Characterization of the Molecular Defects in Rab27a, Caused by RAB27A Missense Mutations Found in Patients with Griscelli Syndrome*

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Received for publication, November 25, 2002, and in revised form, January 7, 2003, Published, JBC Papers in Press, January 16, 2003, DOI 10.1074/jbc.M211996200

Rab27a plays a pivotal role in the transport of melanosomes to dendritic tips of melanocytes and mutations in RAB27A, which impair melanosome transport cause the pigmentary dilution and the immune deficiency found in several patients with Griscelli syndrome (GS). Interestingly, three GS patients present single homozygous missense mutations in RAB27A, leading to W73G, L130P, and A152P transitions that affect highly conserved residues among Rab proteins. However, the functional consequences of these mutations have not been studied. In the present report, we evaluated the effect of overexpression of these mutants on melanosome, melanophilin, and myosin-Va localization in B16 melanoma cells. Then we studied several key parameters for Rab27a function, including GTP binding and interaction with melanophilin/myosin-Va complex, which links melanosomes to the actin network. Our results showed that Rab27a-L130P cannot bind GTP, does not interact with melanophilin, and consequently cannot allow melanosome transport on the actin filaments. Interestingly, Rab27a-W73G binds GTP but does not interact with melanophilin. Thus, Rab27a-W73G cannot support the actin-dependent melanosome transport. Finally, Rab27a-A152P binds both GTP and melanophilin. However, Rab27a-A152P does not allow melanosome transport and acts as a dominant negative mutant, because its overexpression, in B16 melanoma cells, mimics a GS phenotype. Hence, the interaction of Rab27a with melanophilin/myosin-Va is not sufficient to ensure a correct melanosome transport. Our results pointed to an unexpected complexity of Rab27a function and open the way to the search for new Rab27a effectors or regulators that control the transport of Rab27a-dependent vesicles.

Griscelli syndrome (GS) is a rare genodermatosis, characterized by pigmentary dilution of skin and hair, presence of large clumps of pigment in hair shafts, and accumulation of melanosomes in melanocytes. These symptoms are associated with either neurological abnormalities in type 1 GS or immunodeficiency in type 2 GS (1). Morphological and biochemical studies have shown that pigmentary disorders are related to a defect of melanosome transport in melanocytes, whereas neurological abnormalities are associated with a defect in axonal transport in neurons, and immune diseases are associated with a reduced lytic granules secretion in T lymphocytes.

GS has been mapped to 15q21, the region where MYOVA (myosin-Va gene) is located (2). Genetic and biochemical data have identified MYOVA as the first gene involved in GS. Mutations in MYOVA and in its murine counterpart are responsible for GS type 1 (2) and dilute mouse phenotype (3), respectively. Myosin-Va is a molecular motor that binds to actin filaments through its N-terminal head domain and to the cargo through its C-terminal globular tail (4–6). In melanocytes, myosin-Va participates in the transport of melanosomes to the distal part of the dendrites (7, 8). However, several GS patients had no mutation in MYOVA, suggesting that other genes may be responsible for this disease (9). Confirming this point, mutations in the Rab27a gene have been identified in patients with type 2 GS (1) and the corresponding mouse model ashen (10).

Recently the gene mutated in leaden mice, which presents the same pigmentary features as dilute and ashen mice, has been discovered. The leaden locus encodes for a synaptotagmin-like protein, Mlp (melanophilin) (11). However, to date, no human disease has been related to MLPH mutation. Very recent studies have reported that melanophilin, also called Slac2a, interacts through its N-terminal domain with GTP-bound Rab27a whereas the C-terminal domain interacts with myosin-Va (6, 12–14). Moreover, it has been shown that the myosin-Va interaction with melanophilin is dependent on the presence of the alternatively spliced exon F at the C-terminal globular tail of myosin-Va, expressed in melanocytes (6, 15, 16). Thus, melanophilin, Rab27a, and myosin-Va constitute a molecular tripartite complex that controls melanosome distal transport and dendrite targeting. A simplified scheme is shown on Fig. 1A.

Among the 18 previously described GS patients, 16 had mutations in RAB27A. Most of these mutations were nonsense or frameshift mutations leading to a premature stop codon that predicted a truncation of the C-terminal geranylgeranylation motif, which is required for correct vesicular targeting. Therefore, the resulting Rab27a proteins could not be functional.

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* This work was supported by the Institut National de la Santé et de la Recherche Médicale, CERIES, and Université de Nice Sophia-Antipolis. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: GS, Griscelli syndrome; GFP, green fluorescent protein; PBS, phosphate-buffered saline; WT, wild-type.
Alternatively, some of these mutations altered mRNA or protein stability and resulted in the absence of Rab27a protein (1). Interestingly, three GS patients had single homozygous missense mutation in RAB27A, leading to W73G, L130P, or A152P transitions that affect highly conserved residues among Rab proteins (Fig. 1B). To understand the mechanisms by which these mutations cause a defect in melanosomal transport, we have analyzed the behavior of these mutants at molecular and functional levels.

The results gathered in the present report allowed the identification of the molecular defect caused by W73G and L130P transitions in Rab27a. Neither of these mutants bind melanophilin and, consequently, cannot connect melanosomes to myosin-Va and the actin cytoskeleton. Therefore, these mutants are not able to support melanosomal transport to the dendrite tips. Although we have not been able to identify the molecular defect caused by A152P transition in Rab27a, our results point to the crucial role of alanine 152 in a Rab27a function other than GTP binding or melanophilin interaction.

MATERIALS AND METHODS

Plasmids—Rab27a was cloned into the EcoRI/BstYI sites of pGFP-N1 (Clontech). The different mutant constructs Rab27a-W73G, Rab27a-L130P, Rab27a-N133I, and Rab27a-A152P were obtained by site-directed mutagenesis (Stratagene). The myosin-Va-V5 construct was obtained by cloning the exon F-containing C-terminal tail of human myosin-Va into the pCDNA topo plasmid containing the V5 tag at the C terminus (16). The myosin-Va-GFP construct was obtained by cloning the exon F-containing C-terminal tail of human myosin-Va into the Kpn1/Stma1 sites of pGFP-C1 (16). The T7-melanophilin/slc2a construct was described previously (12).

Cell Culture and Transfection—Murine melanoma B16 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 7% fetal bovine serum and penicillin/streptomycin (100 IU; 50 µg/ml) in a humidified atmosphere containing 5% CO2 at 37 °C.

Antibodies—A polyclonal anti-melanophilin antibody was immunized by rabbits with two peptides within the C-terminal domain of mouse melanophilin and was used at 1/5000 dilution for Western blot and 1/50 dilution for immunofluorescence. Anti-Tyrp1 (PEP1) and anti-tyrosinase (PEP7) antibodies (from Dr. Hearing) were used at 1/2,000 dilution for Western blot and 1/50 for immunofluorescence. The monoclonal antibody to Tyrp1, B6G3 (provided by Dr. Parson), was used at 1/1000 dilution for Western blot and 1/10 for immunofluorescence. The monoclonal anti-Rab27a antibody (Transduction Laboratories), the polyclonal anti-Rab27a antibody (provided by Dr. Seabra), and the anti-myosin-Va monoclonal antibody (provided by Dr. Mooseker) were used at 1/2,000 for Western blot and 1/100 for immunofluorescence. The monoclonal antibodies to GFP (Chemicon), to V5 tag (Stratagene), and to T7 tag (Novagen) were used at 1/3,000 for Western blot. Fluorescein isothiocyanate-conjugated goat anti-rabbit antibody and Texas Red-conjugated goat anti-mouse antibody were used at 1/1,000 (Molecular Probes), and peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies (Dako) were used at 1/4,000 dilution.

Immunofluorescence Study—B16 cells cultured on glass coverslips were washed three times in PBS, fixed for 20 min in 3% paraformaldehyde, washed again, and incubated for 10 min in 50 µm NH4Cl after three more PBS washes. Cells were incubated with the primary antibody diluted in PBS containing 0.5% bovine serum albumin and 0.02% saponin for 1 h, washed abundantly in PBS, and incubated for another hour with the appropriate conjugated secondary antibody. After washing, coverslips were mounted on glass slides and viewed either with an Axioshot fluorescent microscope (Zeiss) or a confocal microscope (Leica).

Immunoblotting—For Western blot analysis, samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). Membranes were saturated in a saline buffer containing 5% nonfat dry milk and then incubated with the corresponding primary antibody diluted in the saturation buffer for 1 h at room temperature. After three 10-min washes in the buffer containing 0.05% Triton X-100, 0.5% nonfat dry milk in a saline buffer, blots were incubated for 1 h at room temperature with the corresponding peroxidase-conjugated secondary antibody and washed again as described. The antigen/antibody complex was detected with the ECL kit (Amersham Biosciences).

GTP Binding Assays—Human embryonic kidney 293 cells transfected with the different GFP-Rab27a constructs were scraped in a lysis buffer containing 50 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P-40, and a mixture of protease inhibitors. Lysates were incubated with protein A-Sepharose beads coated with a polyclonal anti-GFP antibody. After 1 h at 4 °C, the beads were washed four times in the same lysis buffer and twice in a binding buffer containing 20 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, and 0.5% bovine serum albumin. For each condition, beads were resuspended in the binding buffer containing 10 µCi of [3H]GTP and divided in four identical samples. 1 µl of unlabeled GTP was added in two samples to evaluate the nonspecific binding. After 2 h of incubation at room temperature, the beads were washed six times in the binding buffer and re-suspended in water, and the associated radioactivity was counted. Total lysates (30 µg of proteins) were analyzed by Western blot to evaluate the expression level of the mutants.
Molecular Defect in Rab27a Mutants

RESULTS

Rab27a, Melanophilin, and Myosin-Va Associate with Melanosomes—First, we studied the expression and localization of melanophilin in B16 mouse melanoma cells. We performed double immunofluorescence experiments using an antibody to melanophilin, together with antibodies to Rab27a, myosin-Va, and to Tyrp-1 to identify melanosomes (Fig. 2). Melanophilin was strongly expressed throughout the cell with a marked pattern was similar to the one observed for Tyrp-1 (Fig. 2, c). Arrowheads indicate the co-localization in discrete cytoplasmic structures. Upper panels (a–c) correspond to immunofluorescence microscopy (bar, 20 μm); lower panels (d–f) correspond to confocal microscopy (bar, 3 μm). D, B16 cells were lysed in buffer without detergent and subjected to immunoprecipitation with pre-immune serum (Mlph), or myosin-Va. These data demonstrate that melanosome transport was blocked in more than 90% of the cells expressing Rab27a-Q78L. As it is the case for Rab27a-N133I, the diffuse distribution of Rab27a-Q78L, which is in a GTP-bound state, showed that melanosome transport was blocked in more than 80% of the transfected cells (white arrows). The merged images showed a diffused co-localization of Rab27a-N133I with melanosomes (Fig. 3B, f). In transfected cells, melanosomes (Fig. 3B, f) and melanophilin (Fig. 3B, h) labeling appeared predominantly localized in the cell body without accumulation at dendrite tips as observed in more than 80% of the non-transfected cells (white arrows). The merged images showed a diffused co-localization of Rab27a-N133I with melanosomes (Fig. 3B, c, e, and f). In transfected cells, melanosomes (Fig. 3B, c, e, and f) and melanophilin (Fig. 3B, h) labeling seemed to be predominantly localized in the cell body without visible vesicular structures. Thus, Rab27a-N133I acts as a dominant negative mutant, because analysis of 200 transfected cells showed that melanosome transport was blocked in all the cells expressing this mutant.
cytoskeleton. This GFP-tagged construct clearly (Fig. 3D, a, d, and g) co-localized with vesicular structures at the perinuclear area of the cell. Accumulation of melanosomes (Fig. 3D, b), Rab27a (Fig. 3D, e), and melanophilin (Fig. 3D, h) to the dendrite extremities was affected in transfected cells. The vesicular structures decorated by myosin-Va tail were also labeled by antibodies to Tyrp-1 (Fig. 3D, c), Rab27a (Fig. 3D, f), and melanophilin (Fig. 3D, i). These results indicate that overexpression of non-functional Rab27a or myosin-Va proteins impairs melanosome and melanophilin transport to the distal part of the dendrites in B16 melanoma cells expressing endogenous wild-type Rab27a.

Overexpression of Rab27a Mutants, Found in Three Patients with Type 2 Griscelli Syndrome, Affects the Subcellular Distribution of Melanosomes, Myosin-Va, and Melanophilin—Next we investigated the behavior of three Rab27a mutants, W73G, L130P, and A152P, found in type 2 GS patients. GFP-Rab27a-W73G presented a diffuse intracellular localization. No peripheral labeling of dendrite extremities was observed (Fig. 4A, a, d, and g). In all the cells overexpressing this mutant, we did not observe major changes in the distribution of melanosomes, myosin-Va, or melanophilin to dendrite tips (Fig. 4A, b, e, and h). As shown by the merged images, melanosomes, myosin-Va, and melanophilin did not co-localize with Rab27a-W73G, especially at the cell periphery (Fig. 4A, c, f, and i). The mutants Rab27a-L130P (Fig. 4B, a, d, and g) and Rab27a-A152P (Fig. 4C, a, d, and g) also showed a diffuse cytoplasmic pattern that overlapped with of melanosomes, myosin-Va, and melanophilin labeling (Fig. 4, B and C, c, f, and i). However, in contrast with Rab27a-W73G, both Rab27a-A152P and Rab27a-L130P blocked the transport of melanosomes, myosin-Va, and melanophilin to dendrite tips (Fig. 4, B and C, b, e, and h). These results have been observed in more than 90% of the cells overexpressing these mutants. Thus the L130P and A152P mutations in Rab27a produce dominant interfering Rab27a proteins that impair the function of endogenous Rab27a and block melanosome transport to dendrite extremities when overexpressed in B16 melanoma cells.

GTP Binding Activity and Subcellular Localization of the Different Rab27a Mutant Proteins—Because Rab GTPases bind to their effectors when they are in their GTP-bound state, we studied the GTP binding capacity of the different Rab27a mutants. Therefore, we transfected human embryonic kidney 293 cells with the different GFP-Rab27a mutant constructs. Transfected Rab27a proteins were immunoprecipitated with an anti-GFP polyclonal antibody, and the immunoprecipitates were incubated with [35S]GTP as described under “Materials and Methods.” The results shown in Fig. 5 indicate that Rab27a-WT, as well as Rab27a-Q78L, bound GTP very efficiently. The GS mutants Rab27a-W73G and Rab27a-A152P were also capable to bind GTP. As expected, the dominant negative mutant Rab27a-N133I was unable to bind GTP. Interestingly the GS mutant Rab27a-L130P presented a very low GTP binding capacity.
Association of Rab27a Mutant Proteins with Myosin-Va and Melanophilin—Rab27a connects melanosomes to the actin network through its interaction with melanophilin and myosin-Va. Hence, we investigated the ability of Rab27a mutants to interact with melanophilin and myosin-Va. To address this point, we co-transfected B16 melanoma cells with each of the GFP-Rab27a mutants, together with either a plasmid encoding a V5-tagged C-terminal fragment of myosin-Va containing exon F (Fig. 6A) or a plasmid encoding a T7-tagged melanophilin (Fig. 6B). The cell extracts were immunoprecipitated using a polyclonal antibody to GFP. Then, total cell extracts (T) or immunoprecipitates (IP) were analyzed by Western blot with monoclonal antibodies to GFP (Fig. 6, A and B, lower panel), to V5 tag (Fig. 6A, upper panel), or to T7 tag (Fig. 6B, upper panel).

We observed that Rab27a-WT, as well as the Rab27a-Q78L mutant and the GS mutant, Rab27a-A152P, co-precipitated with the myosin-Va tail at 80 kDa and with melanophilin at 70 kDa. In contrast, the dominant negative mutant Rab27a-N133I and the GS mutants Rab27a-W73G and L130P co-precipitated neither with myosin-Va nor with melanophilin.

**DISCUSSION**

Rab27a, which links melanosomes to the actin network, plays a crucial role in melanosome transport. Therefore, it is easy to understand that in the absence of Rab27a, melanosomes cannot be transported and accumulated at dendrite tips. However, three patients with type 2 GS have missense mutations in RAB27A that allow the expression of the wild-type Rab27a.
tein but not the targeting of melanosomes to dendrite tips, because these patients have a pigmentedary dilation (17). To gain more information on the role and the function of Rab27a in melanosome transport, we performed a detailed study of Rab27a mutants W73G, L130P, and A152A found in type 2 GS patients.

In the first part of this report, we performed a basic study of melanosomes and associated molecules in B16 melanoma cells. Melanosomes labeled with an anti-Tyrp1 antibody, as well as Rab27a and myosin-Va, showed a strong co-localization with melanosomes. Furthermore, intact melanosome immunoprecipitation demonstrated a physical association between these vesicles and Rab27a, melanophilin, or myosin-Va. As demonstrated previously (6, 15, 18) in other melanocyte cell systems, we observed that the overexpression of the myo- sin-Va C-terminal tail acts as a dominant negative mutant that impairs melanosome recruitment at dendrite extremities. Indeed, this myosin-Va tail construct is able to bind melanosomes, but not to actin, and thus cannot support the recruitment of melanosomes at dendrite tips by peripheric actin filaments. Transfection of Rab27a mutants N133I and Q78L also induced a mislocalization of melanosomes. The effect of Rab27a-N133I, which has been described as a dominant negative, GDP-bound Rab27a mutant (19), was somehow expected. However, the effect of the Q78L mutant was more intriguing. This mutant was supposed to be a GTP-bound Rab27a and act as a constitutive active mutant. Indeed, we demonstrated that Rab27a-Q78L bound GTP and melanophilin but was not targeted to the membrane compartment (not shown) and thus cannot reach the melanosome. This mutant acts as a dominant negative mutant by preventing the interaction of the endogenous Rab27a with its effector, melanophilin.

Interestingly, we observed that overexpression of the myo- sin-Va tail also impairs Rab27a and melanophilin localization at dendrite tips. These results are in agreement with the perinuclear re-localization of Slp2, another Rab27a effector member of the melanophilin/slac2a family, observed in Ser-91 cells derived from dilute mice (13). In the same way, overexpression of Rab27a-N133I and Rab27a-Q78L induced a relocation of melanophilin and myosin-Va to the perinuclear area. These observations indicate that the tripartite molecular complexes are transported together with melanosomes to the dendrite tips and suggest that the interaction of Rab27a/melanophilin with myosin-Va would allow or favor the interaction of the molecular motor with the actin network.

Next, we focused our attention on Rab27a mutants found in GS patients. The proximity of Leu-130 and Asn-133 residues incited us to predict that both mutants would have the same behavior. Nevertheless, the sequence comparison between Rab27a and Rab3a shows that Leu-130 in Rab27a is situated in very close proximity to the third GTP binding site, but it is not exactly situated within this consensus sequence. The overexpression of the Rab27a-L130P mutant blocked melanosome, melanophilin, and myosin-Va transport to the dendrite tips. This mutant was unable to bind GTP and did not associate with melanophilin or myosin-Va. Therefore, Rab27a-L130P cannot connect its cargo with the actin network. Further, Rab27a-L130P did not show a vesicular distribution, thus suggesting that it might not be targeted to the melanosome membrane. Rab27a-L130P, like Rab27a-N133I, acts as a dominant negative mutant of Rab27a, because both mutants are predominately in a GDP-bound form and are supposed to bind avidly to their exchange factor, thereby impairing the activation of the endogenous Rab27a.

Further, the Trp-73 is in the vicinity of the Gln-78 that plays a crucial role in the GTPase activity of Rab27a. This observa-
fect and in B16 melanoma cells that express endogenous wildtype Rab27a. The dominant negative effect can be observed only when the mutants are strongly overexpressed. Indeed, Griscelli syndrome is a recessive genodermatosis. In heterozygous subjects, the expression of one Rab27a wild-type allele is sufficient for correct melanosome transport.

Interestingly, it should be noted that the patient with the mutation A152P developed the first episode of hemaphagocytic syndrome at 8 years old, whereas in most of the other patients with Rab27a mutations, the first episode occurred before 6 months (1). Thus, the A152P mutation in Rab27a might be less detrimental for the immune homeostasis. This Rab27a region would therefore play a more important role in melanosome transport than in cytotoxic granule exocytosis, suggesting that melanocyte- or melanosome-specific effectors could interact with this region. At present, no experimental results have brought evidence for this hypothesis, and further studies will be needed to understand the mechanisms by which the A152P mutation within the Rab27a protein leads to the observed clinical and biological defects.

In summary, the study of these three GS Rab27a mutants allows us to explain the molecular mechanisms that lead to the abnormal pigmentation phenotypes of these GS patients. Further, our results point to an unexpected complexity of the Rab27a function, because, at least for the Rab27a-A152P mutant, interaction with melanophilin/myosin-Va, GTP binding is not sufficient to ensure a correct melanosome transport. These observations open the way to the search for new Rab27a effectors or regulators that might control the transport of Rab27a-dependent vesicles.

Acknowledgment—We are grateful to Dr. Bertolotto for critical reading of the manuscript.

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