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ARTICLE

A new CARD15 mutation in Blau syndrome

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The caspase recruitment domain gene CARD15/NOD2, encoding a cellular receptor involved in an NF-κB-mediated pathway of innate immunity, was first identified as a major susceptibility gene for Crohn's disease (CD), and more recently, as responsible for Blau syndrome (BS), a rare autosomal-dominant trait characterized by arthritis, uveitis, skin rash and granulomatous inflammation. While CARD15 variants associated with CD are located within or near the C-terminal leucine-rich repeat domain and cause decreased NF-κB activation, BS mutations affect the central nucleotide-binding NACHT domain and result in increased NF-κB activation. In an Italian family with BS, we detected a novel mutation E383K, whose pathogenicity is strongly supported by cosegregation with the disease in the family and absence in controls, and by the evolutionary conservation and structural role of the affected glutamate close to the Walker B motif of the nucleotide-binding site in the NACHT domain. Interestingly, substitutions at corresponding positions in another NACHT family member cause similar autoinflammatory phenotypes.
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Introduction

Blau syndrome (BS, MIM 186580) is a rare Mendelian trait with autosomal-dominant inheritance characterized by multi-organ granulomatous inflammation of the skin, eyes and joints.[1] The BS locus was mapped by Tromp *et al*[2] to the chromosomal region 16q12.1–13, which also contains one of several inflammatory bowel disease (IBD) susceptibility loci.[3–5] The caspase recruitment domain gene CARD15, previously named NOD2, was subsequently identified in that region both as a major determinant of susceptibility to Crohn's disease (CD)[6–8] and as the gene responsible for BS.[9] The encoded multidomain protein of 1040 amino acids is implicated in an NF-κB-mediated pathway of inflammation and apoptosis and is mainly expressed in monocytes, granulocytes and dendritic cells, which are major components of BS granulomas. CD-associated variants mostly affect the C-terminal protein domain consisting of leucine-rich repeats (LRRs) and cause decreased NF-κB signaling.[10,11] In contrast, mutations up to now identified in BS patients are missense changes affecting residues 334 and 469 of the nucleotide-binding domain (NBD) and have been shown to result in increased basal NF-κB activity, consistent with the dominant inheritance of the syndrome.[10,11] Here, we describe a different mutation that substitutes glutamate E383 adjacent to the Mg^{2+}-binding (Walker B) motif with lysine. Our finding extends the mutational spectrum of CARD15 in BS and contributes to a better understanding of its pathogenesis in the context of a common molecular mechanism shared with other evolutionarily related proteins containing nucleotide-binding and LRR domains involved in human inflammatory disorders[12–14] and plant disease resistance.[15,16]

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Patients and methods

The proband (II4 in Figure 1), now 51 years, was first seen at the age of 10 years and diagnosed on the basis of described criteria for BS,[1,17] including typical ultrastructural findings in dermal and synovium granulomas. Main signs were skin rash at the extensory aspect of upper and lower limbs, arthritis of the small hand joints and severe bilateral uveitis. The daughter (III2) showed similar skin rash and arthritis since the age of 5 years without ocular involvement. Sibs II1, II2, II3 and the other daughter (III1) showed no symptoms of BS. Both patients and the relatives indicated in Figure 1 gave written consent to genetic investigation on the CARD15 or other candidate BS genes. DNAs of 100 healthy medical students belonging to the Italian population and enrolled according to the rules of our institution were used as normal population controls.

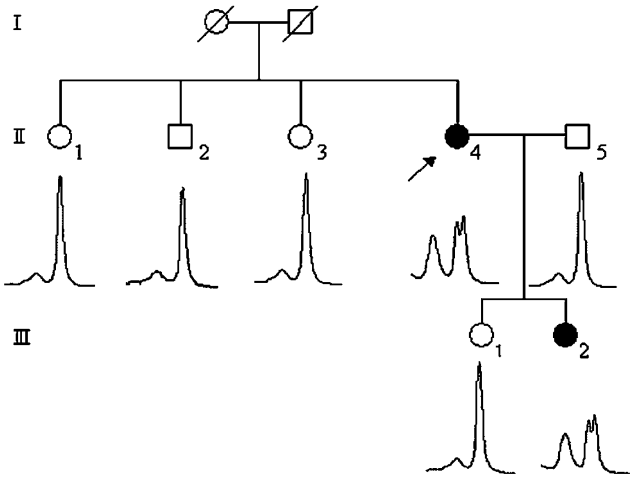

Figure 1 Pedigree of the family. Subjects II4 and III2 are affected with BS. DHPLC shows a clear difference between the three-peak pattern of affected E383K heterozygotes and the single-peak pattern of healthy family members.

Coding sequences of the CARD15 gene (exons 2–11, NCBI accession number NM_022162) were amplified from genomic DNA, using the primers and polymerase chain reaction (PCR) conditions shown in Table 1. Amplified products were size controlled by electrophoresis on 2% agarose, directly sequenced in the two affected subjects and analyzed in all family members by denaturing high-performance liquid chromatography (DHPLC) using the Wave system (Transgenomic). The three CD-associated variants R702W, G908R and L1007fs were genotyped by restriction analysis of exons 4, 8 and 11 as described previously.[18]

We used the PSI-BLAST suite[19] to search for homologs (E-value cutoff 0.005) in the UniProt database.[20] Multiple sequence alignments were assembled by T-COFFEE[21] and improved manually by minor adjustments. Protein structures and secondary structure assignments were obtained

Table 1 CARD15 primers, PCR and DHPLC conditions of exons 2–11

Exon	*Domain*	*Sense (F); reverse (R)*	*T PCR*	*T DHPLC*
2	CARD1+2	(F) 5′-GCATCTGGCTTCTGGAGAAGT (R) 5′-ACCCCTTTCCTGAGAACTCTG	61	63.5
3	—	(F) 5′-CCATCAGCCTTCCTGGAAG (R) 5′-CATGGATCTGCACTGACTGC	57	59.8
4a	NBD	(F) 5′-GGCTGGCTCTCCTATCCCTT (R) 5′-TTGACCAACATCAGGCCAACAG	61	61.5–63.5
4b	NBD	(F) 5′-CTGCAGGGGAAGACTTCCA (R) 5′-AGGGCTGAGGTCTCTTGGA	61	61.1–63.1
4c	NBD	(F) 5′-TCCGCACCGAGTTCAACCTC (R) 5′-CGCGGCAAAGAAGCACTGGA	61	62.4–63.2
4d	—	(F) 5′-GCCAAGAGCGTGCATGCCAT (R) 5′-CACCAGACCCAGCACATAGG	61	62.2
5	LRR	(F) 5′-GGAAGCACAGATGCTGGCAC (R) 5′-TGAGACCAGGGCACTTGGAG	62	57.0–61.0
6	LRR	(F) 5′-GGTGCTCACTGTCCAATGTGC (R) 5′-GACTGACTCAGGAATGGGCC	57	59.6–60.6
7	LRR	(F) 5′-CGCTGTGTTCTCTCAGCCT (R) 5′-CTGAAGAGTTTCACCTGCCC	58	62.0–65.0
8	LRR	(F) 5′-CACTGACACTGTCTGTTGACTC (R) 5′-AAGACCTTCATAAGTGGCCCC	60	61.3–63.3
9	LRR	(F) 5′-GGTTAGCTCATCTCTCGAGGTC (R) 5′-GATTGTGATCCACTTCCCCAG	59	60.2
10	LRR	(F) 5′-GGGGCATGTGAGTTCATCATC (R) 5′-GCGGCCCAGATCTTAATCAG	59	58.3–59.3
11	LRR	(F) 5′-CTGGCTAACTCCTGCAGT (R) 5′-ACTGAGGTTCGGAGAGCT	58	58.9–61.4

Products amplified with a standard PCR protocol using the indicated sense (F) and reverse (R) primers at the annealing temperature indicated in column 'T PCR' and analyzed in DHPLC at the elution temperature indicated under 'T DHPLC'.

from the PDB and DSSP databases,[22,23] and the web servers PSIPRED, SAM-T99 and SSpro2 predicted the secondary structure of proteins to form a consensus prediction by majority voting.[24] The sequence alignments depicted in Figure 2 were illustrated using the online service ESPript.[25] UniProt accession numbers and synonymous names of selected proteins are as follows: F_1-ATPase, Q9T2U4; I-2, Q9XET3; CARD15/NOD2, Q9HC29; CARD4/NOD1, Q9Y239; PYPAF1/NALP3/CIAS1/Cryopyrin, Q96P20; PYPAF2/NALP2, Q9NX02; PYPAF3/NALP7, Q8WX94; PYPAF4/NALP4, Q96MN2; PYPAF5/NALP6, P59044; PYPAF6/NALP11, P59045; PYPAF7/NALP12/MONARCH-1, P59046; and PYPAF8/NALP5/MATER, P59047. The PDB identifier of the crystal structure of bovine F_1-ATPase is 1bmf, and we used chain F that corresponds to the β-subunit.

Results

Direct sequencing of the PCR-amplified segment of exon 4 encoding the NBD (codons 273–577) of CARD15 in the two affected subjects identified a heterozygous G→A substitution at nucleotide 1147, changing glutamate to lysine (E383K). DHPLC analysis confirmed a heteroduplex pattern segregating from the mother to the affected

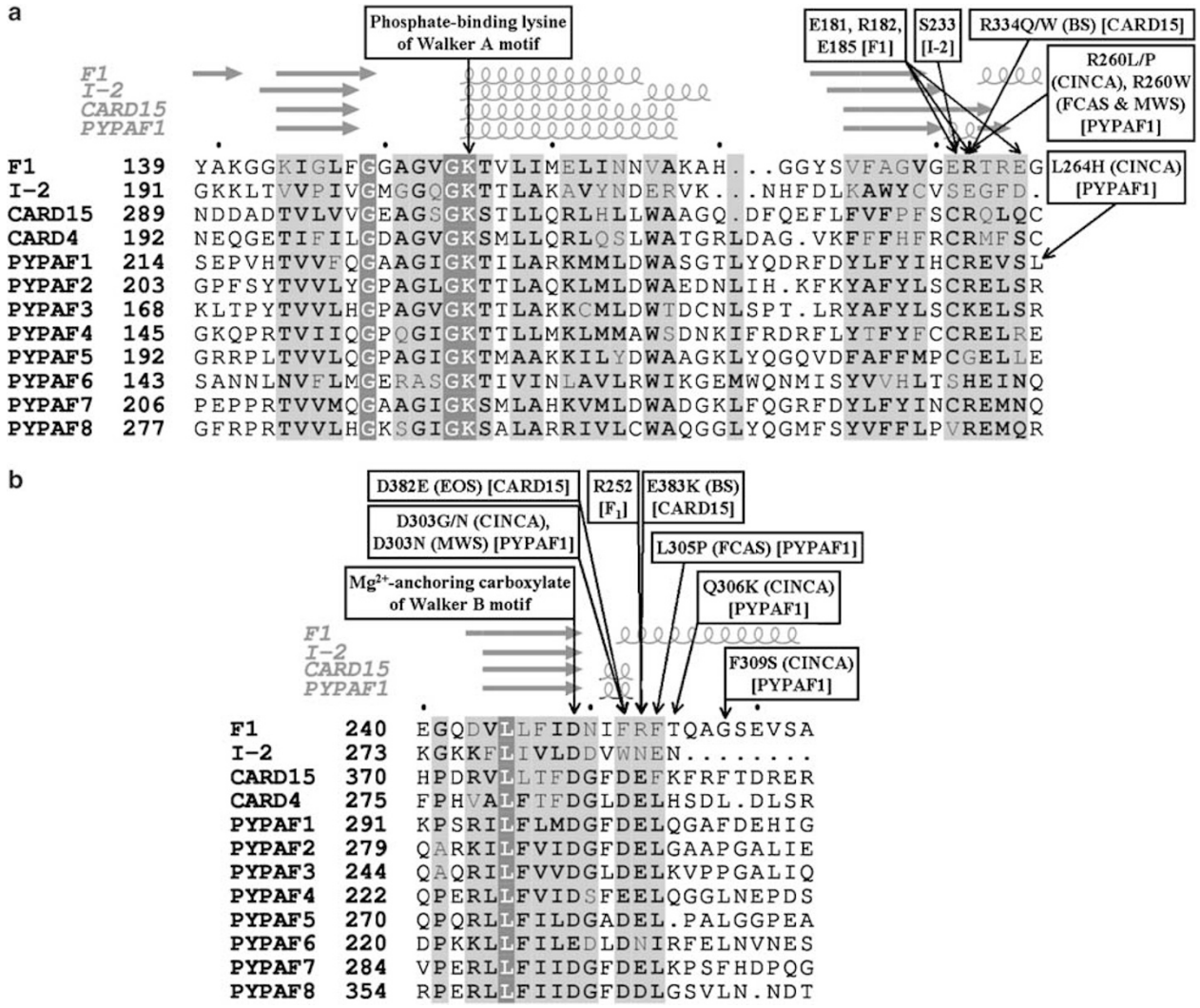

Figure 2 Structure-based multiple sequence alignment of nucleotide-binding sites in NACHT ATPase domains of human CARD15/4 and PYPAF1-8, in the ATPase domain of tomato disease resistance protein I-2 and in the β-subunit of bovine F_1-ATPase. (**a**) Sequence region with Walker A motif including the phosphate-binding lysine. (**b**) Sequence region with Walker B motif including the Mg^{2+}-anchoring aspartate. The secondary structure of F_1-ATPase and the corresponding consensus predictions for I-2, PYPAF1 and CARD15 are depicted in the upper part (α-helices as curled lines, β-strands as horizontal arrows). Alignment columns with strictly conserved residues are highlighted in dark gray boxes, and those in which more than 60% of all residues are physicochemically equivalent are shown in light gray boxes. Text labels point to functionally relevant residues and disease-associated missense variants.

daughter and to none of the healthy sibs (Figure 1). This variant pattern was not found in any of 100 controls. Sequencing and DHPLC analysis of the entire CARD15 coding sequence in the affected subjects did not detect any other variant of probable pathogenic significance. Typing by restriction analysis excluded the presence of the three recurrent CD-associated variants in the family.

A PSI-BLAST search was performed with residues from 273 to 577 of the CARD15 gene product against the UniProt database. This search returned many members of the NACHT domain subfamily.[26,27] E383 is conserved in 85 of the 138 sequences (61.6%) included in the complete NACHT domain sequence alignment of the Pfam database (release 15.0),[28] which indicates a higher evolutionary conservation than that of other residues mutated in BS: R334, conserved in 67 sequences (48.6%), and L469, contained in an unconserved loop after the C-terminus of the Pfam NACHT alignment. For the sake of simplicity, we selected the closely related CARD15, CARD4 and PYPAF1-8 proteins containing the CARD and PYD domain, respectively, for further analyses (Figure 2).[12–14] In addition, we compared these protein sequences with the corresponding segments of more distantly related proteins such as the tomato resistance gene product I-2[29] and the bovine F_1-ATPase[30] in order to gain insights on the structural and functional role of mutated residues. A multiple sequence alignment of the selected segments is shown together with the relevant structural motifs around R334 mutated in previous BS cases (Figure 2a) and E383 mutated in this case (Figure 2b).

Discussion

The CARD15 gene encodes a cellular recognition molecule mediating NF-κB activation in response to the muramyl dipeptide component of bacterial peptidoglycan.[13,31] The identification of CARD15 as a major gene involved in susceptibility to CD[6–8] opened new insights on deregulated natural immunity in IBD and corroborated the long-held hypothesis that its pathogenesis involves abnormal reaction to intestinal microorganisms by showing that it is caused by the failure of the C-terminal LRR receptor domain to recognize bacterial products.[13,31] A distinct group of mutations in the same CARD15 gene causes, in heterozygous state, the rare autoinflammatory BS.[9,32] The Mendelian pattern of inheritance indicates that BS mutations are more penetrant and may affect CARD15 function more severely than the common CD-associated variants. Actually, the latter are thought of as recessive, or gene-dose-dependent, predisposing factors, with an estimated penetrance $<4\%$ in homozygotes/compound heterozygotes and even lower in heterozygotes.[33] Moreover, the inflammatory manifestations of BS affect multiple organs and tissues expressing the CARD15 gene, without any apparent dependence on intestinal stimuli.

Known BS mutations are missense changes in specific positions of the NBD named NACHT, which characterizes a distinct protein subfamily of the STAND NTPases.[27] In most proteins, the NACHT domain is centrally located between a C-terminal sensor domain (LRRs in CARD15/4 and PYPAF1-8) and an N-terminal adaptor domain (CARD/PYD). According to current models, the NACHT domain undergoes, in response to a specific ligand–LRR interaction, stereotyped, NTPase-mediated conformational changes that involve homophilic oligomerization, recruitment of downstream factors, and assembly and dissolution of a signaling complex eventually leading to NF-κB activation.[11–14,27,34] A similar switch changing between an inactive ADP-bound and an active ATP-hydrolyzing conformation functions in the NACHT-related NBDs of apoptotic protease-activating factor 1 (APAF1), with a WD40 repeat sensor domain and a CARD adaptor domain regulating apoptosome activation,[35] and of plant disease resistance proteins, activated by pathogen recognition through their LRRs.[15,16] This regulatory NTPase switch can be compared with that described in detail for other ATPases such as the valosin-containing protein p97,[36,37] a member of the NACHT/STAND-related AAA+ family,[27] and trimeric G proteins.[37–39] In all cases, NTPase domains are generally assumed to work in signaling cascades and to provide scaffolds for NTP-dependent assembly of protein complexes that integrate signals transmitted by different sensor domains.[27,36]

NACHT domain proteins, besides containing the functionally relevant Walker A and B sequence motifs common in many other nucleotide-binding proteins, are characterized by the presence of two tiny residues downstream of the magnesium binding aspartate, followed by two acid residues. Previously known mutations from BS patients affect arginine R334 and leucine L469 of the NACHT domain. The first is located downstream of the nucleotide-binding Walker A motif. L469 is predicted, on the basis of the crystal structure of the related NBD of F_1-ATPase, to be located in the flexible linker downstream of the NACHT domain wrapped around the nucleotide-binding site, and its substitution presumably affects ATP hydrolysis.[30,36,37] Moreover, a mutation converting aspartate D382 to glutamate has been recently reported in early-onset sarcoidosis (EOS).[40] In functional assays, all three BS mutations and D382E exhibited enhanced basal NF-κB activity compared to wild-type CARD15.[10,40]

The new mutation in our patients changes a different residue of the NACHT domain, glutamate E383 to lysine. Despite the lack of a direct functional assay, its pathogenicity is strongly supported by several, although indirect, pieces of evidence. First, it clearly cosegregates with the disease in a dominant manner since it is transmitted from the affected mother to the affected daughter and not to the healthy sib. Second, it was not detected in a sample of 100 unrelated individuals, that is, 200 alleles.

Third, evolutionary conservation within the NACHT domain family[27] is stronger for E383 than for the BS-associated positions R334 and L469. It should be noted that the evolutionary conservation is not absolute, for instance, an aspartate is present in place of E383 in PYPAF8 of man (Figure 2b) and mouse (not shown), and an arginine in F1-ATPase (Figure 2) and human telomerase-associated protein 1 (not shown). However, evolutionary conservation in paralogous proteins cannot always be taken as an absolute criterion of pathogenicity, and the observed conservation already points to the functional importance of the residue E383 in the NBD.

Fourth, structural modeling of the NACHT domain of CARD15 indicates that D382 and E383 are a couple among several acidic residues in the Walker B motif that are involved in anchoring a Mg^{2+} ion by hydrogen bonds through a coordinated water molecules.[36] Since E383 is one of the distinguishing features of the NACHT subfamily,[27] its substitution with a positively charged lysine is expected to alter a fine-tuned, and possibly negative, regulatory mechanism that is specific of it, rather than drastically affect ATP hydrolysis.[11,27,36,41]

Fifth, all three residues affected by BS mutations correspond to the position of pathogenic mutations in the closely related protein PYPAF1 that cause three other autosomal-dominant autoinflammatory diseases, chronic infantile neurological cutaneous and articular syndrome (CINCA), familial cold autoinflammatory syndrome (FCAS) and Muckle–Wells syndrome (MWS). As previously noted, the BS-associated CARD15 mutations R334Q/W correspond to the PYPAF1 mutations R260L/P and R260W associated with CINCA and FCAS/MWS (Figure 2a).[13,14,36,41,42] This correspondence also holds true for E383 that is located next to D382, which is mutated in EOS, and between the positions of the pathogenic mutations D303G/N and L305P in PYPAF1 (Figure 2b). Like the BS-associated missense changes R334Q/W and L469F, D303N and D382E also cause a substantial increase of basal NF-κB activity.[10,40,43] Interestingly, significantly reduced catalytic rates of ATP hydrolysis have been observed experimentally when mutating the corresponding residues E181, R182, E185 and R246 of F_1-ATPase[44,45] and S233 of I-2 (Tameling, personal communication).

In conclusion, all BS variants lie close to the ATP-binding site and are predicted to either directly affect ATP hydrolysis or indirectly modulate the conformational switch through side chain movements of adjacent amino acids. This might in turn allow excessive signaling due to a reduced disassembly of CARD15 signaling complexes, and result in constitutively increased NF-κB activation, even in the absence of the TLR2-mediated bacterial stimuli that occur in the intestine and have recently been implicated in the pathogenesis of CD.[46] Owing to the common inflammatory pathophysiology of patients with BS and MWS, as well as the apparent functional similarities between CARD15 and PYPAF1, treatment with an interleukin-1β antagonist, which has been successful for FCAS and MWS,[47,48] might also be proposed for BS patients.

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