Mutations in *NALP12* cause hereditary periodic fever syndromes

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NALP proteins, also known as NLRPs, belong to the CATERPILLER protein family involved, like Toll-like receptors, in the recognition of microbial molecules and the subsequent activation of inflammatory and immune responses. Current advances in the function of NALPs support the recently proposed model of a disease continuum bridging autoimmune and autoinflammatory disorders. Among these diseases, hereditary periodic fevers (HPFs) are Mendelian disorders associated with sequence variations in very few genes; these variations are mostly missense mutations whose deleterious effect, which is particularly difficult to assess, is often questionable. The growing number of identified sporadic cases of periodic fever syndrome, together with the lack of discriminatory clinical criteria, has greatly hampered the identification of new disease-causing genes, a step that is, however, essential for appropriate management of these disorders. Using a candidate gene approach, we identified nonambiguous mutations in *NALP12* (i.e., nonsense and splice site) in two families with periodic fever syndromes. As shown by means of functional studies, these two *NALP12* mutations have a deleterious effect on NF-κB signaling. Overall, these data identify a group of HPFs defined by molecular defects in *NALP12*, opening up new ways to manage these disorders. The identification of these first *NALP12* mutations in patients with autoinflammatory disorder also clearly demonstrates the crucial role of NALP12 in inflammatory signaling pathways, thereby assigning a precise function to this particular member of an emerging family of proteins whose putative biological properties are currently inferred essentially through *in vitro* means.

genetics | Mendelian disorder | NLRP | autoinflammatory disorder | CATERPILLER

The hereditary periodic fevers (HPFs) consist of a group of Mendelian autoinflammatory disorders characterized by recurrent episodes of fever and systemic inflammation, sometimes complicated by amyloidosis. Based on clinical criteria, six different disorders have been defined: familial Mediterranean fever (FMF), TNF receptor-associated periodic syndrome (TRAPS), hyper IgD with periodic fever syndrome (HIDS), familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and chronic infantile neurological cutaneous and articular (CINCA) syndrome, also known as neonatal-onset multisystem inflammatory disease. The diagnosis of these disorders is often difficult to establish; in addition, the clinical distinction between such related disorders may be problematic. It is, however, crucial to ascertain their diagnosis to avoid unnecessary invasive investigations and to start appropriate medical treatments. Thanks to the current nosology, progress has been made in elucidating their molecular basis. Indeed, the *MEFV*, *TNFRSF1A*, and *MVK* genes have been shown to be involved in FMF (1, 2), TRAPS (3), and HIDS, respectively (4, 5). Mutations of *NALP3* (also called *NLRP3*, *CIAS1*, or *PYPAF1*) are associated with FCAS, MWS, and CINCA (6, 7), highlighting that three distinct clinical entities can now be seen as a single disorder with variable phenotypic expression. The term cryopyrin-associated periodic syndrome (CAPS) has been proposed to designate this spectrum of autoinflammatory diseases. In patients with FCAS who usually have the mildest phenotype, inflammatory attacks are often triggered by generalized exposure to cold. In MWS, attacks are frequently complicated by progressive hearing loss and renal amyloidosis. CINCA corresponds to the most severe phenotype with CNS manifestations (e.g., chronic aseptic meningitis, mental retardation, sensorineural hearing loss, and visual impairment) associated with severe arthropathy. In addition, intrafamilial phenotypic heterogeneity has been observed in several families with CAPS (8–10), which further supports the hypothesis that modifier genes and/or environmental factors influence the expression of the disease phenotype.

The great majority of mutations identified so far in HPF genes correspond to missense mutations, the deleterious effects of which are very difficult to assess in functional assays. Indeed, many conflicting data on the function of HPF proteins and associated mutations have been generated by different teams even when using similar experimental systems (e.g., 11, 12). In addition, HPFs remain genetically unexplained in a very large number of patients (13–16), raising the question of whether one or several other genes are responsible for these periodic fever syndromes. Although positional cloning has been of great help to identify HPF genes, 6 years have passed since the last gene was shown to be involved in these disorders. The identification of new genes through linkage studies is becoming difficult because (*i*) the great majority of patients referred recently correspond to sporadic cases, and (*ii*) many patients present with a combination of symptoms that overlaps different HPFs and do not fit in any classification (8, 17–20), precluding the constitution of homogeneous groups of patients. Despite these difficulties, the identification of new disease-causing genes would greatly facilitate

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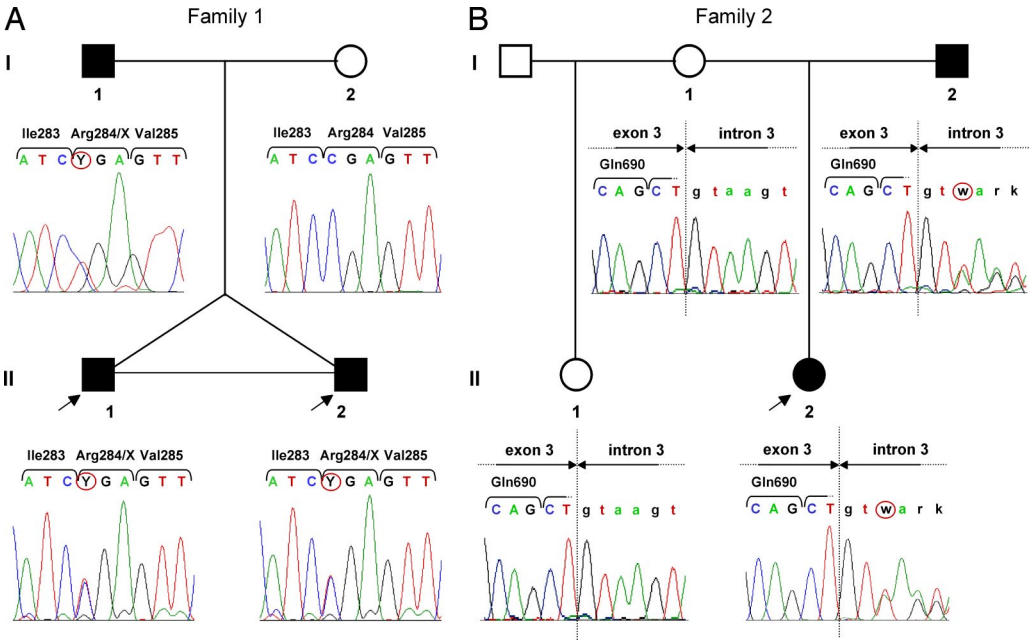

Fig. 1. Genealogical trees and mutational analysis of two families with periodic fever syndromes. Filled symbols represent patients with periodic fever syndromes, and open symbols indicate unaffected family members. Sequencing chromatograms are presented. Red circles indicate the positions of mutations. Upper and lowercases correspond to exonic and intronic sequences, respectively. Index cases are shown by arrows. (*A*) Family 1. (*B*) Family 2.

the management of HPFs in terms of genetic counseling and the development of effective therapies. In the present study, we undertook a position-independent candidate gene approach to determine the molecular basis of a disorder in twin brothers with no mutations in all known HPF genes, yet presenting with symptoms overlapping FCAS and MWS. The results obtained prompted us to perform similar molecular analyses in a second family with an unexplained periodic fever syndrome.

Results

Clinical Report. In family 1, originating from Guadeloupe, 10-year-old monozygotic twin brothers (patients II.1 and II.2) have presented with similar periodic fever syndromes since the first days of life. They have more than one episode per month, lasting 2–10 days, characterized by high fever (>40°C), arthralgias, and myalgias. Episodes are triggered by generalized exposure to cold, and urticaria was observed two times in each patient. Both children have bilateral sensorineural hearing loss. Headache and lower limb pain occur during and between episodes. C-reactive protein (CRP) levels are normal during and between attacks; levels of serum IgD also are normal. Daily treatment with colchicine has no impact on the frequency of episodes, but reduces the fever (≈38°C). The father had attacks lasting 2–3 days during childhood; he currently presents episodes of fever triggered by mild physical injury and urticaria. His audiogram revealed no abnormalities. The two children also were treated by splenectomy for a pyropoikilocytosis due to mutations in the α-spectrin gene.

Patient II.2 (family 2) is a 9-year-old girl who has been presenting with episodes of fever since the first year of life. Until the age of 6, she had one attack every 3 weeks lasting ≈1 week. Episodes associated high fever (39–41°C), abdominal pain, vomiting, and arthralgia. Attacks, which were triggered by generalized exposure to cold, also were characterized by buccal aphthous ulcers and lymphadenopathy. Headache was rarely observed. CRP levels were found to be elevated during attacks (CRP = 120/160 mg·liter^{-1}) and normal between episodes. Levels of serum IgD were normal. Colchicine had no effect on the frequency or severity of episodes and was stopped after several months. Oral corticosteroids reduced the length of febrile episodes to 3–5 days. At the age of 6, patient II.2 moved with her family to the South of France; the warmer climate seems to have reduced the frequency of episodes, with one attack every 2–3 months. Her father had one attack every 2 months from the age of 5 until the age of 12; these episodes, lasting several hours, associated fever and abdominal pain.

Identification of Mutations in *NALP12*. Patients II.1 and II.2 (family 1) display two clinical signs consistent with a diagnosis of CAPS: sensorineural hearing loss and the triggering effect of cold. We therefore looked for mutations in *NALP3*. Nevertheless, sequencing of all coding exons and flanking intronic regions did not reveal any mutation. The subsequent search for mutations in *MEFV*, *TNFRSF1A*, and *MVK*, the three other genes known to be involved in HPFs, also was negative.

These observations prompted us to look for a new HPF gene after a candidate gene approach. Given the disease phenotype, *NALP* genes could be considered as good candidates. Among the 14 members of this gene family, we focused on *NALP12* (also known as *NLRP12*, Monarch-1, or *PYPAF7*). Screening of all exons and flanking intronic sequences revealed that the two children carry the same nonsense mutation in the heterozygous state: c.850C>T (p.Arg284X) located in exon 3 of *NALP12* (Fig. 1*A*). Another sequence variation (c.1352G>A) located downstream was observed on the same allele (data not shown). Sequencing of the parents' DNA showed that the mutated allele was inherited from the affected father, whereas the healthy mother displayed a normal sequence (Fig. 1*A*). Because family 1 originates from an island, we could expect to find the same mutation in other Guadeloupean patients as a result of a founding effect. The DNA samples of eight additional Guadeloupean patients, all but one corresponding to sporadic cases of genetically unexplained periodic fever syndromes, were then screened for mutations in *NALP12*. This analysis revealed in a young girl (patient II.2, family 2) a sequence variation that was different from that found in family 1. This variation

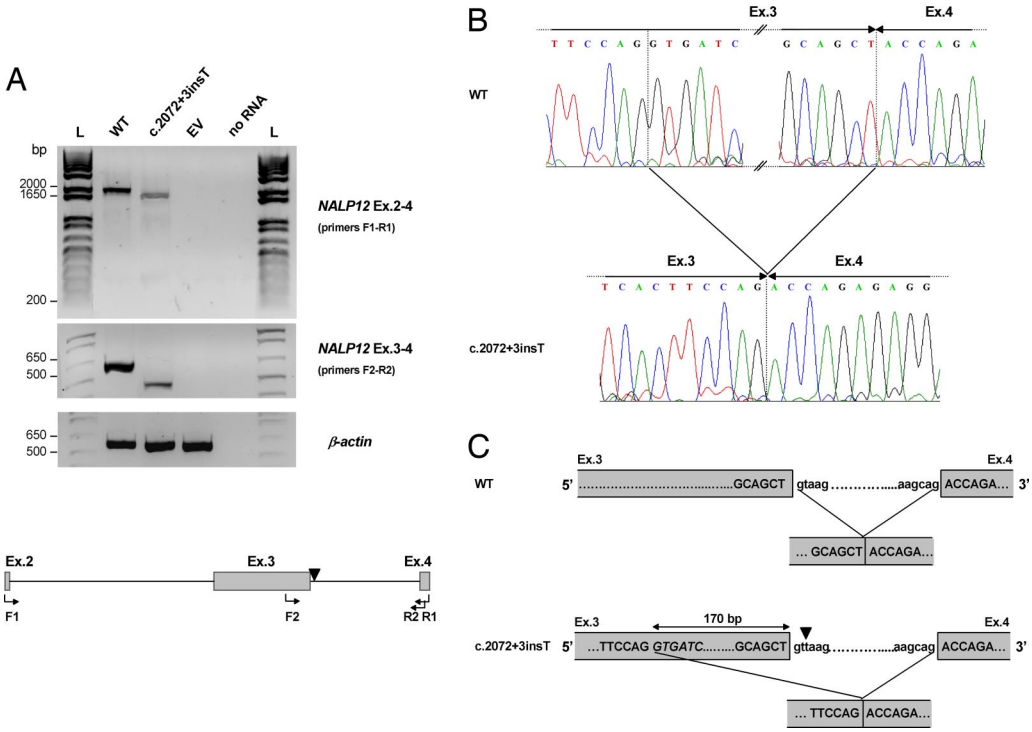

Fig. 2. Impact of the c.2072+3insT mutation on *NALP12* splicing. (*A*) (*Upper*) RT-PCR amplifications of *NALP12* transcripts from HEK293T cells transfected with p*NALP12*g-WT and p*NALP12*g-c.2072+3insT mini-genes. β-actin was amplified on the same sample as control. L, 1-kb+ marker; EV, empty vector. (*Lower*) The diagram indicates the location of the primers used in RT-PCR experiments (arrows). Exons and introns are represented by gray boxes and thin lines, respectively. The c.2072+3insT insertion is indicated by an arrowhead. (*B*) Chromatograms obtained after sequencing of the RT-PCR products presented in *A* (middle gel) and corresponding to the exon 3–exon 4 junction. (*C*) Schematic representation of the effect of c.2072+3insT on *NALP12* splicing. (*Upper*) Normal splicing of intron 3. (*Lower*) Splicing resulting from the c.2072+3insT mutation, indicated by a black arrowhead. Gray boxes correspond to *NALP12* exons; upper and lowercases correspond to exonic and intronic sequences, respectively. The cryptic donor splice site is indicated in italics.

(c.2072+3insT) is a single-base insertion within the donor splice site of intron 3 (Fig. 1*B*). Screening of *NALP3*, *MEFV*, *TNFRSF1A*, and *MVK* did not reveal any other defect in the patient. This mutation also was found in the heterozygous state in her father (patient I.1, family 2), who presented with milder clinical manifestations, but was not identified in her mother or half-sister (Fig. 1*B*). The nonsense and splice-site mutations were not found in 104 chromosomes from Guadeloupean controls.

Impact of c.2072+3insT on Splicing. Because the mutation identified in patient II.2 (family 2) involves a donor splice site and no fresh blood sample was available for this patient, we studied the splicing of *NALP12* transcripts after transfection of WT and mutated *NALP12* minigenes in HEK293T cells. RT-PCR amplification of *NALP12* transcripts spanning exons 2–4 from cells transfected with p*NALP12*g-WT yielded an ≈1,900-bp amplicon, which is consistent with normal splicing of introns 2 and 3 (Fig. 2*A Top*). In contrast, the c.2072+3insT mutation produced a smaller product (Fig. 2*A Top*). To better characterize each isoform, we amplified cDNA fragments encompassing the end of exons 3 and 4. The WT construct generated a molecular species of 632 bp, whereas a single molecular species of ≈450 bp was generated with p*NALP12*g-c.2072+3insT (Fig. 2*A Middle*). Direct sequencing of the latter PCR product revealed that the transcript is deleted from the last 170 bp of exon 3 (Fig. 2*B*), thereby unveiling the activation of a cryptic donor splice site located upstream in exon 3 (Fig. 2*C*). This splicing defect results in a frameshift that introduces 11 new amino acids, followed by a termination codon at position 646 (NALP12-Val635ThrfsX12).

Functional Consequences of the Identified *NALP12* Mutations. NALP proteins comprise an N-terminal pyrin domain (PYD), a central nucleotide-binding site (NBS), and C-terminal leucin-rich repeats (LRRs) (Fig. 3*A*). The p.Arg284X mutation, located within the NBS of NALP12, and the p.Val635ThrfsX12 mutation, located between the NBS and LRRs, would generate proteins deleted of regions highly conserved throughout evolution (Fig. 3*A*), strongly suggesting that these defects are deleterious.

This observation prompted us to study the role played by WT NALP12 on NF-κB signaling and to assess the functional consequences of the two identified *NALP12* mutations. *NALP12* is expressed primarily in cells of the myelomonocytic lineage (11, 21). Because it is known that endogenous NALP12 is difficult to detect in those cells (22), we transfected HEK293T cells with p*NALP12*-WT, p*NALP12*-Arg284X, or p*NALP12*-Val635ThrfsX12. The data obtained revealed that NALP12-WT strongly inhibits the NF-κB activation induced by p65 (Fig. 3*B*). Most importantly, under similar experimental conditions, the inhibitory properties of the protein carrying the p.Arg284X mutation are dramatically reduced (Fig. 3*B*). Similarly, we observed that the NF-κB-suppressive property of NALP12-Val635ThrfsX12 is lower than that of the WT protein (Fig. 3*B*). Similar results were obtained when we induced NF-κB activity by TNFα treatment or through the coexpression of NALP12 with IRAK1 (data not shown).

Discussion

This study identifies the first disease-causing mutations in *NALP12* in patients with periodic fever syndromes. Based on *in vitro* studies and *in silico* analyses, we elected *NALP12* as a relevant candidate gene for HPFs: (*i*) it is expressed in my-

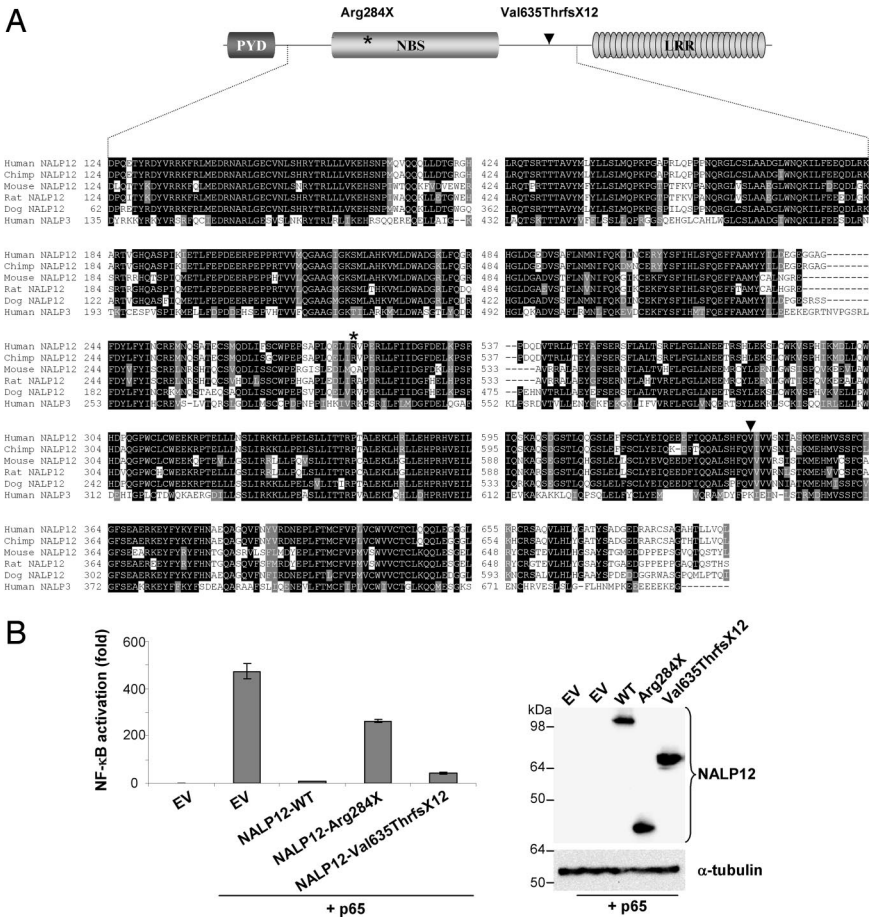

Fig. 3. Functional consequences of the identified mutations on NALP12 function. (*A*) Schematic representation of human NALP12 and partial alignment of its amino acid sequence (GenBank accession no. NP_653288) with corresponding sequences in other species (chimpanzee, XP_524387.2; mouse, XP_978890.1; rat, XP_001066862.1; dog, Ensemble accession no. ENSCAFP00000003989). Alignment with human NALP3 (GenBank accession no. NP_004886.3) is also presented. The Arg-284 residue is indicated by an asterisk, and the Val-635 residue is indicated by an arrowhead. Black shading indicates identical residues, and gray shading indicates similar residues. PYD, pyrin domain; NBS, nucleotide-binding site; LRR; leucin-rich repeats. (*B*) Effect of NALP12-WT and NALP12-mutated proteins on NF-κB signaling. HEK293T cells were transfected with 100 ng of the pNF-κB-LUC luciferase reporter and with 800 ng of plasmids encoding NALP12-WT, NALP12-Arg284X, or NALP12-Val635ThrfsX12 as indicated. The NF-κB signaling pathway was induced by transfection of 100 ng of p65. Then, 24 h after transfection, luciferase activities were determined on cell lysates; results of one representative experiment done in triplicate are expressed as means ± SD. NALP12 proteins were quantitated by Western blot analysis on the same protein lysates. Membranes were reprobed with anti-α-tubulin antibodies as a loading control. EV, empty vector.

elomonocytic cells (11); (*ii*) its nucleotide sequence is highly similar to that of *NALP3*, with 58% identity; (*iii*) among NALPs, the NBS of NALP12 is the most closely related to the one of NALP3 (23), which contains most mutations identified in patients with FCAS, MWS, and CINCA; and (*iv*) NALP12 is believed to regulate inflammatory and immunological pathways (11, 21, 22).

Sequencing of *NALP12* revealed a heterozygous nonsense mutation (c.850C>T, p.Arg284X) in identical twin brothers presenting with symptoms overlapping FCAS and MWS, thereby providing a clear-cut answer to the suspected genetic heterogeneity of these disorders (15, 24). This nonsense mutation also was found in their father, who displays a milder phenotype. A second *NALP12* mutation (c.2072+3insT) was identified in a patient presenting with a periodic fever syndrome, including clinical manifestation of FCAS. This mutation, affecting the donor splice site of intron 3, activates a cryptic splice site located upstream in exon 3 and results in a frameshift, followed by a premature stop codon. This splice mutation also was found in her father, who presented with periodic fever symptoms during childhood. Altogether these data show in two independent families the segregation of the identified mutations with the disease, such data being consistent with an autosomal-dominant mode of inheritance of *NALP12*-associated periodic fever syndromes. Noteworthy, the three affected children investigated herein share several phenotypic similarities: (*i*) the disease started during the first year of life; (*ii*) the patients present more than one attack per month; (*iii*) episodes are triggered by generalized exposure to cold; and (*iv*) attacks that last several days associate high fever (>40°C), arthralgia, and headache in the two families. However, the patients also have distinguishing clinical manifestations: urticaria and sensorineural hearing loss were only observed in family 1, in whom acute-phase reactants were not elevated during crises, whereas aphthous ulcers and lymphadenopathy were noticed in patient II.2 from family 2. Such interfamilial phenotypic heterogeneity is frequently observed in HPFs (19, 20, 25–27); in this regard, it has even been shown that a same mutation (in *NALP3*) can be associated with different CAPSs (8, 27, 28). Furthermore, phenotypic heterogeneity also has been shown to be intrafamilial in this pathology, especially in *NALP3*-associated disorders (8–10). As for acute-phase reactants, although the majority of studies describes a marked increase in CRP levels in a patient with HPFs (29), Hoffman *et al.* (30) reported normal CRP concentrations in a

patient presenting with FCAS. Other cases of patients with slight to moderate elevation of inflammatory markers have been described (8, 31). Overall, such clinical and biological differences in HPFs may reflect the effect of distinct mutations and/or result from different genetic backgrounds or environmental factors. In addition, even if the similarities between NALP3 and NALP12 are numerous, our data argue against functional redundancy because a single mutation in *NALP12* is sufficient to induce an autoinflammatory disease.

The demonstration of the involvement of *NALP12* in HPFs is supported by additional lines of evidence. (*i*) The two identified sequence variations were not found in a population sample of the same origin. (*ii*) Most importantly, such nonsense and splice mutations are nonambiguous defects; in this regard, it is important to emphasize that nearly all mutations reported so far in HPF genes are missense mutations, the deleterious effects of which are particularly difficult to assess. (*iii*) The two mutations affect evolutionarily conserved domains. (*iv*) Both mutations induce a clear reduction of the inhibitory properties of NALP12 on NF-κB signaling, in keeping with a loss of function. In addition, coexpression studies revealed that NALP12-mutated proteins do not interfere with the function of the normal isoform (data not shown). Taken together, these observations are consistent with a dominant mode of expression through haploinsufficiency. Consistent with our observation that NALP12 acts as an NF-κB inhibitor, Williams *et al.* (22) demonstrated suppressive properties of NALP12 on NF-κB signaling induced by various agents (including TNFα and IRAK1). We also showed an inhibitory effect of NALP12 on NF-κB activity induced by p65, a result that, for unknown reasons, was not observed by these authors.

NALP proteins are part of the larger family of CATERPILLER proteins (also known as NLRs) characterized by NBS and LRR domains located downstream of various N-terminal regions. Like TLRs, NLRs have recently been identified as proteins involved in host defense against pathogens: TLRs are primarily cell-surface receptors characterized by extracellular LRRs sensing pathogen molecules and triggering inflammatory and immune responses, whereas NLRs are believed to be intracellular LRR-containing proteins involved in similar signaling pathways, although information about their function is still relatively scarce (32).

Five *NLR* genes have been involved in human pathology: *NALP7* in hydatidiform mole (33), *NALP1* in vitiligo-associated autoimmune diseases (34), *NALP3* in HPFs (7, 35), *CIITA* in bare lymphocyte syndrome (36) and multiple sclerosis (37), and *NOD2* in Crohn's disease and Blau syndrome (38, 39). Our study, which establishes *NALP12* as a gene responsible for periodic fever syndromes, underlines the role of the *NALP* gene family in the pathogenesis of the recently recognized continuum of autoimmune–autoinflammatory diseases (40). The identification of *NALP12* mutations in patients with autoinflammatory disease also demonstrates the crucial role of NALP12 in inflammatory signaling pathways, thereby assigning a precise function to this particular member of the emerging family of NALP proteins, whose putative biological properties are currently inferred essentially through *in vitro* means. However, given the documented role of *NOD2* and *CIITA* in both Mendelian and multifactorial disorders, our data, which should reveal ways to manage periodic fever syndromes, also raise the question of the possible role of *NALP12* in common multifactorial diseases.

Materials and Methods

Patients. This study was approved by the local ethics committee (Comité Consultatif de Protection des Personnes se prètant à une Recherche Biomédicale–Henri Mondor, Créteil, France). Informed written consent for genetic studies was given by all patients or their parents. Clinical features were recorded on a standardized form.

Molecular Analysis. gDNA was extracted from peripheral blood leukocytes. All coding exons and flanking intronic sequences of *MEFV*, *TNFRSF1A*, *MVK*, *NALP3*, and *NALP12* were amplified by PCR and sequenced. The presence of mutations was confirmed by forward and reverse sequencing.

Plasmid Constructs. The full-length NALP12 cDNA coding sequence was cloned into pcDNA3 to generate pNALP12-WT. Site-directed mutagenesis (Stratagene) was performed to generate pNALP12-Arg284X and pNALP12-Val635ThrfsX12.

Because no fresh blood sample was available for the study of NALP12 transcripts, we constructed NALP12 mini-genes. Genomic fragments spanning exons 2–4 and flanking intronic sequences were amplified by PCR; normal gDNA (Roche) and DNA of patient II.2 (family 2) were used as a template. The resulting products were cloned into pcDNA3.1-V5HisTopo (Invitrogen) to generate pNALP12g-WT encoding the normal sequence and pNALP12g-c.2072+3insT corresponding to the mutated allele identified in patient II.2 (family 2). The NF-κB p65 expression plasmid has been described previously (41). All constructs were checked by sequencing.

Splicing Assay. p*NALP12*g-WT and p*NALP12*g-c.2072+3insT were transfected into HEK293T cells. Then 24 h after transfection, total RNA was extracted (RNA PLUS kit; Qbiogene). cDNAs were synthesized by random-primed reverse transcription (Sensiscript kit; Qiagen). PCR amplifications of *NALP12* and β-actin transcripts were performed by using different combinations of forward and reverse primers. Primer sequences for PCR amplifications are available on request.

NF-κB Luciferase Assay. HEK293T cells were transfected (LipofectAMINE; Invitrogen) with 100 ng of pNF-κB-LUC (Stratagene), 100 ng of p65, 100 ng of pIRAK1, and 800 ng of *NALP12* expression plasmids as indicated. Cells were treated with 10 ng/ml TNFα for 24 h or left untreated. Luciferase activities were determined on cell lysates from triplicate cultures (Promega assay) and normalized to protein concentration (Coomassie Plus Protein Assay; Pierce). Western blotting was performed with anti-NALP12 (Tebu-Bio), anti-α-tubulin antibodies (DM1A, Sigma–Aldrich), and HRP-conjugated anti-mouse antibodies (Sigma–Aldrich). Detection was performed with chemiluminescence reagents (GE Healthcare).

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