Multiple Factors Contribute to Inefficient Prenylation of Rab27a in Rab Prenylation Diseases*

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Post-translational geranylgeranylation of Rab GT-Pases is essential for their membrane association and function as regulators of intracellular vesicular transport. The reaction is catalyzed by Rab geranylgeranyl-transferase (RGGT) and is assisted by the Rab escort proteins (REP), which form stable complexes with newly synthesized GDP-bound Rabs. Two genetic diseases involve the Rab geranylgeranylation machinery: choroideremia, an X-linked retinal degeneration resulting from loss-of-function mutations in REP1, and gunmetal, a mouse model of Hermansky-Pudlak syndrome resulting from mutations in the a-subunit of RGGT. A small subset of Rab proteins is selectively under-prenylated in both diseases, most notably Rab27a. Here we analyze why Rab27a is selectively affected in diseases of Rab geranylgeranylation. Semi-quantitative immunoblotting suggests that mass action, i.e. the amount of Rab27a relative to other Rabs, is unlikely to be a factor as the expression level of Rab27a is similar to other Rabs not affected in these diseases. In vitro binding assays and fluorescence resonance energy transfer detected by fluorescence lifetime imaging microscopy in intact cells demonstrate that Rab27a binds equally well to both REP1 and REP2, suggesting differential affinity of Rab27a for REP isoforms is not an important factor. However, steady-state kinetic analysis of the geranylgeranylation reaction indicates that REP2-Rab27a has lower affinity for RGGT compared with REP1-Rab27a. Furthermore, we show that Rab27a has relatively low GT-Pase activity, presumably decreasing the affinity of the REP interaction in vivo. We suggest that the restricted phenotypes observed in these diseases result from multiple contributing factors.

Protein prenylation is a common post-translational lipid modification, which consists of the covalent addition of the C15 farnesyl or more commonly the C20 geranylgeranyl to C-terminal cysteine residues in specific cellular substrates (1–3). The largest family of prenylation substrates is the Rab GTPases, which consist of the major GTPases, Rab5, Rab7, Rab10, and Rab27a. Functional Rab27a associates with specific subcellular compartments where they regulate trafficking pathways (4–6). Geranylgeranylation of Rabs involve a series of steps in a complex reaction that is incompletely understood (2). The reaction is catalyzed by a heterodimeric enzyme, Rab geranylgeranyltransferase (RGGT). The protein substrate for RGGT is a 1:1 complex between any given newly synthesized Rab protein and Rab escort protein (REP) (7, 8). RGGT first binds the lipid substrate, geranylgeranyl pyrophosphate, then binds the REP-Rab complex (8). Alternatively, RGGT binds REP first and then Rab binds the REP-RGGT-GGPP complex (9). RGGT then catalyzes the transfer of geranylgeranyl groups to one or two cysteines present in the C termini of Rab proteins (10). Because the enzyme may bind only one lipid at once, the addition of two geranylgeranyl groups to Rabs bearing two cysteines is likely to be sequential (8, 11–14). After lipid transfer, the enzyme dissociates from the REP-Rab complex, and REP is believed to deliver the prenylated Rab to cellular membranes (15, 16).

Defects in Rab prenylation cause genetic disease (17, 18). Choroideremia (CHM) is an X-linked slow-onset retinal degeneration affecting the retinal pigment epithelium (RPE), the choroid, and the photoreceptors, which results from loss-of-function mutations in REP1 (19). REP function in CHM cells is provided by REP2, a protein functionally redundant with REP1. Nevertheless, REP2 is not able to fully compensate for the loss of REP1, and a selected subset of Rab proteins is found under-prenylated in CHM cells (20, 21). The most prominent protein was subsequently identified as Rab27a and shown to be highly expressed in the same layers of the rat eye that are affected by CHM (20). These observations suggested that defects in Rab27a function, resulting from the loss of REP1, might underlie the retinal degeneration observed in CHM.

Another disease in which Rab prenylation is affected is the gunmetal (gm) mouse, a model for Hermansky-Pudlak syndrome. The gm mutation was identified as a Gly to Ala substitution in a splice acceptor site in the gene encoding the murine RGGT a-subunit (22). As a consequence, the major RGGT transcript in the gm mutant lacks a start codon and is nonfunctional. However, the gm mutant contains a reduced level of RGGT activity due to the low frequency activation of a cryptic splice acceptor site within RGGT-a RNA, which results in the production of a full-length transcript. This observation sug-

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1 The abbreviations used are: RGGT, Rab geranylgeranyltransferase; REP, Rab escort proteins; CHM, choroideremia; DTT, dithiothreitol; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; EGFP, enhanced GFP; GMPP, geranylgeranyl pyrophosphate; FLIM, fluorescence lifetime imaging microscopy; Bicine, N,N-bis(2-hydroxyethyl)glycine; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.
gested that deficiency of Rab(s) prenylation results in the Herms-}

Pudlik syndrome phenotype, an hypothesis remarkably

similarly to one proposed for CHM. Consistent with this

possibility, a subset of RabS accumulate unprenylated in the

gm cytosol, including Rab27a (22, 23). Interestingly, Rab27

isofoms are highly expressed in the tissues affected in the

gm mouse suggesting that the gm phenotype is at least in part

result of reduced Rab27 function.

In the present study, we analyze why Rab27a is selectively

affected in diseases of Rab geranylgeranylation. We dem-

onstrate that Rab27a binds equally well to both REP1 and

REP2. However, the REP2-Rab27a substrate has lower affinity for

RGGT. Further, we show that Rab27a is likely to be mostly

GTP-bound due to low intrinsic GTPase activity, pre-

sumably decreasing the affinity of the REP interaction in vivo.

EXPERIMENTAL PROCEDURES

Constructs—Bacterial expression constructs pET14b-Rab1a (24),
pET14b-Rab27a (20), and pET14b-K-Ras (25) were described previ-

ously. The chimeric Rab1a-loop in pET14b (pET14b-Rab1a-li) was con-

structed by inserting a sequence encoding a 10-amino acid stretch

(residues 54–63 of rat Rab27a) between residues 56 and 57 of canine

Rab1a using PCR mutagenesis in two stages. The flanking primers

tained an NdeI restriction site (primer 1, 5′-GCTCTAGACATG

TCCAGCATGAATCCCGAATATGAT) and a BamHI restriction site

(primers 1, 5′-TTAAGETTGGATCCCTGGAACCTCCCTACGGAC

TGCTT) for cloning into pET14b, and the inner primers contained the

extra sequence (primer 3, 5′-GGCATGGTGCTACCATCCGCACTTG

GCGATGGGAAAACAAATCAGCATCATCTTCAAAT). The chimeric Rab27a-loop out in pET14b (pET14b-

Rab27a-lo) was constructed by deleting the sequence encoding 10 amino

acids corresponding to residues 54–63 of canine Rab27a and intro-

ducing an NdeI restriction site (primer 5, 5′-GCTC

CTT) and a BamHI restriction site (primer 6, 5′-TTAAGCTTGATCCCTGCAACCATG

GCCCTTCTTCTTCT) for cloning into pET14b, and the inner primers contained a SnaB1 restriction site

(primers 5′, 5′-GGAGCTTGCTACTAGGTAGAGATAGTAC

CTCCATGGATGGGAAAACAAATCAGCATCATCTTCAAAT). A detailed description of the FRET monitored by

FLIM detection in the frequency (phase) domain can be found elsewhere

(31, 32). Phase methods provide an average lifetime where sinusoidally

modulated light is used to excite the sample. The lag in the emitted

fluorescence signal permits measurement of phase (τp) and modulation

depth (τm) of the fluorescence. The lifetime, τ, is the average phase shift and relative modulation depth (τp + τm)/2 of the emitted fluorescence signal. The average EGFP lifetime without acceptor (τp) was calculated from 10 cells per experiment, and the average EGFP lifetime with acceptor (τp + τm) was deduced from 7 cells per experiment. Each experiment was repeated three times. The cumulative lifetimes of EGFP-Rab27a, EGFP-Rab1a, and EGFP-Hras alone and that measured with acceptor fluorophore were plotted on two-dimensional histograms. The average population variation, a concomitant decrease in τp and τm, indicates a reduction in EGFP lifetime due to FRET. GraphPad InStat software (version 3.0 for Mac-2001) was used for the statistical analysis. A two-tailed t test with Welch correction was used to determine the significance of variation in lifetime (7 or more cells were analyzed per experiment). The cumulative lifetimes of EGFR-Rab27a, EGFR-Rab1a, and EGFR-Hras alone and that measured with acceptor fluorophore were plotted on two-dimensional histograms. The average population variation, a concomitant decrease in τp and τm, indicates a reduction in EGFP lifetime due to FRET. GraphPad InStat software (version 3.0 for Mac-2001) was used for the statistical analysis. A two-tailed t test with Welch correction was used to determine the significance of variation in lifetime (7 or more cells were analyzed per experi-
mantion of appropriate horseradish peroxidase-conjugated secondary anti-

body (Dako) diluted in solution 1 and washing as before. Bound anti-

body was detected using the Western Supersignal System (Pierce).

Blots were calibrated with prestained molecular weight standards (Bio-

Rad). For immuno-fluorescence, coverslip-grown cells were washed

in PBS and then fixed in 3% paraformaldehyde in PBS for 15 min. Excess

fixative was removed by washing with PBS; cells were permeabilized by

incubation with 0.2% Triton X-100 for 5 min and washed as before. Cells

were then incubated in 1 mg/ml sodium borohydride in PBS for 10 min to

quench and washed twice with PBS and once with PBS containing 1%

BSA (PBS/BSA). Cells were then incubated with 0.22-μm filtered Cy3-

conjugated J905 anti-REP rabbit polyclonal antibody diluted in PBS/

BSA for 2 h. Coverslips containing fixed cells were then mounted in

Innomo Fluor medium (ICN).

FRET Methods—A detailed description of the FRET monitored by

FLIM detection in the frequency (phase) domain can be found elsewhere

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corded at a modulation frequency of 80.218 MHz. The GFP-Rab/Ras was excited using the 488 nm line of an argon/krypton laser and the

fluorescence signal at 500–550 nm was imaged using a 1.4 numerical aperture, phase 3 oil objective, with images re-
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fluorescence signal at 500–550 nm was imaged using a 1.4 numerical


REP1 and REP2 were KD and 3.6

Instrument Co.).

exposing for 2 h. A Cyclone storage phosphor multipurpose screen and developing solvent. Radioactive GTP and GDP spots were revealed by

bated in buffer A containing 250 mM ammonium sulfate for 10 min at room temperature with gentle agitation. The beads were then incubated in buffer A (20 mM Tris-HCl, pH 8, 2 mM EDTA, 1 mM DTT, 5 mM GTP, 5 mM GDP) by heating at 65 °C for 2 min. Eluted nucleotides were separated by thin layer chromatography on polyethylenimine-cellulose sheets (J. T. Baker Inc.) with 0.75 M KH₂PO₄, pH 3.5, as the developing solvent. Radioactive GTP and GDP spots were revealed by exposing for 2 h on a Cyclone storage phosphor multipurpose screen and quantified using the Cyclone storage PhosphorImager System (Packard Instrument Co.).

RESULTS

At the outset, we tested whether Rab27a accumulated in an unprenylated state in CHM cells due to differences in its ability to bind REP1 and REP2. We first measured the affinity of Rab27a toward REP1 and REP2 by using an in vitro fluorescence assay. In this assay, recombinant Rabs were fluorescently labeled with dansyl chloride. The binding of REP to dansylated-Rabs changed the local chemical environment and resulted in fluorescence emission quenching. Fig. 1 shows the binding curves for Rab27a and Rab1a for REP1 and REP2.

The observed dissociation constants (KD) between Rab27a and REP1 and REP2 were KD REP1 = 223 ± 51 nM and KD REP2 = 240 ± 39 nM, respectively. The KD values for Rab1a were not significantly different from Rab27a (KD REP1 = 106 ± 39 nM; KD REP2 = 229 ± 47 nM). These values are similar and suggest that the affinities of Rab1a and Rab27a for REP1 and REP2 are not significantly different.

To examine the interactions in intact cells between REPs and Rab27a, we exploited FRET measured by fluorescence lifetime imaging microscopy (FLIM). Fig. 2 illustrates the specific interaction of REP and Rab proteins in intact COS-7 cells. Cells were transiently transfected with EGFP-Rab1a or EGFP-Rab27a. The fixed cells were then labeled with Cy3-conjugated J905 (anti-REP antibody which recognizes both REP1 and REP2), and the fluorescence lifetime of EGFP-Rab (donor) was measured with and without Cy3-J905 (acceptor). In the presence of acceptor, the average EGFP-Rab1a and EGFP-Rab27a lifetimes decreased from 2.10 to 1.72 ns and 2.00 to 1.65 ns, respectively. Moreover, the two-dimensional histograms illustrate a simultaneous decrease in both the τo and τm indicating a reduction in EGFP lifetime due to FRET (Fig. 2C). Parallel experiments were performed with EGFP-Hras as a specificity control. The EGFP-Hras lifetime remained unchanged with or without Cy3-J905, suggesting that the results obtained with the Rab proteins were specific.

Concomitantly, experiments were performed with CHM fibroblasts (Fig. 3). CHM fibroblasts exhibit loss-of-function mutations in REP1 and do not express detectable amounts of REP1 (29). In these cells, we observed a similar extent of decrease in EGFP-Rab27a lifetime (2.1 to 1.5 ns) and EGFP-Rab1a lifetime (2.1 to 1.55 ns) in the presence of Cy3-J905 acceptor. The significant decrease in EGFP lifetime due to FRET is also represented by two-dimensional histograms (Fig. 3C). Furthermore, no changes were detected with EGFP-Hras as observed in COS-7 cells. Altogether, these results suggest that the affinity of Rab27a for REP1 is similar to its affinity to REP2 in vitro and in intact cells. Furthermore, Rab27a exhibits comparable affinities to other Rabs for REPs (8).

These results indicate that differences in the affinity of Rab27a versus Rab1a for the REP proteins do not explain the accumulation of non-prenylated Rab27a in the cytosol of CHM patient lymphoblasts. Thus, another possibility is that Rab27a is expressed at much higher levels than other Rabs in lymphoblastoid cells, and thus its selective accumulation could be explained by mass action. We undertook comparative immunoblotting of Rab proteins in control and CHM lymphoblast cell lines by using antibodies reactive to Rab27a, Rab5a, and Rab6a together with known amounts of recombinant hexahistidine-tagged Rab proteins. By using this semi-quantitative technique, we observed that Rab27a was not significantly over-ex-
pressed relative to Rab5 or Rab6 in lymphoblast cells indicating that the accumulation of unprenylated Rab27a in CHM lymphoblasts is not simply due to the high expression of Rab27a.²

We then considered the possibility that the REP2-Rab27a complex could be a poorer substrate for RGGT. By using an established in vitro prenylation assay (8, 36), we determined the steady-state kinetic parameters for the substrate complexes REP1 or REP2 in complex with either Rab27a or Rab1a (Fig. 4, A and B). Table I summarizes the kinetic parameters obtained. Our results indicate that the $K_m$ value for the REP1-
Rab27a is significantly lower than either REP2-Rab27a or any REP-Rab1a complex. The differences in $K_m$ values obtained for Rab27a in complex with REP1 and REP2 were highly reproducible, consistent with a previous result (20), and suggest that the REP1-Rab27a is a better substrate for prenylation than REP2-Rab27a. We hypothesized that a 10-amino acid insert present in loop 3 of Rab27 proteins (residues 54–63 in human Rab27a), but not in other Rabs, could be responsible for this effect. We prepared two mutant recombinant proteins, one where the loop was deleted in Rab27a (Rab27a loop-out) and one where the Rab27a loop was inserted into Rab1a (Rab1a loop-in). The kinetic analysis is shown in Fig. 4 and Table I. The significant changes in the kinetic parameters induced by the mutagenesis were a decrease in the $K_m$ value of Rab1a loop-in compared with Rab1a and an increase in $V_{\text{max}}$ value of Rab27a loop-out compared with Rab27a. These results suggest that the loop insert in Rab27a affects the affinity of the REP-Rab27a complex for RGGT.

REPs binds preferentially Rabs in the GDP-bound state, but Rabs are presumably GTP-bound after synthesis (24). Therefore, intrinsic GTP hydrolysis rates of Rab proteins could affect the efficiency of prenylation. To test this possibility, we analyzed the intrinsic rate of GTP hydrolysis using an established thin layer chromatography assay (Fig. 5). We used Rab5a and Rab3a as controls given the extensive data available on the enzymatic properties of these Rabs. At 37°C, we obtained a rate of 0.164 min$^{-1}$ for Rab5a, 0.082 min$^{-1}$ for Rab3a, and 0.0058 min$^{-1}$ for Rab27a (Fig. 5). These data suggest that Rab27a exhibits a slow rate of intrinsic hydrolysis.

DISCUSSION

The molecular basis of CHM and other genetic diseases affecting Rab prenylation appears to result from defects in prenylation of a subset of Rabs, most notably Rab27a. In this paper we suggest two reasons that might contribute to the selectivity of the defect. One is that Rab27 is among the slowest hydrolyzing Rabs, suggesting that a high proportion of newly synthesized Rab27a is in the GTP-bound form and therefore not able to bind to REP. The second reason is that the REP1-Rab27a complex is recognized by RGGT with greater affinity.
than REP2-Rab27a. In the absence of REP1 in CHM, all Rab27a is processed by REP2.

We have hypothesized previously that REP1 exhibited higher affinity for Rab27a than REP2, but the present results do not support this possibility (19). We used a fluorescence-based in vitro binding assay for REP and Rab and determined the association constants for the interaction between REP1 and REP2 and different Rab proteins. Analysis suggests that both REP1 and REP2 exhibit the same affinity toward Rab27a and, furthermore, that the affinity for Rab27a is similar to that of other Rabs such as Rab1a. The values measured using this assay were comparable with those obtained previously using another fluorescence-based in vitro binding assay (8).

In vivo studies using FRET detected by FLIM confirmed the results obtained in the in vitro binding assays. We were able to detect FRET of similar efficiency (reflected by a comparable variation in the donor (GFP) lifetimes) between REP1/2 and Rab1a, and REP1/2 and Rab27a in COS-7 cells. Furthermore, we used a CHM cell line, which only expresses REP2 to test specifically the interactions between REP2 and Rab27 and REP2 and Rab1a detected by the variation in donor lifetimes. We then studied the kinetics of the prenylation reaction. We found that the REP1-Rab27a substrate exhibited a higher affinity for RGGT than any other substrate analyzed to date. In contrast, the REP2-Rab27a substrate exhibited an affinity comparable with other REP-Rab complexes studied previously. Interestingly, the addition of a stretch of 10 amino acids characteristically present in the putative loop 3 of Rab27a into the corresponding location in Rab1a altered the kinetics of the fusion protein. The Rab1 loop-in substrate exhibited lower \( K_m \) values than Rab1 wild type, suggesting that this stretch of amino acids is involved in the recognition by RGGT. Conversely, the removal of this 10-amino acid stretch from Rab27a increased the apparent \( K_m \) value for the reaction.

These differences may account for the selective defect in CHM but not for those in gm where Rab27a under prenylation also features pre-eminently. In gm cells REP1 and REP2 expression is unaffected, instead it is a quantitative reduction in the amount of RGGT that leads to the prenylation defect. Here we tested the hypothesis that the intrinsic hydrolysis rate could affect prenylation efficiency, given that we have previously established that REPs have a marked affinity for the GDP-bound state of Rabs (24). Our analysis suggests that, among Rabs, Rab27a has one of the slowest rates of GTP hydrolysis. Similar GTPase rates were observed previously for Rab7 and Rab9 (37, 38). We suggest that in vivo, in the absence of other factors, e.g. Rab GAPs, Rabs that...
have slow intrinsic GTP hydrolysis rate may be disadvantaged in their ability to become prenylated relative to other, faster hydrolyzing, Rabs. Interestingly, Rab7 has been reported to bind REP in vitro with higher affinity than Rab27a (39). This may explain why other slow hydrolyzing Rabs do not accumulate unprenylated in CHM patients. Future studies will be required to further understand the events leading to REP-Rab complex formation in vitro. Altogether, our in vitro and in situ studies suggest that the molecular basis for the selective under-prenylation of certain Rabs in genetic disease associated with defects in Rab prenylation machinery are complex and probably multifactorial.

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