sequences, and one of them reduced ASC expression in a dose-dependent manner (Fig. 5A). At a dose of 60 pmol of ASC siRNA, there was no detectable amount of ASC protein in THP-1 cells, whereas the same amount of control siRNA did not lower the level of ASC protein either in the presence or absence of AP1510 (Fig. 5A). The ASC siRNA did not influence cell viability2 and was specific in that expression of tubulin was not affected (Fig. 5A). Importantly, inhibition of ASC expression by siRNA decreased the amount of IL-1β secretion in cryopyrinPD-Fpk3 THP-1 cells in a dose-dependent manner in the presence of AP1510. In contrast, the same cells retained their ability to secrete IL-1β when incubated with control siRNA (Fig. 5B). In addition, the three cryopyrin siRNAs, which were unable to alter ASC expression, did not reduce IL-1β secretion, providing further evidence for the specificity of the results.2 These findings indicate that ASC is required for cryopyrin-mediated secretion of IL-1β in THP-1 monocytes.

In conclusion, we have shown that the cryopyrin disease-associated missense mutations R260W, D303N, and E627G found within the NOD exhibit a gain-of-function phenotype as shown by NF-κB activation and IL-1β secretion. The observed phenotype is consistent with the dominant mode of genetic transmission of FCAS, MWS, and NOMID (1). The molecular mechanism that confers constitutive activation to the disease-associated mutations is unclear. NOD family members are thought to be kept in an inactive conformation by interactions with the LRRs, a state that is relieved by recognition of stimulating ligands via the LRRs. For example, exposure of HeLa cells to Shigella activates Nod1, resulting in the recruitment of RIP-like interacting CLARP kinase (RICK) and NF-κB activation (23). In this regard, the disease-associated mutations are constitutively active and exhibit an increased binding to ASC, an adaptor molecule shown here to be essential for cryopyrin-mediated IL-1β secretion in THP-1 cells. Thus, the mutations in cryopyrin associated with disease may mimic activation induced by a microbial ligand, which remains to be identified. In addition to dysregulated IL-1β secretion, disease-associated cryopyrin mutants exhibited enhanced NF-κB activation. An important role for IL-1β in cryopyrin-mediated inflammation is suggested by clinical improvement induced by treatment of MWS patients with IL-1 receptor antagonist (18, 19). The latter observation suggests that secretion of IL-1β is more relevant than NF-κB activation in cryopyrin-mediated inflammatory disease. However, enhanced NF-κB activity is likely to contribute to overproduction of IL-1β in MWS patients. For example, IL-1β expression is induced transcriptionally through NF-κB sites located in the IL-1β promoter (24).

Our current studies have focused on NF-κB and IL-1β secretion induced by cryopyrin. There is also evidence that cryopyrin and ASC promote apoptosis (10). Thus, it is also possible that abnormal apoptosis induced by disease-associated cryopyrin mutations could contribute to inflammatory disease. Further studies are needed to fully understand the role of NF-κB, IL-1β secretion, and apoptosis in cryopyrin-mediated autoinflammatory syndromes.

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