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A novel missense mutation (G43S) in the switch I region of Rab27A causing Griscelli Syndrome

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Abstract

The autosomal recessive Griscelli syndrome type II (GSII) is caused by mutations in the *RAB27A* gene. Typical clinical features include immunological impairment, silver-gray scalp hair, eyelashes and eyebrows, and hypomelanosis of the skin. Rab proteins help determine the specificity of membrane trafficking steps within cells. In melanocytes, the GTP-bound form of Rab27A associates with the membranes of mature fully-pigmented melanosomes through its geranylgeranyl group. Once attached, Rab27A recruits the downstream effector Melanophilin (Mlph) and the actin-dependent motor protein Myosin Va (MyoVa). The molecular Rab27A/Mlph/MyoVa tripartite complex, which links melanosomes to the peripheral actin network, is required to achieve melanosome transfer to surrounding keratinocytes in the epidermis. Here we report a novel homozygous missense mutation c.127G>A, p.G43S in exon 2 of the *RAB27A* gene of an Afghani GSII patient. Laser scanning confocal microscopy showed that the G43S mutation, which is located in the highly conserved switch I region of Rab27A, induces perinuclear localization of melanosomes in normal melanocytes, and fails to restore melanosomes to the actin-rich periphery in GSII melanocytes. Co-immunoprecipitation studies showed that Rab27A(G43S) fails to interact with its effector Melanophilin, indicating that the Switch I region functions in the recruitment of Rab effector proteins.

Keywords

Griscelli syndrome; Rab27A; Melanosome; Melanophilin; Myosin Va; Switch I region

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Introduction

Griscelli syndrome (GS) is an autosomal recessive disorder characterized by various degrees of skin hypopigmentation and silver-colored hair accompanied by immunologic impairment and/or defects in the central nervous system [1,2,3]. Three different genes are associated with GS in humans [4,5,6].

GSI is caused by mutations in the *MYO5A* gene on chromosome 15q21; *MYO5A* is the human homologue of the mouse *dilute* gene [4,7]. The Myosin Va (MyoVA) protein product is an actin-dependent motor protein involved in trafficking of various organelles; its role in melanosome transport has been described in great detail [8,9,10,11,12,13,14]. The clinical manifestations in GSI include neurological impairment [15,16,17,18]. The most common GS subtype is GSII, caused by mutations in *RAB27A*, whose murine homologue is the *ashen* gene [6,19]. Like *MYO5A*, *RAB27A* is located on chromosome 15q21 [6,19]. Rabs cycle between an inactive GDP-bound state and an activated GTP-bound state that associates with membranes by virtue of C-terminal geranylgeranyl groups bound by ester linkages to cysteine residues [20]. The Switch I and II regions of Rabs are highly conserved motifs located on the surface of the Rab protein. They undergo GDP or GTP-dependent conformational changes that determine their affinity for the Rab-effectors and regulators they recruit [21,22,23]. Rab27A, in its GTP-bound form, interacts with the melanosome membrane where it serves as a receptor for specific effectors and for the actin-dependent motor protein Myosin Va [10,11,26,14,24, 25,26]. In addition to its well-known role in pigmentation, the Rab27A protein functions in granule release within cytotoxic T lymphocytes. Consequently, mutations in *RAB27A* leave patients with an immune system unable to successfully combat infection [27].

The *Melanophilin* (*MLPH*) gene on chromosome 2q37 is the human homologue of the mouse *leaden* gene [6,28] and encodes a member of the Rab effector family. Mutations in *MLPH* cause GSIII; only one such patient has been reported [6]. Clinical manifestations are restricted to the hypopigmentation that results because melanosomes, which synthesize melanin [29], cannot be transferred to surrounding epidermal keratinocytes [30]. In order for melanosome transfer to keratinocytes to occur, melanosomes must be captured in the actin-rich periphery of melanocytes, a process mediated by the Rab27/MyoVA/Mlph tripartite complex. Rab27A recruits its effector Mlph by a direct interaction while the C-terminal globular tail of MyoVA binds directly to Mlph [10,11,14,25,31,32,33]. Mutations in the corresponding genes of the Rab27a, MyoVA and Mlph proteins result in an aberrant tripartite complex, with perinuclear aggregation of melanosomes and, consequently, skin hypomelanosis in GSII patients [4,6,7, 10,11,19,25,28].

Here we describe a novel disease-causing missense mutation (G43S) in the Switch I region of Rab27A that impairs recruitment of Mlph to form the tripartite complex.

Materials and Methods

Patients and cells

Two GSII patients were enrolled in clinical protocol 76-HG-0238, "Diagnosis and Treatment of Inborn Errors of Metabolism," approved by the NHGRI Institutional Review Board. Written informed consent was obtained from the parents of both patients. Patient 1 is a five-year-old girl from non-consanguineous Danish parents who was molecularly diagnosed with GSII by Dr. G. de Saint Basile (Paris, France) at the age of three months in 2001; the mutations were confirmed by our lab. The girl was admitted to the NIH Clinical Center after a successful bone marrow transplant in Denmark. A few years earlier her older sister had died from what histologically appeared to be cerebral hemophagocytic lymphohistiocytosis. The mother had noticed the unusual silver blond hair of the deceased girl and when the younger sister was a

born with similar hair appearance, GS was suspected. The diagnosis was supported by microscopy of the child's hair showing characteristic clumping of melanin in the hair shaft. A stem cell transplant from an unrelated donor was conducted successfully at the age of 6 months before the girl showed any signs of disease acceleration.

Patient 2 is a six-year-old Afghan boy clinically diagnosed and treated in Denmark. His parents are first cousins and he is the fifth of six siblings; the other siblings are in good health. Patient 2 had slightly gray hair from infancy (Fig. 1A). His development was normal until three years of age when he experienced a stroke-like attack followed by squinting of the eyes, hemiplegia, and difficulty speaking. The condition remained relatively stable but unexplained. MRI scans of his brain showed progressive white matter abnormalities.

At age six he had fever, progressive pancytopenia, coagulopathy, elevated serum levels of liver enzymes, hepatosplenomegaly, and marked increase in ferritin. NK-cell activity was markedly reduced. The cerebrospinal fluid was normal. The patient was clinically diagnosed with Griscelli syndrome and treated in Denmark according to the HLH-94 protocol [34]. He responded to therapy with normalization of biochemical abnormalities and improvement of motor function and speech. The patient received an unrelated bone marrow transplantation. Currently, he is eleven years old and doing well with moderate but stable developmental delay including slight hemiplegia.

A skin biopsy was obtained from patient 1 but not from patient 2, and primary epidermal melanocytes were cultured as described [11]. Normal human primary epidermal melanocytes were purchased from Cascade Biologics (Portland, OR). HEK cells (gift of Dr. Jan Tavernier, Gent University, Belgium) were cultured in DMEM (Invitrogen, Carlsbad, CA) and 10% fetal bovine calf serum (Gemini Bio-Products, West Sacramento, CA).

Molecular mutation analysis

Genomic DNA was isolated from blood using the Puregene DNA purification kit according to the manufacturer's guidelines (Gentra Systems, Minneapolis, Minnesota). Exon six and all six *RAB27A* coding exons were PCR amplified, using the primers illustrated in Table 1, from genomic DNA for patient 1 and patient 2, respectively. Sequencing was performed on a Beckman CEQ 8000 sequencer, using the CEQ Dye Terminator Cycle Sequencing kit according to the manufacturer's protocol (Beckman Coulter, Fullerton, CA). Variant sequences were cross-referenced against GenBank and the Single Nucleotide Polymorphism database. The genomic DNA of fifty healthy individuals was sequenced for exon 2 of *RAB27A* to rule out a naturally occurring polymorphism. Conservation analysis was performed by using the online bioinformatics tool MultAlin (Multiple Sequence Alignment, <http://bioinfo.genopoleoulouse.prd.fr/multalin/>)

plasmids, mutagenesis and electroporation

The Mlph I.M.A.G.E. Consortium [LLNL] cDNA clone (I.M.A.G.E. Consortium CloneID 2958853) was purchased from ATCC (Manassas, VA). Mlph cDNA was subcloned into the pDsRed-Monomer-C1 vector (BD Biosciences, Palo Alto, CA) using the EcoRI and BamHI restriction sites. Rab27A(G43S)-GFP was generated by PCR-based site-directed mutagenesis using the Quickchange mutagenesis kit according to the manufacturer's guidelines (Stratagene, La Jolla, CA). We used the previously described Rab27A(WT)-GFP plasmid [34] as a template and the following primers: forward: CAAATTTATCACAACAgTgAgCATTgATTTCAGggAAAA; reverse: TTTTCCCTgAAATCAATgCTCACTgTTgTgATAAATTg. Electroporation of normal epidermal primary and GSII human melanocytes was performed as described [11]. Briefly, a mixture of 100 µl Nucleofector solution (Amaxa GmbH, Cologne, Germany) and 1 µg plasmid

DNA was added to approximately 700,000 melanocytes. The cells were electroporated in the Nucleofector electroporator (Amaxa GmbH) with the U₁₆ specific Nucleofector program.

Laser scanning confocal microscopy

Normal or GSII melanocytes electroporated with Rab27A(WT)-GFP or Rab27A(G43S)-GFP were grown for 48 h on glass slides. The cells were fixed in 3% paraformaldehyde, blocked in PBS containing 0.1% saponin, 100 μ M glycine, and 2% donkey serum followed by incubation with the HMB45 mouse monoclonal antibody (Lab Vision, Fremont, CA) (1:100 dilution). Cells were co-stained with BODIPY 633 phalloidin to visualize the cell boundary (Invitrogen, Carlsbad, CA). The cells were washed and incubated with the donkey anti-mouse antibodies conjugated to ALEXA-555 (Invitrogen), washed again, and mounted in VectaShield (Vector Laboratories, Burlingame, CA). Cells were imaged with a Zeiss 510 META (Zeiss, Thornwood, NY). Images were acquired using a Plan Apochromat 63X/1.4 oil DIC objective. All images are 1D projections of confocal z-sections.

Immunoprecipitation

Twenty hours after transfection of HEK cells with Mlph-Dsred and Rab27A(WT)-GFP or Rab27A(G43S)-GFP or GFP, cells were washed with PBS and lysed with 1% Nonidet P40, 50 mM TRIS pH 8, 150 mM NaCl and a protease inhibitor mix (Roche Diagnostics, Indianapolis, IN). After coupling the mouse monoclonal anti-GFP (Millipore, Billerica, MA) or the rabbit polyclonal anti-GFP antibodies (Abcam, Cambridge, UK) to M-280 magnetic dynabeads (Invitrogen), cell extracts were mixed with the GFP coupled dynabeads in lysis buffer, and incubated overnight at 4°C. The beads were washed six times in lysis buffer and boiled in Laemmli buffer (Biorad, Hercules, CA) for 5 min. The entire eluates were loaded onto a 4–12 % Tris-Glycine gel (Invitrogen) for Western blotting.

Western blot

Equivalent amounts of total protein, as determined by BCA assay (Pierce Biotechnology, Rockford, IL), from HEK cell extracts or normal and GSII melanocytes extracts, were loaded onto 4–12 % Tris-Glycine gels. After blotting, the nitrocellulose membranes (Invitrogen) were probed with a mouse monoclonal antibody against Rab27A (BD Biosciences, San Jose, CA), a mouse monoclonal α -tubulin antibody (loading control, Sigma, St. Louis, MO) and a rabbit polyclonal Mlph antibody (Proteintech Group Inc., Chicago, IL). HRP-labeled anti-mouse antibody or anti-rabbit antibody (Amersham Biosciences, Piscataway, NJ) was used as a secondary antibody. The antigen–antibody complexes were detected with an ECL kit (Amersham Biosciences, Piscataway, NJ).

Results

c.127G>A, p.G43S is a novel *RA27A* missense mutation

PCR amplification of the six exons of the *RAB27A* gene was performed on the genomic DNA of patient 2 using *RAB27A*-specific primers (Table 1). cDNA Expression levels and PCR product size were comparable to a control sample (Fig. 1B). Sequencing revealed a homozygous missense mutation c.127G>A, p.G43S in exon 2 (Fig. 1C); the mutation was confirmed using reverse primers as well (data not shown). Sequence analysis of 50 healthy individuals confirmed that c.127G>A is not a common polymorphism (data not shown); none of the hundred alleles showed this variant.

The p.G43S mutation is located in the Rab27A switch I region. As illustrated for Rab3A (Fig. 2A), Switch I regions are located on the surface of Rabs. Conservation analysis revealed that G43 was conserved amongst several human Rabs (Fig. 2B). Moreover, within Rab27A, G43

was conserved amongst several species (Fig. 2C). These findings indicate that G43S in Rab27A is disease causing. To verify this, we studied the effect of the mutation on melanosome localization in normal and GS melanocytes.

Rab27A(G43S) induces perinuclear distribution of melanosomes in normal melanocytes

Melanocytes of GSII patients and their murine counterpart, *ashen*, exhibit a clustered perinuclear distribution of melanosomes that is recapitulated when mutant Rab27A is expressed in normal melanocytes [10,25,35,36]. To test whether Rab27A(G43S) is disease causing, we electroporated normal human epidermal melanocytes with Rab27A(WT)-GFP (Fig. 3a, 3d, 3e) or Rab27A(G43S)-GFP (Fig. 3f, 3i, 3j). Laser scanning confocal microscopy was used to evaluate the distribution of Rab27A and the melanosome-specific protein Pmel17 (HMB45), which forms the melanosomal striations upon which melanin I deposited [29]. In melanocytes transfected with Rab27A(WT)-GFP (Fig. 3a), both Pmel17 (Fig. 3c) and Rab27A (WT)-GFP (Fig. 3a) showed a punctuate pattern in the cell periphery and in dendrites, with accumulation and co-localization in dendritic tips (Fig. 3d, arrow, 3e). In melanocytes transfected with Rab27A(G43S)-GFP (Fig. 3f), both the mutant Rab27A(G43S) (Fig. 3f) and Pmel17 (Fig. 3h) accumulated and co-localized in the perinuclear area but were absent from the dendrites and dendritic tips (Fig. 3e, 3g, 3h, arrows, 3j); this pattern of distribution is typical for melanosomes in GSII melanocytes [10,25,35,36]. Phalloidin-633 staining visualized cell boundaries (Fig. 3b, 3g). These data show that Rab27A(G43S) co-localizes with Pmel17, indicating that Rab27A(G43S) targets to melanosomes, and that Rab27A(G43S) induces a GSII cell biological phenotype in normal melanocytes.

Rab27A(G43S) does not rescue the perinuclear distribution of melanosomes in GSII melanocytes

The perinuclear accumulation of melanosomes in GSII or *ashen* melanocytes can be rescued by expressing wild type Rab27A protein in the mutant cells [37,38]. As a source of mutant GSII cells, the cultured melanocytes of patient 1, who has two heterozygous nonsense mutations, R200X and R184X, in Rab27A (Fig. 4A), were employed. Western blot analysis showed that, compared to normal cells, the melanocytes of patient 1 had a decreased amount of Rab27a protein with a lower molecular weight (Fig. 4B). The R200X and R184X premature stop codons prevent translation of 21 and 37 C-terminal amino acids, respectively. The truncated C-terminal CXC motif is necessary for posttranslational geranylgeranyl modification and correct targeting to the melanosome membrane [20].

The melanocytes from patient 1 were used to determine if introduction of other Rab27A proteins could correct the perinuclear clustering of melanosomes (Fig. 4C). In GSII melanocytes transfected with Rab27A(WT)-GFP (Fig. 4C.a), both Pmel17 (Fig. 4C.c) and Rab27A(WT)-GFP (Fig. 4C.a) showed a vesicular pattern and co-localization in the cell periphery and in dendrites and dendritic tips (Fig. 4C.d, 4C.e), while non-transfected GS melanocytes showed accumulation of Pmel17 in the perinuclear region (Fig. 4C.c, arrows). In GSII melanocytes transfected with Rab27A(G43S)-GFP, however, the (Rab27A(G43S)-GFP (Fig. 4C.f) and Pmel17 (Fig. 4C.h) accumulated and colocalized in the perinuclear region (Fig. 4C.i, 4C.j); there was no correction of the GSII cellular phenotype by the mutant Rab27A. A phalloidin-633 stain visualized the cell boundaries (Fig. 4C.b, 4C.g). Although Rab27A(G43S) colocalized with Pmel17 and, therefore, melanosomes in GSII melanocytes, it did not induce relocalization of melanosomes from the perinuclear area to the actin-rich cell periphery.

Rab27A(G43S) does not interact with Melanophilin

In normal human and murine melanocytes, Mlph is recruited to melanosome-associated, GTP-bound Rab27A; the two proteins interact in a direct fashion [10,11,14,25,31,32,33]. To study the protein-protein interaction between Rab27A(G43S) and Mlph, we used a co-

immunoprecipitation assay. Protein extracts were made from HEK293 cells that were transfected with Mlph-Dsred and either Rab27A(WT)-GFP, Rab27A(G43S)-GFP, or GFP alone. Pull-down assays were performed with two different anti-GFP antibodies and the Western blot was probed with anti-Mlph antibody or anti-Rab27A antibody (Fig. 5). Mlph coimmunoprecipitated with Rab27A(WT)-GFP (Fig. 5, lane 6 and 7) but not with Rab27A (G43S)-GFP or GFP alone (Fig. 5, lane 4, 5, 8, and 9). These data indicate that the conserved switch I domain of Rab27A is involved in binding of effectors such as Mlph.

Discussion

Most reported *RAB27A* mutations associated with GSII are nonsense or frameshift mutations leading to premature stop codons and resulting in absent Rab27A or in a nonfunctional protein product lacking a C-terminal geranylgeranylation site. [3,5,39,40]. Only four disease-causing *RAB27A* missense mutations have been described in GSII patients. Rab27A (W73G) and Rab27A(L130P) could not be targeted to melanosomes or interact with Mlph; Rab27A(L130P) also had a very low GTP-binding activity [35]. Through protein-protein interaction studies, Rab27A(A87P) was shown to be unable to recruit effector proteins [41]. Rab27A(A152P) was not targeted to melanosomes but was able to interact with Mlph [35]. None of these missense mutations are located in the switch I region of Rab27A.

We have shown that the missense mutation c.127G>A, p.G43S in exon 2 of *Rab27A* causes GSII and that the mutant protein product cannot interact with its effector Mlph. This is the first reported Rab27A mutation located in the switch I region. Fukuda studied the interaction between Mlph and Rab27A in great molecular detail, focusing on amino acid residues in the switch I and switch II regions that are unique to Rab27A. His mutagenesis study showed that a Rab27A switch I mutant, E48V, could bind to Mlph in a normal fashion; he proposed that switch II was the region primarily responsible for recruitment of Mlph to Rab27A [42]. Fukuda did not study other switch I mutations, but mutagenesis data showed that amino acid K82 in the switch I region of the Golgi-associated Rab34 is involved in recruitment of its effector RILP [21].

Our data indicate that conserved amino acids in the switch I region of Rab27A are also involved in recruitment of effector proteins such as Mlph. Detailed molecular studies of each amino acid in the switch I region are needed to determine the role of the switch I region in assembly of the Rab27A/Mlph/ MyoVa tripartite complex on the melanosomal membrane. Naturally occurring switch I missense mutations in Rab27A, such as G43S, can serve as tools for future studies.

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Figure 1. G43S (GGC>AGC) is a novel missense mutation in the *RAB27A* gene

(A) GS patient 2, before bone marrow transplant, with pigmented skin and a mix of silvery gray and dark brown hair. (B) Agarose gel electrophoresis of the amplified DNA fragments representing exons 1–6 and the exon/intron boundaries of the *RAB27A* gene from the patient (p) and a healthy control (c). The patient's bands are similar to control bands. (C) Chromatograms of the forward sequence of a normal individual (left panel) and the homozygous alteration of G>A leading to a G43S (GGC>AGC) missense mutation in *RAB27A* in patient 2 (right panel). M: marker.

Figure 2. G43 is highly conserved and located in the switch I region of Rab27A

(A) Crystal structure of the Rab3A conserved switch I region (highlighted in yellow), which is located on the surface and affected by the mutation. (B) The mutated amino acid (G43S) is part of the motif T(I/V)G(I/V/A)(D/E)F; which is highly conserved among human Rabs (green arrow). (C) Comparison of the Rab27A amino acid sequence in several species shows evolutionary conservation of G43.

Figure 3. The mutant Rab27A(G43S) induces perinuclear melanosome accumulation in normal melanocytes

Normal human melanocytes transfected with Rab27a(WT)-GFP (a–e) or Rab27a(G43S)-GFP (f–j). Phalloidin-633 visualizes the cell boundaries (b and g). Melanocytes transfected with Rab27A(WT)-GFP show a normal punctuate peripheral distribution (a) resembling that of HMB45 (against the melanosomal protein Pmel17) (c), with co-localization in the dendritic tips (d, arrow), clearly demonstrated by the high zoom image (e). Melanocytes transfected with Rab27A(G43S)-GFP show a perinuclear distribution of GFP (f, arrow) and induction of a perinuclear accumulation of HMB45 as well (h, arrow). Rab27A(G43S)-GFP and HMB45 co-localize in the perinuclear area (i, arrow), the high magnification image demonstrates this as well (j). Bars: 50 μ m

Figure 4. The mutant Rab27A(G43S) does not rescue perinuclear melanosome accumulation in GSII melanocytes

(A) Sequence chromatograms of patient 1 that is heterozygous for two nonsense mutations c. 550C>T, p.R184X (left panel) and c.598C>T, p.R200X (right panel). (B) Western blot of protein extracts from normal melanocytes and patient 1 melanocytes stained with an anti-mouse Rab27A Ab and antimouse alpha-tubulin Ab. The GSII melanocytes express a decreased amount of Rab27A protein of lower molecular weight (compare Rab27A band intensities with those of alpha-tubulin). (C) GSII melanocytes (from patient 1) transfected with Rab27A(WT)-GFP (a–e) or Rab27A(G43S)-GFP (f–j) and stained with phalloidin-633 (b and g), or antimouse HMB45 Ab (c and h). Transfection with Rab27A(WT)-GFP induced a punctuate peripheral distribution of HMB45 (c), while untransfected cells retained the perinuclear accumulation of HMB45 (c, arrows); co-localization of both markers is shown in (d, e). Transfection with Rab27A(G43S)-GFP shows a perinuclear accumulation of GFP signal (f) and HMB45 (h); both Rab27A(G43S)-GFP and HMB45 co-localize in the perinuclear area (i, j). Bars: 50 μm

Figure 5. Rab27A(G43S) does not interact with its effector Melanophilin

Coimmunoprecipitation performed on protein extracts from HEK cells transfected with Mlph-Dsred and Rab27A(G43S)-GFP, Rab27A(WT)-GFP or GFP. The immunoprecipitation was performed with the Millipore anti-mouse GFP Ab (lane 4, 6, and 8) or the Abcam anti-rabbit GFP Ab (lane 5, 7, and 9) while the blot was stained with an anti-rabbit Mlph Ab and an anti-mouse Rab27A Ab. Rab27A(WT)-GFP did pull down its effector Mlph (lane 6 and 7) while Rab27A(G43S)-GFP and GFP alone did not (lane 4, 5, 8, and 9). The same amount of each protein extract was loaded on the gel as an internal loading control (lane 1, 2, and 3).

Table 1

Rab27A sequencing primers

RaB27A-F1	gtgccatgacaaaacagctg
RaB27A-R1	ctccgtggtcactgaaaatgc
RaB27A-F2	cccgtagacatacaggttaac
RaB27A-R2	cctttgtcctctaattcctac
RaB27A-F3	ccagatggcattctcttgg
RaB27A-R3	ggccaccattctcatctcag
RaB27A-F4	ggtttaactgaccattctgc
RaB27A-R4	gtcacagaagtagagcataag
RaB27A-F5	ctgategctttgaaagcattatg
RaB27A-R5	gcattgtcaatggatggtag
RaB27A-F6	agcaaaagtcactctccttt
RaB27A-R6	gtgccatgtatcaatcataga