Interaction between Pyrin and the Apoptotic Speck Protein (ASC) Modulates ASC-induced Apoptosis*

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Patients with familial Mediterranean fever suffer sporadic inflammatory attacks characterized by fever and intense pain (in joints, abdomen, or chest). Pyrin, the product of the MEFV locus, is a cytosolic protein whose function is unknown. Using pyrin as a “bait” to probe a yeast two-hybrid library made from neutrophil cDNA, we isolated apoptotic speck protein containing a caspase recruitment domain (CARD) (ASC), a proapoptotic protein that induces the formation of large cytosolic “specks” in transfected cells. We found that when HeLa cells are transfected with ASC, specks are formed. After co-transfection of cells with ASC plus wild type pyrin, an increase in speck-positive cells is found, and speck-positive cells show increased survival. Immunofluorescence studies show that pyrin co-localizes with ASC in specks. Speck localization requires exon 1 of pyrin, but exon 1 alone of pyrin does not result in an increase in the number of specks. Exon 1 of pyrin and exon 1 of ASC show 42% sequence similarity and resemble death domain-related structures in modeling studies. These findings link pyrin to apoptosis pathways and suggest that the modulation of cell survival may be a component of the pathophysiology of familial Mediterranean fever.

Familial Mediterranean fever (FMF)1 is a genetically recessive disease characterized by sporadic attacks of fever and pain (1). Attacks typically last 1–3 days and are accompanied by a massive influx of neutrophils into specific anatomical sites, most commonly in the abdomen (peritoneal membranes), chest (pleural membranes), and joints (synovial membranes). The gene responsible for FMF is called MEFV, and its protein product has been named pyrin (2) or marenostrin (3). Pyrin is a 781-amino acid protein that is expressed in a tissue-restricted manner in neutrophils, monocytes, and eosinophils and in fibroblasts of skin, synovium, and peritoneum (2–5). Based on the nature of FMF attacks, it has been speculated that wild type pyrin acts as a negative regulator of the inflammatory response (1). Pyrin mutations, it is proposed, could compromise this function, resulting in inappropriate inflammatory responses to otherwise innocuous triggers. However, the nature of this regulatory pathway and the identity of the trigger(s) that incite attacks remain to be identified.

The C-terminal half of the pyrin molecule (amino acids 300–781) harbors several motifs found in other proteins including a B-box zinc finger (thought to be a protein-protein interaction domain), an α helical region, which may adopt a coiled coil configuration, and a C-terminal rfp or B30.2 domain speculated to function in ligand binding (6) or signal transduction (7). This tripartite structure is found in a variety of genes, including transcription factors (STAF50 and estrogen-responsive finger protein), Mid1 (a cytosolic protein important in development of midline structures), and Ro52 (an autoantigen) (6). What common molecular functions or pathways (if any) might be attributed to these shared domains in these apparently diverse proteins is still unclear. However, a recent report suggests that some of these elements define a family (TRIM) and are important in subcellular localization (8). In the case of pyrin, of the 21 mutations now identified, 66% fall in the C-terminal rfp domain, a fact that attests to the functional importance of this domain.

At the time that the MEFV gene was cloned, the N-terminal portion of pyrin (amino acids 1–300) appeared to be unique; there were no characterized proteins with homology to this region. Recently, however, several proteins have been found to contain a sequence domain with similarity to the N terminus of pyrin; this domain has been called a pyrin domain (PyD) (9, 10), DAPIN domain (11), or PAAD domain (12). These proteins include ASC (apoptosis speck protein with caspase recruitment domain (CARD), Ref. 13), zebrafish caspase 13 (14), nucleotide-binding protein 1 (NBS1, N-terminal nucleotide-binding protein 1; FADD, Fas-associated death domain; TRIM, tripartite motif; HA, hemagglutinin; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; FITC, fluorescein isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole; TUNEL, terminal dUTP-mediated nick-end labeling; CMV, cytomegalovirus; GFP, green fluorescent protein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Hsp27, heat shock protein 27; DIC, differential interference contrast.

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†† The abbreviations used are: FMF, familial Mediterranean fever; rfp, Ret finger protein; PyD, pyrin domain; CARD, caspase recruitment domain; ASC, apoptotic speck protein containing a CARD; NBS1, nucleotide-binding protein 1; FADD, Fas-associated death domain; TRIM, tripartite motif; HA, hemagglutinin; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; FITC, fluorescein isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole; TUNEL, terminal dUTP-mediated nick-end labeling; CMV, cytomegalovirus; GFP, green fluorescent protein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Hsp27, heat shock protein 27; DIC, differential interference contrast.
that these function in apoptosis, inflammation, and innate immunity (9, 10, 12, 14, 18).

To begin to explore the molecular function of pyrin, we carried out a yeast two-hybrid screen using the entire wild type pyrin molecule as a bait. Screening of a cDNA library generated from human neutrophils identified ASC (also known as TMS1) as one of several pyrin-interacting proteins. ASC was first identified as a protein that aggregates into a cytosolic “speck” during apoptosis of HL-60 cells, a human promyelocytic cell line (13). ASC is a 195-amino acid protein that contains two domains, a C-terminal CARD and an N-terminal PYD. In 293 cells, speck formation occurs upstream of caspase 9 activation and eventual apoptosis, suggesting that ASC is a proapoptotic protein (19). In HL-60 cells, anti sense ASC oligonucleotides block etoposide-induced apoptosis, confirming that endogenous ASC is proapoptotic (13).

The interaction between pyrin and ASC was confirmed using immunoprecipitation and cellular co-localization studies. By transient transfection, we show that speck-positive HeLa cells transfected with ASC are destined for apoptosis. When cells are co-transfected with both pyrin and ASC, speck formation is accelerated but fewer speck-positive cells succumb to apoptotic death. Thus, pyrin alters the ASC death machinery. Pyrin co-localizes with ASC in specks, and exon 1 of pyrin is necessary and sufficient for this targeting phenomenon. However, exon 1 of pyrin is not sufficient to modulate ASC-induced death; this requires additional regions of the pyrin molecule.

Apoptotic proteins often share structural domains that provide interaction interfaces (e.g. the death domain, the death effector domain, and the CARD, Ref. 20). Here we present structural modeling data to show that the homologous first exons of both pyrin and ASC encode a six-helix structure closely related to a death domain. When the exon 1 sequences for pyrin and ASC are threaded onto the FADD death domain structure, analysis of the resulting electrostatic charge distribution reveals probable interaction surfaces. These findings in combination suggest that pyrin functions in an apoptotic pathway.

**EXPERIMENTAL PROCEDURES**

**Plasmids—**All myc-tagged pyrin constructs were cloned into pCMVTag3a (Stratagene). β-Galactosidase-myc (lacZ-myc) and heat shock protein 27- myc (hsp27-myc) were gifts from Yi-feng Jia and Dr. Michael Welsh (University of Michigan). A plasmid expressing DEFCAP-HA was kindly provided by Dr. Gabriel Nunez (University of Michigan). All FLAG-tagged constructs were cloned into pCMVTag2B (Stratagene). HA-tagged constructs were engineered from pcDNA3. YFP- and GFP-tagged constructs were produced by cloning the appropriate cDNA into eYFP or eCFP vectors (CLONTECH).

**Antibodies—**Mouse anti-FLAG antibody (M2 monoclonal) was obtained from Sigma. Mouse anti-Myc, rabbit anti-Myc, rabbit anti-HA, and goat anti-mouse horseradish peroxidase were obtained from Santa Cruz Biotechnology. Goat anti-mouse AF568, goat anti-rabbit AF688, phalloidin AF488, and mouse anti-tubulin were purchased from Molecular Probes. Goat anti-mouse-FITC was purchased from Jackson Immunoresearch.

**Yeast Two-Hybrid Screen—**A yeast two-hybrid library was constructed using poly(T)-primed Poly(A)+ RNA isolated from cells recovered from the knee of a patient with gout. Microscopic examination of the aspirate confirmed that the majority of cells were neutrophils. The library consisted of 2 × 10⁹ clones. Full-length wild type pyrin was cloned into the “bait” vector pAS2-1 (CLONTECH), which directs expression of pyrin as a fusion product with the GAL4 DNA binding domain, and transformed into Y187 cells. The prey library was then transformed into pyrin-expressing Y187 cells; putative interactants were selected as colonies that grew in triple dropout selection, His(−) LacZ(+) and were blue upon testing with Xgal. Individual prey plasmids were isolated from His(−) LacZ(+) clones and retransformed into yeast in the absence of the pyrin bait to determine that they did not directly activate the His and lacZ genes. Candidates were also retested in the presence of pyrin to confirm their interaction with pyrin. Individual prey plasmids were also co-transformed with heterologous baits (p53 and lamin) to rule out nonspecific interactions. Clones passing these tests were sequenced. A total of 132 clones were further analyzed. DeF CAP-HA was identified as a protein that aggregates into a cytosolic “speck” during apoptosis of HL-60 cells, a human promyelocytic cell line (13). ASC is a 195-amino acid protein that contains two domains, a C-terminal CARD and an N-terminal PYD. In 293 cells, speck formation occurs upstream of caspase 9 activation and eventual apoptosis, suggesting that ASC is a proapoptotic protein (19). In HL-60 cells, anti sense ASC oligonucleotides block etoposide-induced apoptosis, confirming that endogenous ASC is proapoptotic (13).

**Immunoprecipitation—**1 × 10⁶ 293T cells were plated on 10-cm plates and transfected via the CaPO₄ method. A total of 6 μg of DNA was used for transfections. Cells were harvested at 48 h and lysed in a buffer containing 50 mM HEPS, 150 mM NaCl, 10% glycerol (w/v), and 2 mM EDTA to which 0.5% (v/v) of various detergents were added (detailed in Fig. 1). Lysate and pellet fractions were saved. Proteins were immunoprecipitated from lysates as follows, 2–10 μg of antibody were added to each lysate and incubated for 1 h on ice. Lysates were then incubated with Protein A/G Plus (Santa Cruz Biotechnology) overnight at 4 °C. After washing, immunoprecipitates were run on SDS-polyacrylamide gels and transferred to polyvinyldene difluoride membranes overnight. Western blots were probed with the antibodies indicated in the text.

**Cellular Transfection and Co-localization Studies—**293T or HeLa cells were grown in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum. Cells were plated onto microscope coverslips placed in six-well culture plates. Cells were either transfected (with 6 μg of DNA) via the CaPO₄ method or (with 5 μg of DNA) via the LipofectAMINE 2000 transfection reagent (Life Technologies, Inc.). 293 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 200 ng/ml phosphorol 12-myristate 13-acetate to induce the macrophage phenotype. Under these conditions, cells adhere to glass coverslips placed in six-well plates. After 24 h, most cells were attached and the culture medium was replaced. Cells were transfected with the LipofectAMINE 2000 transfection reagent (Life Technologies, Inc.) using a total of 4 μg of plasmid DNA and 20 μl of transfection reagent. After 24 h, cells were washed twice with RPMI 1640 medium, fresh culture medium was added with or without 10 μg/ml etoposide or 1 μg/ml camptothecin (Sigma), and cells were incubated for an additional 8–12 h.

For co-localization studies, cells were fixed in 4% paraformaldehyde, phosphate-buffered saline; permeabilized using 0.2% Triton X-100, phosphate-buffered saline; and blocked in 5% goat serum, 0.1% Tween20, phosphate-buffered saline for 1 h. Primary and secondary antibody incubations were done with antibodies as described in the text, and nuclei were counterstained with DAPI. Cells were visualized using a Zeiss Axiohot 2 microscope or a Zeiss LSM 510 confocal microscope.

**Terminal dUTP-mediated Nick-end Labeling (TUNEL) Assay—**HL-60 cells were cultured in 24-well culture plates with or without 10 μM camptothecin (Sigma), and cells were incubated for an additional 8–12 h.

**Immunoprecipitation—**1 × 10⁶ 293T cells were transfected with CMV-GFP and either (a) myc-pyrin, (b) ASC-FLAG, or (c) ASC-FLAG plus myc-pyrin. Several subfragments of the myc-tagged pyrin cDNA were also tested, including exon 1 alone. At several time points after transfection (24, 48, and 72 h) immunofluorescence (to detect the FLAG epitope) and DAPI staining (to stain nuclei) were carried out. Low power microscopy was used to randomly select multiple fields on the DAPI channel (where specks are not detectable), and a picture of the field was recorded. The GFP channel was then selected, and another picture was taken to visualize transfected cells. Finally the rhodamine channel was used to visualize specks (via staining of FLAG-ASC), and a third picture was taken. Each channel was then superimposed, and total cells (DAPI), transfected cells (GFP), and speck-positive cells (rhodamine) were counted. At least 500 cells/well were scored in this way for each condition tested. The percentage of 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. The statistical significance of differences between means was determined using the Student’s t test.

**Prediction and Modeling of PyD-containing Proteins—**The N-terminal amino acid sequences of PyD-containing proteins (including pyrin, ASC, CARD7, NBS1, and zebrafish caspase) were submitted to the protein fold recognition server (22) at Ben Gurion University (www.cs. bgu.ac.il/~bioinbg/) as well as the 3D-PSSM fold server (23). Scores >5 were considered significant for the Ben Gurion server; lower scores were considered more accurate than higher scores for the 3D-PSSM server.
immunoprecipitations (pyrin-HA was used as a mobility standard with anti-HA, pyrin was visualized in the immunoprecipitate. However, after co-transfection with HA-pyrin and FLAG-ASC, immunoprecipitation with anti-FLAG, Western blotting, and visualization with anti-HA, pyrin was visualized in the immunoprecipitate (arrow). Immunoprecipitations were done using a variety of detergents for the preparation of lysates as detailed in the text. n-AcβD gluc, N-acetyl-β-D-glucopyranoside; Zvit, Zwittergent 3-14.

solution from both servers, the structure of the PyD of pyrin was modeled onto the coordinates of the solution structure of the FADD death domain. Due to the high sequence similarity between the PyDs of pyrin and ASC (42%), the structure of ASC was derived from the modeled pyrin structure. The sequence alignment of FADD, pyrin, and ASC revealed a 5-residue insert in a loop region of the pyrin and ASC structures. This insert could not accurately be modeled; its position is shown as a // on the resulting ribbon diagram. Modeling was completed using the graphics program O (24) and molecular dynamics program XPLOR (25).

RESULTS

Isolation of ASC in the Yeast Two-hybrid Screen and Confirmation of Interaction by Immunoprecipitation—The yeast two-hybrid screen resulted in the identification of several putative pyrin-interacting proteins. One of these, ASC, is described here. ASC clones represented 6 of the 132 final isolates. To confirm an interaction between ASC and pyrin, immunoprecipitation assays were carried out. C-terminal HA-tagged pyrin was co-transfected into 293T cells in the presence or absence of N-terminal FLAG-tagged ASC. Cells were lysed, and immunoprecipitation was accomplished with anti-FLAG. Immunoprecipitates were separated in SDS-polyacrylamide gels and Western blotted. Blots were probed with anti-HA to recognize immunoprecipitated pyrin. Fig. 1 shows that pyrin is co-precipitated by antibodies to epitope-tagged ASC. A variety of different detergents were used in the immunoprecipitation assays. Co-precipitation of pyrin was detectable with 0.5 and 0.1% Triton X-100, N-acetyl-β-glucopyranoside, CHAPS, and Zwittergent 3-14 (Fig. 1) as well as N-acetyl-β-d-thioglycopranoside (not shown). However, in the presence of deoxycholic acid, no immunoprecipitation was detectable (not shown). The fact that the immunoprecipitation is less pronounced or not seen under more stringent conditions (deoxycholic acid and 0.5% Triton X-100) suggests that the interaction may be relatively weak. We note, however, that a significant amount of immunoprecipitated pyrin remains in the pellet (not shown). Since the relative insolubility of both pyrin and ASC makes it difficult to accurately assess the efficiency of the immunoprecipitation, we turned to cellular co-localization assays.

Co-localization of Pyrin and ASC in Specks—Transfection of HeLa cells with full-length pyrin tagged with several different epitope tags (myc-pyrin, YFP-pyrin, and pyrin-HA) revealed a cytosolic distribution of the transfected pyrin protein (Fig. 2, A and B). No staining was visible in the nucleus by confocal microscopy (data not shown). No speck-like structures were ever observed after transfection of cells by epitope-tagged pyrin alone. Cytosolic distribution of full-length pyrin has been observed previously (26, 27).

Previous studies have shown that when HL-60 cells are induced by a variety of different agents to undergo apoptosis, the native ASC protein in these cells aggregates into large hollow-centered structures called specks (13). The number of specks is directly correlated with the number of apoptotic cells, indicating that the formation of specks is associated with the apoptotic process.

We transfected HeLa cells with FLAG-ASC alone and observed two distinct types of staining (Fig. 2, C and D). In some cells, ASC exhibited a nuclear staining pattern. In other cells, FLAG-ASC was found in cytosolic specks similar to those reported earlier (13, 19). In every cell in which specks were seen, the nuclear staining of ASC was no longer visible as if all of the protein had collected into the speck structure. Spontaneous
speck formation after transfection of ASC in the absence of chemical induction of apoptosis has also been observed after transfection of COS cells (28) and 293T cells (see below).

When cells were co-transfected with myc-pyrin and FLAG-ASC, both proteins were co-localized in specks 24 h after transfection (Fig. 2, E–G). While ASC appeared to be localized exclusively in specks (Fig. 2E), pyrin staining was observed to be concentrated in specks, but additional diffuse staining was observed in the cytoplasm (Fig. 2F). A similar pattern of co-localization was observed with eYFP-pyrin and eCFP-ASC, although eCFP-ASC specks seem to lose the hollow quality of the speck by confocal microscopy (data not shown). This finding may indicate that the relatively large CFP moiety disrupts the ability of the ASC to form normal speck architecture. Consequently we used small epitope-tagged constructs for further experiments.

**Exon 1 of Pyrin Is Necessary and Sufficient for Localization to Specks**—A set of deletion constructs was used to determine the region of pyrin that is necessary for interaction with specks. Constructs were designed using intron/exon borders since such borders often mark functional domains. As shown in Fig. 3, A and B, exon 10 of pyrin fails to localize to specks. This is the region of the pyrin protein that harbors most of the known mutations. However, exon 1 of pyrin (the region that contains the PyD) is clearly targeted to specks (Fig. 3, C–E). In contrast, a pyrin molecule lacking exon 1 (exons 2–10) was not targeted to specks (Fig. 3, F and G). A nonspecific control protein (LacZ-Myc) also failed to localize to specks (data not shown). Constructs encoding exons 1 and 2, 1–4, and 1–9 were also tested, and in each case the epitope-tagged encoded protein was localized to specks (data not shown). These studies confirm that exon 1 is necessary and sufficient for localization of pyrin to specks.

**Exons 1 and 3 of ASC Form Filamentous Structures**—We tested whether exon 1 of ASC (which also encodes a PyD) is sufficient to form specks and whether this domain is sufficient for recruitment of pyrin. However, transfection of exon 1 of ASC does not result in the formation of specks. Rather, long filamentous structures are observed (Fig. 3, H and I). Co-transfection of exon 1 of pyrin and exon 1 of ASC does not result in the recruitment of pyrin to these filaments (Fig. 3, K and L). Thus, it is possible that although exon 1 of pyrin is sufficient for interaction with ASC, the corresponding PyD of ASC (contained within its exon 1) is not sufficient to mediate this interaction. Alternatively, oligomerization of exon 1 of ASC may sterically prohibit its interaction with pyrin. We also observed that the CARD of ASC forms similar oligomerized filamentous structures (data not shown). The formation of filaments by both halves of the ASC protein has been recently demonstrated (29). Interestingly, the filaments seen in our studies were much more extended than those observed by Masumoto et al. (29), who observed more aggregated filamentous structures similar to expanded-appearing specks. This may be a consequence of the different cell types that were used in the two studies, HeLa (this work) and COS7 (29). Additional cell-specific proteins may interact with the aggregates to determine their final morphology.

**Specks Have a Filibrillar Nature**—Specks generated by co-transfection of full-length myc-pyrin and full-length FLAG-ASC were examined at higher magnification at several time points after transfection. Condensations are first visible 6 h after transfection (Fig. 4, A and B); early condensations consist of fibrillar structures at the cell periphery. More mature specks are rounded with a hollow-appearing center. Filamentous structures are seen to flay out from the periphery of the specks (Fig. 4, C and D). A view of the top of the speck reveals interwoven filaments resembling a bird’s nest (Fig. 4E). To determine whether specks consist of aggregates of cytoskeletal material, ASC-transfected cells were stained with anti-tubulin

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**Fig. 3. Exon 1 of pyrin is both necessary and sufficient for targeting of pyrin to specks.** All images were taken 24 h after transfection. A and B, cells were transfected with myc-pyrin exon 10 (visualized on the rhodamine channel, red) and full-length FLAG-ASC (visualized on the FITC channel, green). A shows the overlay of both red and green channels, and an ASC speck is clearly visible. B reveals that exon 10 of pyrin is not localized in this speck. C–E, HeLa cells were co-transfected with myc-pyrin exon 1 (visualized on the rhodamine channel, red) and full-length FLAG-ASC (visualized on the FITC channel, green). C shows the FITC channel overlaid onto the DIC image. An ASC speck is visible. D shows the rhodamine channel for the same cell also overlaid onto the DIC image. E represents overlays of images from C and D, confirming co-localization of pyrin exon 1 and ASC in the speck (white arrow). Nuclear DAPI staining is also shown for red, E, F, and G. Exon 1 of pyrin is required for targeting to specks. Cells were transfected with a construct encoding pyrin exons 2–10. No co-localization of this construct (visualized on the rhodamine channel) was observed with specks. H and I, exon 1 of ASC causes filament formation. HeLa cells were transfected with FLAG-ASC exon 1 alone.osomal staining was observed (Fig. 4G). In J, green fluorescent filaments formed by ASC exon 1 are visible near DAPI-stained nuclei. In K, the FITC (ASC) channel is overlaid onto the rhodamine (pyrin) channel. In L, the pyrin (red) channel for the same cell is shown to demonstrate the absence of co-localization of exon 1 of pyrin with ASC filaments.
exhibit specks at 24 h after transfection. To test the specificity in an increase in the number of speck-positive cells; 21% of cells (Fig. 5A). Co-transfection of ASC alone, 2.6% of cells exhibit specks (Fig. 4, B) and G, specks do not co-localize with actin in the cell. These HeLa cells were transfected with full-length FLAG-ASC and full-length myc-pyrin, and the speck was visualized on the rhodamine (pyrin) channel. In addition, either micro-filamentous actin (F and G) or microtubules (H and I) were examined after staining with AF488-conjugated anti-phalloidin or anti-tubulin, respectively. The images in F and G are confocal images that reveal that actin does not co-localize with the speck. White arrows indicate speck location. H and I show that the speck occupies a hole in the microtubular network.

Pyrin Accelerates Speck Formation When Co-transfected with ASC—The number of cells with specks was examined in HeLa cells co-transfected with CMV-GFP plus either myc-pyrin, FLAG-ASC, or both constructs. At 24 h after transfection, immunofluorescence studies were carried out to assess (a) total number of cells, (b) number of transfected cells, and (c) number of cells with specks as described under “Experimental Procedures.” Specks are never seen after transfection of pyrin alone. After transfection of ASC alone, 2.6% of cells exhibit specks (Fig. 5A). Co-transfection of ASC plus full-length pyrin results in an increase in the number of speck-positive cells; 21% of cells exhibit specks at 24 h after transfection. To test the specificity of this effect, lacZ-myc (Fig. 5A) or hsp27-myc (data not shown) were co-transfected with ASC. Neither resulted in a significant increase in the number of specks present in HeLa cells at 24 h. Examination of cell lysates showed that both LacZ-Myc and Hsp27 were expressed at levels equal to or greater than pyrin, indicating that the functional increase in speck formation is specific to pyrin.

To determine whether the increase in speck formation is a property of PyD-containing proteins in general, ASC was co-transfected with DEFCAP-HA (Fig. 5), a recently characterized CED-4-like protein with an N-terminal PyD (15). DEFCAP-HA was also unable to mediate an increase in the number of specks, indicating that this effect may be specific to pyrin. To rule out effects of the transfection protocol, cell type, or epitope tag, the experiments were repeated using LipofectAMINE-mediated transfection, 293T cells, or C-terminally tagged pyrin-HA. Similar results were observed for all conditions (data not shown). However, when experiments were repeated with eYFP-ASC, we observed a large increase in the initial number of specks relative to FLAG-ASC, and the pyrin effect on the number of specks was abrogated (data not shown). This further suggests that the large YFP (or CFP, see above) moiety functionally alters ASC, perhaps stabilizing the protein and allowing for increased speck formation. We thus did not use eYFP-ASC for any functional assays, exclusively using FLAG-ASC.

Speck formation assays were also done to measure the ability
of various portions of the pyrin molecule to increase speck formation (Fig. 5A). Although Myc-pyrin exon 1 was sufficient for localization of pyrin to specks (see above), it did not increase speck formation. Thus, acceleration of speck formation requires additional regions of the pyrin molecule.

Cells with Specks Undergo Programmed Cell Death—To examine the fate of cells containing specks, HeLa cells were transfected with full-length myc-pyrin and full-length FLAG-ASC and examined at 24, 48, and 72 h after transfection. At these time points, we counted the total number of cells with specks as well as the number of speck-containing cells that were apoptotic (assessed by cell shrinkage, membrane blebbing, and nuclear fragmentation). When ASC is transfected alone, a large proportion of cells that have specks have an apoptotic appearance. At 24, 48, and 72 h after ASC transfection, 30, 55, and 82% of cells, respectively, are apoptotic in appearance (Fig. 5B). However, when ASC is transfected with pyrin, a lower percentage of speck-positive cells appear to be apoptotic at every time point (6, 27 and 65%, respectively), and these differences are statistically significant (p = 0.001). A similar analysis of cells without specks at all time points showed no significant difference in apoptosis between cells transfected with FLAG-ASC alone and FLAG-ASC plus pyrin (Fig. 5B).

Pyrin Localizes to Specks in HL-60 Cells Induced to Undergo Apoptosis—The original observation of ASC-induced speck formation was accomplished in HL-60 cells induced by a variety of agents to undergo apoptosis (13). Thus, we tested whether pyrin would co-localize with ASC in HL-60 cells. Cells were transfected with myc-pyrin and FLAG-ASC. After 24 h, transfected cells were exposed to etoposide. Fig. 6, A–C, shows that specks were formed, ASC and pyrin were co-localized in these specks, and cells with specks were apoptotic-looking by morphology (shrunken with condensed or fragmented nuclei). Thus, in HL-60 cells as in HeLa cells, pyrin and ASC are co-localized to specks, and speck formation is associated with cell death. We also examined TUNEL staining in cells transfected with both ASC and pyrin. Fig. 6, D–I, shows two examples of cells with specks (as visualized by staining for Myc-pyrin) that are also TUNEL-positive (Fig. 6, F and H). Cells transfected with ASC only were also apoptotic and speck-positive (Fig. 6, J and K).

Finally, we tested whether transfected pyrin protein would interact with native ASC in HL-60 cells. In this experiment, only myc-pyrin was transfected into HL-60 cells, and transfected cells were exposed to etoposide as above. Immunolocalization with anti-Myc revealed specks in TUNEL-positive cells (Fig. 6, M–O). This result contrasts with results in HeLa and 293T cells in which specks were never seen after transfection of pyrin alone. On the basis of earlier studies (13), we speculate that the specks result from coalescence of native ASC that occurs during etoposide-mediated killing of HL-60 cells. Myc-pyrin co-localizes with these native ASC specks, indicating that neither transfected ASC protein nor the FLAG epitope tag is required for co-localization.

The Predicted Tertiary Structure of Exon 1 of Pyrin Conforms to a Death Domain—Several proteins in the data base are now documented to contain a 5’ pyrin-like domain or PyD (11, 12). In all cases, the pyrin domain is found on the N-terminal end of the proteins (although CARD7/DEFCAP/NAC contains a second pyrin domain just 5’ to the C-terminal CARD). Interestingly the homology among these proteins ends abruptly at amino acid 92 of pyrin and amino acid 91 of ASC, the precise ending of the first exons of these proteins (data not shown). Thus, this is an example of exon shuffling.

To determine an ab initio three-dimensional structure for exon 1 of pyrin, its amino acid sequence was submitted to protein fold recognition programs at Ben Gurion University (www.cs.bgu.ac.il/~bioinbgu). The server probes a library of over 3000 three-dimensional structures. The hybrid fold recognition method used by this server exploits both structural and evolutionary data in predicting the correct three-dimensional fold for the submitted sequence (22). This analysis revealed that the PyD of pyrin conforms, with high probability, to the FADD death domain (PDB code 1fad, Ref. 30). The score of the match for pyrin and the FADD death domain was 20 on the Ben
Gurion server (where scores >5.0 are considered significant). Table I lists several of the top hits for pyrin as well as several of the other PyD-containing proteins. To confirm these results, the primary sequences of the PyD-containing family members were also subjected to the 3D-PSSM threading program (23).

The results from both programs predict death domain-related folds among the top hits for every member of the pyrin domain family tested (Table I). Members of the death domain class, including death domains, death effector domains, and caspase family tested (Table I). Members of the death domain class, including death domains, death effector domains, and caspase recruitment domains, differ in sequence but have the same three-dimensional fold characterized by six α helical regions (20).

Fig. 7A shows the alignment of pyrin exon 1 and ASC exon 1 with the mouse and human FADD death domains. A core of hydrophobic residues have been defined that are involved in the packing of the helices within a death domain (20, 30). The amino acids that comprise this core are conserved in pyrin (which contains the PyD) is both necessary and sufficient to target pyrin to ASC specks. However, it is not sufficient to increase speck formation, implicating other as yet unknown regions of the pyrin molecule in this function.

The data presented here support the notion that the pyrin domain is a protein-protein interaction domain with close structural similarity to a death domain. Although we observed that the PyD of pyrin is the domain required for ASC interaction, we were not able to positively demonstrate the reverse, that exon 1 of ASC is required for interaction with pyrin. Since both the N- and C-terminal halves of the ASC protein (encoding the PyD and the CARD, respectively) form extended filaments upon transfection into a variety of cell types (Ref. 29 and Fig. 3), lack of interaction of either half of the molecule with pyrin may simply be a consequence of this altered structure. It is interesting that the PyD of ASC forms extensive filaments in transfected cells, while the PyD of pyrin does not. Thus, the two PyDs seem to differ in their ability to self-associate. Masumoto et al. (29) have also shown that the dissected halves of ASC can interact with one another. Interestingly when exons 1 and 2 plus exon 3 of ASC were co-expressed in cells on different constructs, we did not observe specks (data not shown). Filaments were observed, presumably formed by homo- or heterooligomerization of the CARD and/or PyDs. Identical results were observed when ASC exons 1 plus exons 2 and 3 were co-expressed in cells on different constructs. The inability to reconstitute specks when the PyD and CARDs are separated suggests that their proximity is necessary for correct speck formation and that both the PyD and CARD participate in speck formation. It is possible that both halves of ASC are also required for pyrin co-localization. We did not detect co-localization of pyrin with ASC in cells in which two expression vectors encoding the two halves of ASC were simultaneously transfected along with pyrin (data not shown), and this further indicates that ostensibly reassociated ASC is structurally different from native ASC.

Although the interaction between the ASC PyD and pyrin cannot be directly demonstrated in immunolocalization studies, we note that when the PyD of both proteins are projected onto the FADD death domain structure, clear areas of electro-

### Table I

| Structural domain "hits" for the PyD family members |
|-----------------------------------------------|
| **Pyrin** | **ASC** | **Zf. Casp.** | **CARD7** | **NBS1** |
| Bioinbgu Hit no. 1 | 1 | 1 | 1 | 1 |
| (score/% ID) (20.3/19%) | (5.7/6.2%) | (17.6/24.1%) | (8.8/16%) | (7.7/26.4%) |
| Bioinbgu Hit no. 4 | 4 | 2 | 6 | 5 |
| (score/% ID) (4/13%) | (3.3/10%) | (6.0/14.6%) | (2.2/19%) | (1.9/8%) |
| Bioinbgu Hit no. 9 | 9 | 5 | 4 | 8 |
| (score/% ID) (1.6/13%) | (2.0/19%) | (2.9/9%) | (1.7/19%) |
| 3D-PSSM Hit no. 1 | 1 | 1 | 3 | 2 |
| (score/% ID) (4.7/16%) | (7.1/20%) | (1.4/20%) | (5.1/19%) | (11.2/12%) |
| 3D-PSSM Hit no. 4 | 4 | 2 | 8 | 9 |
| (score/% ID) (5.8/12%) | (13.3/19%) | (7.7/9%) | (8.0/19%) | (15.9/24%) |
| 3D-PSSM Hit no. 3 | 3 | 2 | 5 | 10 |
| (score/% ID) (7.3/13%) | (8.6/12%) | (11.1/16%) | (16.3/8%) |
static complementarity can be discerned, suggesting possible interaction surfaces. This information provides targets for future mutagenesis; should mutation of predicted interacting amino acids be found to abrogate co-localization in specks, this will bolster the idea that the PyD of ASC and pyrin indeed interact directly.

Recent studies indicate that ASC also interacts with another of the PyD-containing proteins, DEFCAP, although the responsible domains still need to be mapped (10). We have confirmed this interaction by immunoprecipitation, but by immunofluorescence DEFCAP appears to associate only weakly with specks in HeLa cells (data not shown) relative to pyrin. Furthermore, DEFCAP does not functionally increase the number of specks in cells as pyrin does (Fig. 5). Thus, while DEFCAP and pyrin both interact with ASC, the two proteins may have differing effects on cell survival.

Interestingly the CARD of ASC is closely related to the CARDs of DEFCAP and NBS1 (56% similarity). Thus, it is possible that these proteins could interact via their CARD or via their PyDs. Moreover, recent studies by Chu et al. (16) reveal that the CARD of DEFCAP can interact with the CARDs of Apaf1 and Nod1/CARD4, while Hlaing et al. (15) have documented the ability of DEFCAP to interact with caspase 2. It has also recently been demonstrated that the apoptotic activity of ASC functions via caspase 9 but that speck formation occurs independently of caspase 9-mediated apoptosis (19). The possibility must thus be entertained that pyrin is centrally located in an apoptotic signaling complex that includes ASC, DEFCAP, Nod1/CARD4, Apaf-1, caspase 9, and perhaps caspase 2.

The PyD-containing DEFCAP and NBS1 proteins are structurally related to a large family of so-called plant R proteins that mediate resistance to plant pathogens including bacteria, viruses, and fungi (31). DEFCAP, NBS1, and several of the R proteins contain a CARD, a nucleotide binding domain, and a series of leucine-rich repeats. In plants, the leucine-rich repeats are highly diverse and thought to be involved in the direct recognition of pathogen components in a one gene:one pathogen “arms race” (31). These proteins thus comprise the innate immunity system of plants. Recent work indicates that the leucine-rich repeats of Nod1/CARD4 and Nod2 (which are also structurally related to the plant R proteins as well as to CARD7 and NBS1) act as intracellular lipopolysaccharide receptors (32). Although this has not yet been tested for DEFCAP or for NBS1, the possibility that these related proteins could also encode intracellular receptors for bacterially expressed molecules is intriguing. Through its interaction with these proteins or through secondary interactions among all of the PyD-containing proteins, the pyrin protein could be placed into contact with bacterial products. This could provide an explanation for the finding that pyrin appears to be under positive Darwinian selection in human populations (33, 34) and in primates (35).

The data shown here indicate that pyrin, like DEFCAP, may play a modulatory role in apoptosis signaling. This is the first identification of a possible cellular function for pyrin. In the absence of ASC, pyrin transfection does not cause speck formation and does not appear to promote apoptosis of cells. However, immunofluorescence assays indicate that after co-trans-
The effects of the pyrin-ASC interaction have not yet been directly tested in human neutrophils (due to the lack of a pyrin antibody that functions in immunofluorescence assays). However, it is clear from earlier studies that ASC specks form spontaneously in untransfected HL-60 cells (13), and we show here that transfected pyrin interacts with those native specks. Thus, it is likely that specks play a role in apoptosis and that pyrin modulates that role. Furthermore, the basic elements of a signaling cascade involving pyrin, ASC, and caspase 9 seem to be in place. ASC speck formation occurs independently of caspase 9 activation and can be dissociated from progression to apoptosis via inhibition of caspase 9 (19). If pyrin truly modulates the rate of speck formation, it is likely that pyrin operates upstream of ASC-mediated speck formation. It is clear that pyrin is inducible in neutrophils exposed to proinflammatory mediators such as lipopolysaccharide, interleukin-1β, and tumor necrosis factor-α (4). These same cells also express ASC. Once induced, pyrin may influence the rate of speck formation or speck-mediated cell death, and speck formation/activity may mediate the progression to death via caspase 9.

On the basis of the FMF disease phenotype, it has been speculated that wild type pyrin is an inhibitor of the inflammatory response and that the pyrin mutations that are associated with FMF decrease the efficacy of that inhibitory function. It is not unreasonable to think that this function could be carried out through linkage of pyrin to the apoptotic cascade since it is well known that the inflammatory response of the neutrophil can be mediated by controlling its survival (36). However, signals that prolong neutrophil survival typically enhance rather than inhibit the inflammatory response. If pyrin is found to retard ASC-induced apoptosis in neutrophils as has been shown here in HeLa cells, then the possibility must be entertained that the role of wild type pyrin is to promote rather than to inhibit inflammation. This possibility fits well with our recent evolutionary findings that several of the common pyrin mutations were actually recapitulations of ancient amino acid character states (35). This is consistent with functional evolution of pyrin in primates by alteration of some of the very same amino acids that cause disease in humans; these same mutations also appear to be under positive selection in human populations (2, 34, 37). Together the data suggest the possibility that the mutant forms of pyrin could function better (rather than less efficiently as initially proposed) than wild type pyrin. Thus, it will be important to determine the effects of mutant forms of pyrin on ASC-induced cell death. Further exploration of these links between pyrin, apoptotic signaling, and innate immunity as well as dissection of the pathways involved will be an important next goal.

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