RESEARCH ARTICLE

Mutations That Impair Interaction Properties of TRIM32 Associated With Limb-Girdle Muscular Dystrophy 2H

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TRIM32 belongs to a large family of proteins characterized by a tripartite motif, possibly involved in the ubiquitination process, acting as an E3 ligase. In addition, TRIM32 has six NHL repeats with putative interaction properties. A homozygous mutation at the third NHL repeat (D487N) has been found in patients with limb girdle muscular dystrophy 2H (LGMD2H). This mutation was only identified in the inbred Manitoba Hutterite or their descendants. Interestingly, a mutation in the B-box domain of TRIM32 cosegregates with Bardet-Biedl syndrome type 11 (BBS11). The signs of BBS11 include obesity, pigmentary and retinal malformations, diabetes, polydactyly, and no muscular dystrophy, suggesting an alternative disease mechanism. We aim to ascertain whether D487N is the only pathological LGMD2H allele, limited to Hutterites. We studied the TRIM32 gene in 310 LGMD patients with no mutations at the other known loci. We identified four patients with novel mutated alleles. Two mutations were homozygous and missing in controls. These mutations also clustered at the NHL domain, suggesting that a specific (interaction) property might be abolished in LGMD2H patients. No mutations were found at the B-box region where the BBS11 mutation is found. We tested TRIM32 and its mutants by yeast-two-hybrid assay, developing an interaction test to validate mutations. All LGMD2H mutants, but not the BBS11, lost their ability to self-interact. The interaction of TRIM32 mutants with E2N, a protein involved in the ubiquitination process, was similarly impaired. In conclusion, the mutations here reported may cause muscular dystrophy by affecting the interaction properties of TRIM32. Hum Mutat 0, 1–8, 2007.

KEY WORDS: TRIM32; LGMD2H; muscular dystrophies; yeast two-hybrid

INTRODUCTION

Limb-girdle muscular dystrophies (LGMDs) include a broad group of genetically determined progressive muscle disorders [Angelini, 2004]. By definition, patients should present primary or predominant symmetrical atrophy of the pelvic and/or shoulder girdle musculature, and a necrotic regeneration pattern [Emery, 2002; Nigro, 2003]. However, the clinical course is characterized by a great variability and the term now includes many different phenotypes ranging from severe forms with onset in the first decade and rapid progression, to milder forms with later onset and atypical presentation [Bushby, 1999]. This determines clinical overlaps with Becker muscular dystrophy, late-onset spinal muscular atrophy, myotonic dystrophy type 2, Bethlem myopathy, and Pompe disease. Thus, a clinical examination cannot clinch the diagnosis [Straub and Bushby, 2006].

LGMD2H (MIM# 254110) is rare, and one of the most intriguing forms of autosomal recessive limb-girdle muscular dystrophies. Homozygous p.D487N mutation of the TRIM32 gene (MIM# 602290) causes LGMD2H in 41 patients from Hutterite families [Frosk et al., 2002]. Haplotype analyses showed that all LGMD2H patients share the same allele at 9q33.1 [Weiler et al., 1998]. This suggests a founder effect arising before the emergence of the Hutterite religion in central Europe in the 16th century [Frosk et al., 2002]. LGMD2H onset is usually within the second or third decade of life, and progression is slow. Most patients remain ambulatory until the sixth decade of life [Shokeir and Kobrinsky, 1976; Shokeir and Rozdilsky, 1985].

The Supplementary Material referred to in this article can be accessed at http://www.interscience.wiley.com/jpages/1059-7794/suppmat.

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This same mutation in TRIM32 has recently been found to cause sarcotubular myopathy (STM) in Hutterite descendants [Frosk et al., 2005; Schoser et al., 2005], indicating that STM and LGMD2H are allelic disorders, with STM having a more severe phenotype. The explanation for this is obscure.

TRIM32 [Fridell et al., 1995] is a ubiquitously expressed protein that belongs to a family known as TRIM or RBCC [Reymond et al., 2001]. These genes are characterized by the tripartite motif (TRIM), which consists of a RING domain, one or two B-box motifs, and a coiled-coil region (RBCC) [Slack and Ruvkun, 1998]. This motif is usually associated with variable C-terminal domains [Torok and Etkin, 2001; Meroni and Diez-Roux, 2005].

Members of this family are involved in a broad range of cellular processes including apoptosis, cell growth, differentiation, transcriptional regulation, and ubiquitination and, as a consequence, when altered, they are implicated in many different disease states. PML [de The et al., 1991; Goddard et al., 1991], RFP [Hasegawa et al., 1996], and EFP [Ikeda et al., 2000] as TRIM32 [Horn et al., 2004] have been linked to tumor initiation and progression. MID1 [Dal Zotto et al., 1998; Trockenbacher et al., 2001], is altered in Opitz syndrome and MUL is associated with Mulibrey nanism [Avela et al., 2000], two developmental genetic diseases, while pyrin/marenostrin is implicated in an inflammatory disease named familial Mediterranean fever [Bernot et al., 1998].

Other studies indicate that TRIM32, consistently with the presence of the RING domain [Joazeiro and Weissman, 2000], has E3 ubiquitin ligase activity, binds to the head and neck region of myosin, and ubiquitinates actin [Kudryashova et al., 2005]. This suggests that TRIM32 may regulate components of the cytoskeleton. Recently, in a small consanguineous Israeli Bedouin family, an SNP microarray genotyping analysis for homozygosity revealed a new mutation (P130S) in the TRIM32 B-box domain, cosegregating with Bardet-Biedl syndrome type 11 (BBS11; MIM♯ 209900). BBS is a pleiotropic disorder characterized by obesity, pigmentary retinopathy, polydactyly, renal abnormalities, learning disabilities, and hypogenitalism [Chiang et al., 2006].

In this report we describe the first cases of LGMD2H in a non-Hutterite population that we linked to the loss of TRIM32 interaction properties [Cao et al., 1998; Cainarca et al., 1999; Reymond et al., 2001].

MATERIALS AND METHODS

Sample Collection

The following criteria were used to recruit patients including limb girdle onset, type of progression, autosomal recessive inheritance, and dystrophic changes on muscle biopsy.

Blood samples from patients diagnosed with LGMD were taken. All the possible causative genes were examined by linkage analysis or mutation detection. About 1,000 DNA samples and 200 biopsies were consecutively collected and stored at the SUN-Naples Human Mutation Gene Bank (Cardiomyology and Medical Genetics) and Telethon Institute of Genetics and Medicine. All samples were analyzed for mutations in the LGMD2A, 2B, 2C, 2D, 2E, 2F, 2G, 2I, 1B, and 1C loci. All exons and intron-flanking regions were studied.

In about 50% of patients no mutation was found in any of the LGMD genes. This indicates that we are including a wider spectrum of muscular phenotype. This can be an advantage to define alternative phenotypes due to a mutation in a single gene [Piluso et al., 2005]. Genomic DNA was extracted by phenol/chloroform to be used for DHPLC analysis. DNA was quantified and diluted for the amplification by PCR [Underhill et al., 1997].

In the current survey, DNA samples from 310 LGMD patients and 600 control chromosomes with similar ethnic background were analyzed by high-throughput DHPLC (HT-DHPLC). Of these samples, 82% were recruited at the Universities of Naples, Padua, and Rome. Other patients were from Argentina (one patient; 0.3%), Croatia (six patients; 1.9%), Slovenia (five patients; 1.62%), Germany (one patient; 0.32%), Greece (two patients; 0.65%), Israel (six patients; 1.94%), France (five patients; 1.62%), Spain (one patient; 0.32%), and Turkey (40 patients; 13%).

Polymerase Chain Reaction

The sequence of TRIM32 gene (NM_12210.2) (one coding exon) and untranslated regions were amplified by PCR from genomic DNA using seven overlapping fragments with the primers described in the Supplementary Table S1 (available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat).

Primers were compared with results of the web-based program Primer3 (PRIMER3; primer3_www.cgi, v 0.2; http://frodo.wi.mit.edu). Each oligonucleotide was also checked by Blastn against the NCBI data bank genome for specificity (BLAST, www.ncbi.nlm.nih.gov/BLAST; NCBI, www.ncbi.nlm.nih.gov).

For PCR analysis, 60 ng of genomic DNA was amplified with a DNA Thermocycler System (MWG AG Biotech, Ebersberg, Germany; MJ Research PTC-100 96 well PCR, Roche, Alameda, CA). An initial denaturation step at 95°C for 7 min was set, followed by 34 cycles (95°C for 30 s, 60–61°C for 1 min and 30 s, and 68°C for 1 min) followed by 95°C and a final extension at 68°C for 10 min. The annealing for the untranslated regions was 61°C.

DHPLC Analysis

The entire TRIM32 gene was analyzed, including the 5′ untranslated region. We performed comparative mutation scanning to select amplicons for aberrant DHPLC profiles not shared by normal controls.

Primers were longer than 25 nucleotides to reduce the allele preference determined by sequence differences located in the region of annealing. DHPLC was performed on a WAVE DNA fragment analysis system (Transgenomic Inc., San Jose, CA) equipped with a DNASep column (3,500 High Throughput [HT]) employing a UV-C scanner to detect eluted DNA [O'Donovan et al., 1998].

Based on DHPLC requirements, special buffer formulations and primer design were used to improve sensitivity and specificity [Underhill et al., 1997; O'Donovan et al., 1998].

Genomic DNA Sequence Analysis

Both strands were sequenced using BigDye® Terminator sequencing chemistry (Applied Biosystems, Foster City, CA). An ABI3130XL automatic DNA sequencer (Applied Biosystems) was used to analyze the product of the sequence reaction. We verified each nucleotide change by direct sequencing of a second amplified PCR product obtained with different primers. Mutations were numbered based on protein (GenBank NP_036342) and cDNA sequence (GenBank NM_012210.2). Nucleotides were numbered according to international recommendation [den Dunnen and Antonarakis, 2001].

In Vitro Interaction Mating

For functional analysis of TRIM32 novel mutations, we used a yeast interaction mating assay. The cDNA bait sequences corresponding to the wild-type and mutant TRIM32 (nt: 134–2096 of

NM_012210.2, aa: 1–654) were cloned into pGBKT7 plasmid vector (Clontech Laboratories Inc., Palo Alto, CA). Prey constructs of fusion genes encoding TRIM32 single or combined domains, or combinations of domains (R; R-BB, R-BB-CC, BB-CC-NHL, CC-NHL, and NHL), were cloned into pGADT7 (Clontech) and were individually transformed into the *Saccharomyces cerevisiae* (*S. cerevisiae*) Y187 strain.

These lines were mated with the bait lines transformed into *S. cerevisiae* AH109 and diploid cells selected on medium lacking Leu and Trp, Interacting clones were selected on synthetic SD minimal medium QDO plates (lack of Adenine, Histidine, Leucine, and Tryptophan) according to the Clontech manufacturer's protocols.

The human p53 and pTD1 proteins were used as positive controls for the assay. The same system was used to check for possible interaction between TRIM32 and some Ubiquitin conjugating enzymes.

The cDNA sequences corresponding to TRIM32 (wild-type and mutated forms) were cloned into pGBKT7 plasmid and transformed in the *S. cerevisiae* AH109 strain.

The different human Ubiquitin conjugating enzymes cloned into pGADT7 vector were cotransformed into the Y187 strain. The positive interaction was observed on selection medium plates, Selective Dropout lacking Leu, Trp, His, Ade (SD-LWHA), after incubation at 30°C.

To test the correct expression and the amount of TRIM32 mutant proteins in yeast system, we performed a Western Blot (WB) analysis using anti-myc-tag 9E10 antibody (Abcam plc, Cambridge, UK) (Supplementary Fig. S1).

Immunoprecipitation

The cDNA corresponding to full-length human TRIM32 in wild-type and mutant forms, were cloned into pcDNA3.1/HAvector. The cDNA for human E2N (aa.:1-152; cds nt: 367-825) was cloned into pcS2 Myc-tag vector (Invitrogen Corporation, Carlsbad, CA).

Expression vectors for Myc-tagged human E2N and HA-tagged human TRIM32 wild-type and TRIM32 mutants were cotransfected into COS7 cells. Cells were harvested after 48 hr and then homogenized in lysis buffer (PBS1X, 0.8% NP40, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, pH 7.8). Cell lysates were centrifuged at 10,000 *g* for 15 minutes, and the supernatants preincubated with protein A sepharose (Sigma-Aldrich, St. Louis, MO) to adsorb nonspecific binding proteins. After centrifugation, the supernatants were incubated with anti-Myc antibody (9E10) or anti-HA (Roche Applied Science, Indianapolis, IN).

After incubation with protein A sepharose for 60 minutes, immunocomplexes were collected by centrifugation. The resin was washed with an excess amount of lysis buffer followed by elution of immunoprecipitated proteins with SDS sample buffer.

Transfection was performed using Polyfect (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

Immunofluorescence Assay

COS7 cells were grown on glass coverslip put into 12-well plates (NUNC A/S, Roskilde, Denmark). They were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and penicillin-streptomycin (Gibco-Invitrogen, Carlsbad, CA) and maintained in a 5% CO_2 incubator at 37°C.

Transfections were carried out using Polyfect reagent with 750 ng of pcDNA3MycEGFP (Invitrogen) null vector or with wild-type TRIM32 or all TRIM32 mutants (D489N TRIM32, R394H TRIM32, 1559delC TRIM32, D588del TRIM32, and P130S TRIM32) subcloned in-frame with green fluorescent protein (GFP). After 36 hr, cells were fixed in 4% paraformaldehyde/PBS for 10 minutes at room temperature. Coverslips were mounted with Vectashield mounting medium with DAPI (Vector Laboratories Inc., Burlingame, CA). Wild-type and mutant TRIM32 proteins were identified by enhanced GFP (EGPF) (green) fluorescence. Cells were examined using a Zeiss microscope (Axio Imager A1, Carl Zeiss S.p.A, Milano, Italy) and analyzed using Axio Vision Rel. 4.5 software. Digital images were saved and managed by Adobe PhotoShop (Adobe Systems Inc., Mountain View, CA).

Molecular Modeling

The effect of the mutations here described on the TRIM32 3-D structure, was investigated using as a template the crystal structure of BRAT. This is homologous to TRIM32 (Protein Data Bank [PDB]; www.rcsb.org/pdb). A model was generated for amino acid residues 371–643 of NHL. The sequence identity between target and template for this region was about 40%. The model was refined using YASARA (Yet Another Scientific Artificial Reality Application; www.yasara.org), which was shown to increase model accuracy [Krieger et al., 2004].

RESULTS

We carried out a mutation analysis using DHPLC and sequencing on a cohort of 310 LGMD patients with different degrees of severity. All patients were previously examined for the other LGMD genes. We identified three novel putative mutations in the TRIM32 gene (p.R394H, p.T520TfsX13, and p.D588del) in

TABLE 1. **Summary of Novel Mutations Detected in the *TRIM32* Gene***

Gene	DNA	Protein	Patients
TRIM32	c.[1180G>A]+[?]	p.R394H	1
TRIM32	c.[1180G>A]+[1180G>A]	p.R394H	1
TRIM32	c.[1761_1763delGAT]+[?]	p.D588del	1
TRIM32	c.[1559delC]+[1559delC]	p.T520TfsX13	1

*Mutation numbering is based on *TRIM32* cDNA (sequence position 1 is the A in the first ATG codon). The reference sequence used is GenBank ID NM_012210.2.

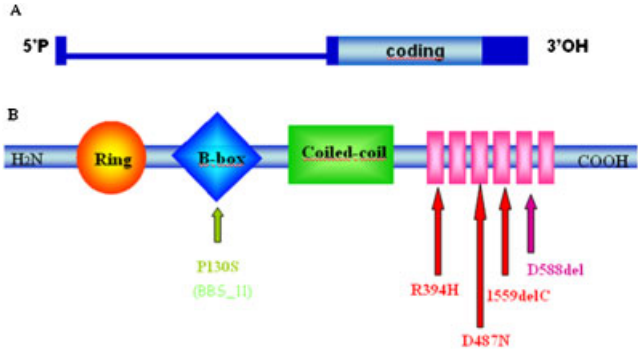

FIGURE 1. ***TRIM32* characteristics. A: The *TRIM32 HT2A* gene maps on chr 9q 33.1 (1–3,160 bp). Only one exon is coding (134–2095 bp). B: TRIM32 protein is composed of 673 amino acids (72 kDa). Here are shown TRIM32 functional domains: RING finger domain, B-box type 1 domain, Coiled coil region, and six NHL repeats. The arrows indicate the mutation location in TRIM32 protein structure. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

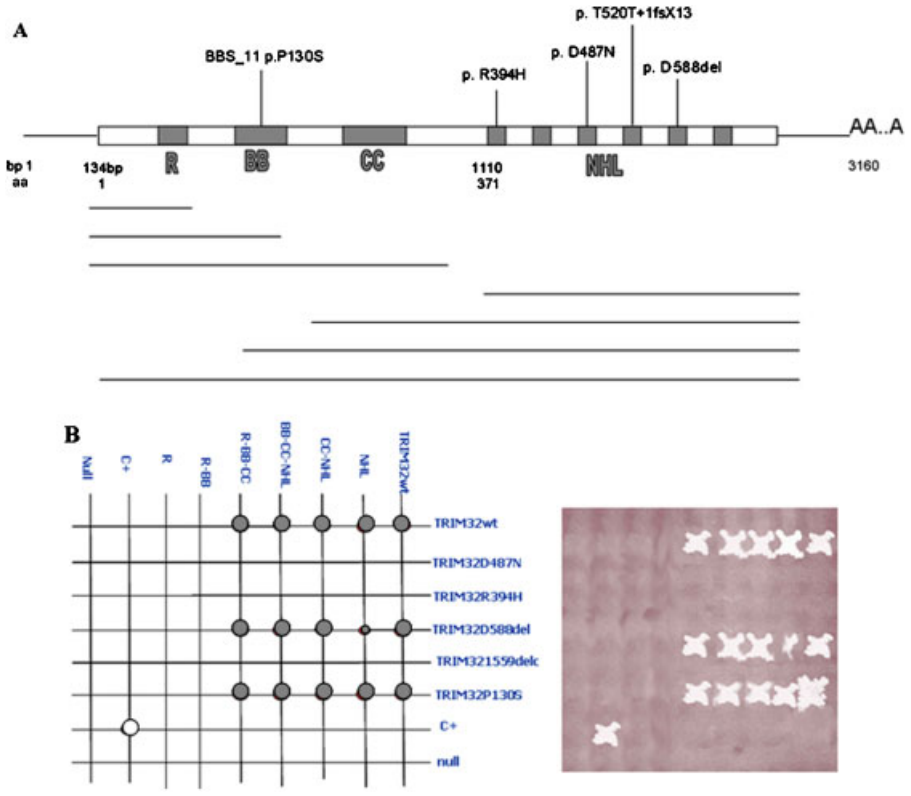

FIGURE 2. **Yeast two-hybrid analysis. The loss of interaction was tested in the yeast two-hybrid assay by cotransformation. A: Schematic structure of human full-length *TRIM32* cDNA. Numbers indicate nucleotide residue numbers (top, labeled bp) and amino acid residue numbers (below the nucleotide number, labeled aa). AAA indicates a poly(A+) tail. The structural/functional domains are shown in gray. Lines below indicate the partial TRIM32 bait constructs that were generated for yeast two-hybrid screens to test for the self-interaction property. B: On the left is the interaction mating outline of the plate picture on the right. The filled circles show the growth on selective media (QDO) compared to the positive control represented by the empty circles. Each variation found in homozygous, compared with p53/pTD1 control growth, makes TRIM32 unable to self-interact. TRIM32_P130S remains able to self interact. NHL domains are responsible for the Coiled-Coil conformation and TRIM self-interaction function. Pictures of the plates were taken after 5 days of culture at 30°C. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

four patients (two homozygous and two heterozygous). These alleles were absent in 600 control chromosomes (Table 1).

Phenotypic Analysis

Patient 3639 (proband #1) is a 44-year-old Croatian woman, who carries a homozygous c.1559delC p.T520TfsX13 variation (Fig. 1; Supplementary Fig. S2). She complained of a slowly progressive proximal weakness and muscle wasting, respiratory weakness, and chronic keratitis; creatine-kinase (CK) levels were in the normal range. A physical examination in 2001 showed marked atrophy of the shoulder and abduction of the arms. Facial muscles were also involved, affecting the ability to close the eyes. Electromyography (EMG) revealed both neuropathic and myopathic elements. It was concluded that the disorder combines phenotypic features of LGMD and facio-scapulo-humeral dystrophy. Her parents had no muscular involvement. Her brother was affected by Wilson syndrome.

A testing method was devised to detect the c.1559delC p.T520TfsX13 mutation based on a natural restriction site for MslI enzyme that is formed by the cytosine deletion. The allele wild-type was digested once by MslI, the allele of the proband had two extra fragments (Supplementary Fig. S2).

Patients 3144 and 3996 (probands #2 and #3, respectively) are from Southern Italy and show the same TRIM32 variation, c.1180G>A p.R394H (Fig. 1; Supplementary Fig. S3).

Proband #2, heterozygous for this mutation, is a 73-year-old man presenting with hypertension and type II-diabetes. A chance occurrence of slightly elevated serum CK values (2.5×), not associated with muscle symptoms or signs, was revealed at the age of 64 years and later confirmed. The patient was the last of nine siblings; five of them died from cardiovascular events.

The last clinical examination showed calf pseudohypertrophy, mild ankle contractions, and scapular winging, at rest and in upper limb adduction, especially on the left side. No respiratory involvement was observed.

Proband #3 is homozygous for c.1180G>A p.R394H TRIM32 allele. Disease onset was in the third decade of life and characterized by weakness and paresthesia. The patient noted progressive difficulty in rising from the floor, in climbing stairs, and in walking. A muscle biopsy, performed at the age of 59 years, showed a muscular dystrophy with a normal dystrophin staining. The patient was diagnosed with LGMD. He lost the ability to walk at the age of 64 years, after a prolonged immobilization for other causes.

Physical examination, showed marked atrophy in both upper and lower limbs (proximal>distal), with muscle weakness. Retractions were present at the ankles, knees, and Achilles tendons. Scapular winging was also observed. Electrocardiography (ECG) revealed a right bundle branch block. Forced expiratory vital capacity (FVC) was reduced to 41%.

Patient 3990 (proband #4) is a 15-year-old boy, heterozygous for c.1761_1763delGAT p.D588del. Two maternal cousins also presented high CK levels.

Elevated CK values (4–5 ×) were found at the age of 8 years before a minor surgery. The only symptoms of this patient were muscle cramps after exercise. Physical examination at the age of 11 years showed scapular winging, no pseudohypertrophy of calves, and normal muscle strength at upper and lower limbs.

This mutation lies at the fifth TRIM32 NHL motif and inserts a new restriction site for HphI (Fig. 1; Supplementary Fig. S4).

TRIM32 mutants do not mislocalize. We generated TRIM-32-GFP fusion proteins to investigate whether TRIM32 mutants display a different subcellular localization to wild-type controls. We transfected COS7 cells by Polyfect reagent, using 750 ng of each plasmid.

TRIM32 localizes to discrete cytoplasmic organelles (Golgi, endoplasmic reticulum [ER]) and in some preparation the fluorescence is associated with a diffusely stained background [Reymond et al., 2001].

No TRIM32 mutant proteins showed any significant difference with the wild-type form. The proteins show a diffusely background, sometimes accompanied with a spot pattern (Supplementary Fig. S5).

LGMD2H mutants lost the ability to self-interact. The novel mutations occur in a protein domain referred to as NHL domain (beta-propeller) that represents a conserved region, possibly involved in protein–protein interaction [El-Husseini and Vincent, 1999]. These mutations could produce a significant alteration in the structure and function of this domain.

The strong self-association properties found for all of the TRIM proteins, are attributed to the Coiled Coil (CC) region that is considered responsible for the formation of higher order complexes, since TRIM32 Coiled-coil deletion mutants confirm these properties of the CC region.

To investigate whether the mutant proteins have lost their ability to self-interact, we utilized the interaction mating technique.

We tested the self-association ability of TRIM32 that carries the D487N mutation. This mutation abolished self-binding of TRIM32. The consequence of this single amino acid replacement was thus similar to the complete deletion of the coiled coil-NHL domains (Fig. 2B).

We tested all the novel alleles using the same method. The homozygous mutations p.R394H and p.1559delC cause the complete loss of TRIM32 homodimerization, while the BBS11 mutation (p.P130S) maintained the self-interaction. The p.D588del, found only in heterozygosity, showed the TRIM32 homodimerization property, albeit with slower growth (Fig. 2B). This demonstrates that in addition to the CC region, the NHL domain is important for self-interaction and mutations abolished this function.

E2N is a specific partner of TRIM32 that is lost by TRIM32 mutants. TRIM32 is an E3 ubiquitin ligase, due to the presence of the RING finger domain in its structure, TRIM32 specifically self-ubiquitinated in the presence of ATP, ubiquitin, E1 enzyme, and E2 enzymes [Kudryashova et al., 2005].

We investigated the ability of different ubiquitin conjugating enzymes to interact with TRIM32 protein.

Results showed that the specific enzyme E2N interacts with the TRIM32 full-length protein, while E1 and E3 do not (Meroni G., Napolitano L., personal communication).

UBE2N gene (Hs.524630; NM_003348.3) encodes a human E2 that is involved in protein degradation, mainly expressed in muscle and testis.

The replacement of the arginine with histidine at position 394, the deletion of the cytosine (nt 1559 of TRIM32 coding sequence) and the substitution of aspartic acid D487 to asparagine N (pGad-TRIM32_D487N) inhibited the binding to full-length E2N.

Aspartic acid deletion in position 588 shows on selective media a reduced growth, while p.P130S (causative of BBS) displays a normal growth (Fig. 3).

Structural changes in NHL domains due to several mutations. We created a computer model of six NHL motifs using the MolIDE program, supported by the BRAT three-dimensional structure (http://dunbrack.fccc.edu/molide/).

The effect of the novel variations on the DNA-binding capacity of NHL domain of TRIM32 was investigated using the crystal structure of BRAT (PDB entry site). A model was constructed for amino acid residues 371–643 of NHL of TRIM32. The sequence identity between target and template for this region was 40%. Subsequently, the model was refined using YASARA, which was shown to increase model accuracy [Krieger et al., 2004].

We observed a predicted structure with a six-bladed β-propeller. Each blade is composed of a highly twisted four-stranded antiparallel β-sheet. When TRIM32 has the mutations p.R394H or p.D487N, it may take on a different shape, masking the coiled-coil region. This could prevent the interaction properties (Fig. 4) [Edwards et al., 2003; Arama et al., 2000]. However, p.D588del seems to be heavily altered in its structure, albeit the functional consequence seems to be weaker. There is possibly a difference between computer prediction and real conformation that could be solved by direct conformation analyses.

This prediction supports our previous studies that have shown that mutations in TRIM32 NHL domains abrogate TRIM32 self-interaction and E2N binding.

DISCUSSION

To date, LGMD2H has only been described in the Manitoba Hutterite ancestry. No other haplotype of LGMD2H/STM has until now been detected and no other mutation has been associated with these diseases. TRIM32 gene has also been involved in a completely different disease: a form of Bardet-Biedl syndrome, BBS11. The allele cosegregating with BBS11 has the missense mutation p.P130S at B-Box domain of TRIM32.

There are many examples where different mutations in the same gene can result in different disorders [Capell and Collins, 2006; Rankin and Ellard, 2006]. To better understand the mechanisms underlying the development of muscle disease, we genetically characterized LGMD2H to determine whether D487N allele identified in Manitoba Hutterites is the only mutation of LGMD2H.

The Hutterites are an Anabaptist sect, who lives in colonies on North America [Nimgaonkar et al., 2000]. The term "anabaptist" comes from the practice of baptizing individuals who had been baptized previously. They constitute a unique religious and genetic group. They migrated from Germany to North America in the 1870 s. This population shows a high prevalence of autosomal recessive disorders. This may be due to founder effects.

LGMD forms are also common in this population. Current estimates exceed 1/400 compared to 1/15,000 of the general population [Piluso et al., 2005]. There is a second type of LGMD in the Hutterite population that maps to chromosome 19q31–q33 and is due to homozygosity for the p.L276I mutation in FKRP. This is also inherited from a common ancestor [Frosk et al., 2005].

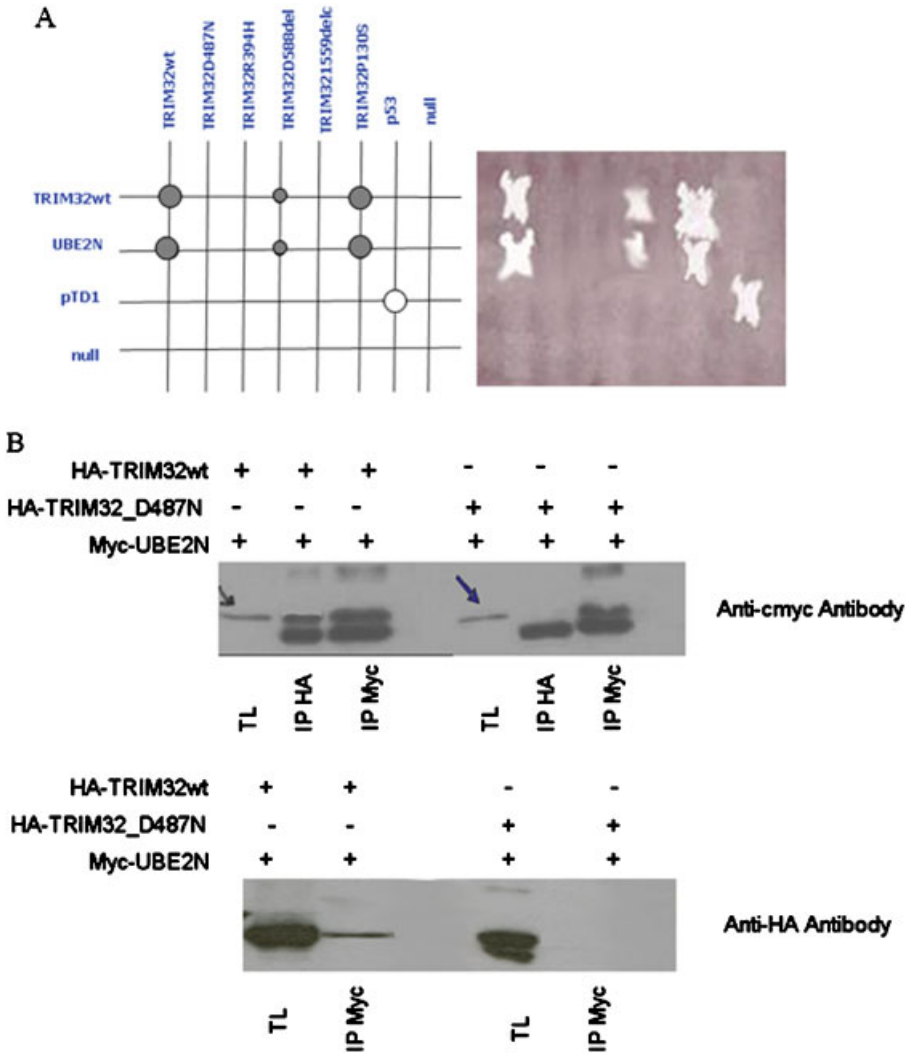

FIGURE 3. **Interaction between TRIM32 (wild-type and mutated forms) and E2N. A:** *Yeast two-hybrid system.* **Full-length UBE2N cDNA was cotransformed with TRIM32 cDNAs into *S. cerevisiae*. The wild-type TRIM32 bait and TRIM32 p.P130S variation show the same grade of interaction with E2N. The TRIM32 variants TRIM32 pR394H, c.1559delC, and pD487N baits do not interact with E2N, indicating that the NHL motif in TRIM32 protein is involved in the interaction with the enzyme. TRIM32 with the aspartic acid deletion in position 588 shows only a retarded growth, a reduced interaction with E2N on selective media. B: Co-IP in COS7 cells to confirm the interaction between E2N and TRIM32 (the arrow points to the myc-E2N protein). Total lysates (TL) of cotransfected cells were immunoprecipitated with anti-HA antibody. The interaction is revealed by Western blot using anti-myc antibody. The same experiment was repeated by immunoprecipitating TL with anti-cmyc antibody and detection by anti-HA antibody. The interaction is lost between the D487N_TRIM32 and E2N proteins. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

In 2005, Frosk et al. [2005] demonstrated that LGMD2H appears to be more frequent in the Schmiedeleut subdivision of the Hutterites, whereas LGMD2I is more frequent in the Dariusleut. The clinical heterogeneity for both LGMD2H and LGMD2I is due to different disease mechanisms. Presumably, the more severe phenotype was due to a double homozygote mutation.

On the basis of our results, we could not exclude a phenotypic heterogeneity based on additional modifying loci in patients with p.D487N mutation. These could be due to variations in other LGMD genes.

This study was conducted to assess whether: 1) LGMD2H is a worldwide disease or is confined to the Hutterites; 2) there is only a single specific TRIM32 allele producing a muscular phenotype in a mutation-specific fashion, as in other genetic disorders (i.e., achondroplasia, progeria, etc); and 3) to develop a functional test to check alleles.

This is the first report of LGMD2H in a non-Hutterite population. We identified three novel alleles (p.R394H, p.D588del, and p.T520TfsX13) in our cohort of European patients. Mutations are all located at NHL repeats and do not modify subcellular localization of TRIM32. Given the self-interaction property of TRIM32, we set up a yeast two-hybrid interaction mating to test this property using TRIM32 mutants. When we introduced the p.D487N mutation, no self-interaction was observed. The same result was obtained using mutations p.R394H and p.T520TfsX13. p.D588del had a weaker interaction compared with the wild-type protein. p.P130S/BBS11 was identical to wild-type TRIM32.

The effect of the homozygous mutations was comparable to the deletion of the entire CC and NHL, suggesting a dramatic effect of these mutations on protein folding. In contrast, BBS is a multisystemic disorder and a different mechanism is likely

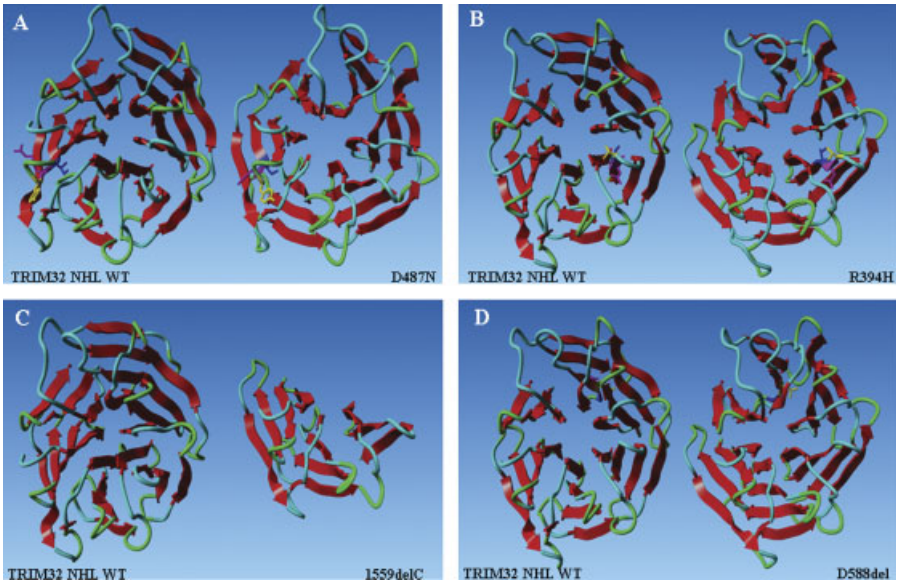

FIGURE 4. **Molecular modeling of the NHL 6 β-propeller structure of TRIM32 wild-type protein compared to the TRIM32 p.D487N; p.R394H; c.1559delC; and p.D588del alleles. Each propeller is constituted by the four antiparallel red β-sheets. The position of the mutated amino acids is colored in magenta, the previous amino acid is blue, the next is yellow. This figure was visualized using YASARA (see YASARA website; www.yasara.org). The mutations would probably cause a local change in the protein scaffold.**

involved, considering that no phenotypic overlap exists between these two conditions. The difference among TRIM32 alleles was evident using the yeast two-hybrid direct interaction mating with TRIM32_D487N and the three novel variations described here. We demonstrated that mutations impairing self-dimerization of TRIM32 are associated with muscular phenotypes. Alleles R394H and T520TfsX13, similar to the p.D487N mutation, prevent TRIM32 self-interaction. They all occur in the conserved NHL domains and may hinder coiled coil homodimerization.

TRIM32 is not a muscle-specific protein. We suggest that the specific damage to the muscle cells could be due to the disruption of a muscle-specific interaction as a consequence of NHL mutations. Since TRIM32 has a putative E3 ligase property, we tested some E2 enzymes for the interaction. E2N, but not E1 and E3, was able to bind TRIM32 and this binding was abolished in mutants. The binding was confirmed by co-immunoprecipitation (co-IP) assay. E2N is a muscle protein and it can confer tissue-specificity to TRIM32 mutations.

We can hypothesize that these mutations in NHL domains create a three-dimensional conformational change in the TRIM32 shape masking the binding. While the two mutations found in homozygosity are effective in abolishing the property of interaction, we have some concerns about the significance of the p.D588del allele since: 1) this allele was not found in homozygosity; 2) the result of the assay was not clear-cut. Nevertheless, the mutation was never found in 600 normal chromosomes. A possible explanation is that we missed the second mutation of TRIM32. It may be located outside the sequenced part of the gene. Alternatively, p.D588del is a rare private variant, with no relationship to muscular dystrophy.

The phenotypes of these LGMD2H patients are mild but they share the characteristics of an irreversible loss of motility after immobilization. We hypothesize that TRIM32 could be linked to the process of atrophic degeneration/regeneration involving massive muscle protein ubiquitination. When TRIM32 is mutated this process could be impaired and muscle regeneration could be insufficient.

Since the phenotype of LGMD2H is produced when a partner of TRIM32 is lost, the observation that there is a great variability in clinical severity in patients carrying the same mutation suggests that other factors may interfere in the course of the disease.

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