The C-terminus of prenylin is important in forming a dimer conformation necessary for endoplasmic-reticulum-to-Golgi transport

Zhimin LIANG, Helga VEERAPRAME, Nami BAYAN and Guangpu LI[1]

Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, 940 S.L. Young Blvd., BMSB 853, Oklahoma City, OK 73104, U.S.A.

Prenylin [or prenylated Rab acceptor 1 (PRA1)] is a multi-pass transmembrane protein that initially inserts into the ER (endoplasmic reticulum) membrane, followed by vesicular transport along the exocytic pathway to the Golgi complex where it may regulate the functions of prenylated proteins. Deletion of the C-terminal 10 amino acid residues of prenylin blocks its export from the ER. We have employed site-directed mutagenesis to investigate the role of each of the C-terminal 10 residues in the ER export of prenylin. This region contains a di-acidic motif (Asp^{176}-Xaa-Glu), but changing either acidic residue to alanine has no effect on the ER export of prenylin. Alanine-scanning mutagenesis of the entire C-terminal region reveals that only the very C-terminal Val^{185} residue is crucial for the ER export of prenylin. Changing the C-terminal Val^{185} to most other amino acids effectively prevents prenylin from exiting the ER. However, deletion of Val^{185} has only moderate effect on the ER export of prenylin, suggesting that this valine residue is not part of an export signal itself; instead, it may affect the folding and conformation of prenylin. We show that the wild-type prenylin can efficiently form a homodimer in the cell by using a cell-permeant cross-linker, whereas the large C-terminal truncation and Val^{185} mutants are defective in forming such a dimer. Thus we have identified a single C-terminal valine residue that is essential for the proper dimerization and ER export of prenylin.

Key words: di-acidic motif, endoplasmic reticulum export (ER export), prenylated Rab acceptor 1 (PRA1), prenylin, protein trafficking, Yip3.

INTRODUCTION

Prenylin [or prenylated Rab acceptor 1 (PRA1)] interacts with prenylated proteins, such as the small GTPases Rab and Ras, and is suggested to regulate their functions in protein transport and signal transduction [1–4], but a detailed mechanism is yet to be established. Studies on prenylin biosynthesis have revealed that it is primarily a Golgi membrane protein [3,5] and may appear in late endosomes [6]. Similarly to other Golgi proteins, it is initially inserted into the ER (endoplasmic reticulum) membrane and is then transported to the Golgi complex via the exocytic pathway. Prenylin is a 20-kDa transmembrane protein that contains four transmembrane segments and adopts a membrane topology with both N- and C-terminal domains in the cytoplasm [7]. However, it is unclear which domain is important for the efficient ER export of prenylin and whether there is an ER export signal in the prenylin sequence.

Two mechanisms have been proposed to explain how proteins are exported from the ER. The first mechanism suggests that ER export is a bulk-flow process [8], and any properly folded/modified protein will be exported without the need for an ER export signal. The second mechanism suggests that proteins to be exported from the ER contain an export signal, such as the di-acidic motif (Asp-Xaa-Glu) found in the cytoplasmic domain of the vesicular-stomatitis virus glycoprotein [9,10]. Previous studies indicate that sequences adjacent to the di-acidic motif are also important for ER export [11], and that an expanded version of the signal plays a role in exporting potassium channels from the ER [12]. However, not all exported proteins contain such an ER export signal, and a compromising view suggests that only minor protein species contain a specific signal for efficient ER export and abundant protein species may exit the ER via the bulk-flow mechanism [13].

To determine if there is a sequence in prenylin involved in its ER export, we truncated the C-terminal 10 residues of prenylin and found that the resulting truncation mutant ($prenylin^{\Delta C10}$) failed to exit the ER. The C-terminal 10 residues of prenylin start with a di-acidic motif Asp^{176}-Gly-Glu, followed by Glu-Leu-Gln-Met-Glu-Pro-Val^{185}. We have used alanine-scanning mutagenesis to determine which of the C-terminal residues is important for the ER export of prenylin. In contrast with a previous report [5], mutations at the di-acidic motif have no effect on ER export. We have thus investigated further the mechanism by which the C-terminal domain of prenylin controls the ER export and found that a dimer conformation involving the ultimate C-terminal residue is essential for prenylin to exit the ER.

EXPERIMENTAL

Plasmids

The mouse prenylin cDNA was cloned at the *Bam*HI restriction site of the plasmid pH2J1 [14,15]. This pH2J1-prenylin construct was used as a template to generate point mutations in the C-terminal 10 amino acid residues by using the QuikChange Site-Directed Mutagenesis method (Stratagene). The entire prenylin cDNA sequence and all mutations were confirmed by direct DNA sequencing (DNA Sequencing Facility, University of Oklahoma Health Sciences Center).

The mutant prenylin cDNAs were then subcloned into the Sindbis virus vector pToto1000:3′2J via the *Apa*I and *Xho*I restriction sites [14,15]. In addition, some of the prenylin cDNAs were

Abbreviations used: BHK, baby hamster kidney; BMH, bismaleimidohexane; DSS, disuccinyl suberate; ER, endoplasmic reticulum; GFP, green fluorescent protein; Man II, mannosidase II; WT, wild-type; D176A etc., $Asp^{176} \rightarrow$ Ala replacement etc.

[1] To whom correspondence should be addressed (e-mail guangpu-li@ouhsc.edu).

also subcloned into the plasmid pH2J1-GFP in a manner that the prenylin cDNA was fused in frame to the C-terminus of GFP (green fluorescent protein). The GFP-prenylin cDNAs were then subcloned into the pToto1000:3′2J vector. Recombinant Sindbis viruses were generated from the pToto1000:3′2J constructs, as described previously [14,15], and utilized to express the prenylin mutants and their GFP-fusion proteins in BHK (baby hamster kidney) cells. In some cases, the prenylin mutants were expressed via the pcDNA3 vector (Invitrogen) without the GFP tag to confirm that different expression vectors and the GFP tag do not affect their intracellular localization.

We also made FLAG-tagged and Myc-tagged prenylin constructs and cloned both in the bi-directional expression vector pBI (BD Biosciences), which can express both proteins simultaneously upon co-transfection with pTet-Off (BD Biosciences).

Expression of recombinant prenylin proteins in BHK cells

BHK cell monolayers were grown in 35-mm culture dishes with α-minimal essential medium containing 10 % fetal bovine serum (Invitrogen). Cells were infected with the recombinant Sindbis virus which is capable of expressing the prenylin proteins at a multiplicity of infection of 50 plaque-forming units/cell and maintained in a 37 °C incubator with 5 % CO_2/95 % air for 4 h. For co-expression of FLAG–prenylin and Myc–prenylin, cells were co-transfected with pTet-Off and pBI/FLAG-prenylin/Myc-prenylin constructs via the Lipofectamine™ 2000-mediated procedure (Invitrogen) and incubated at 37 °C for 24 h. The Lipofectamine™ 2000-mediated procedure was also used for the transfection of pcDNA3 constructs. The expression of the prenylin proteins was confirmed by immunoblot analysis and their intracellular localization was determined by fluorescence microscopy (see below).

Fluorescence microscopy

BHK cells were grown on coverslips and infected with the recombinant viruses or transfected with plasmid constructs as described above. For detection of the GFP–prenylin fusion proteins, cells were rinsed 3 times with PBS and fixed in 4 % paraformaldehyde (in PBS) for 30 min. The coverslips were then mounted in PBS on glass slides and the fluorescence was observed via a Leica confocal laser-scanning microscope. Indirect immunofluorescence microscopy was performed to identify the ER marker calnexin, the Golgi marker Man II (mannosidase II) and several prenylin mutants. In this case, following fixation (see above), the cells were permeabilized with 0.1 % Triton X-100 (in PBS) for 5 min and incubated with the anti-calnexin antibody (Stressgene Biotechnologies), the anti-(Man II) rabbit serum (from Dr Kelley Moremen, Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA, U.S.A.) or our anti-prenylin goat serum for 60 min in PBS containing 1 % BSA. Cells were rinsed 3 times with PBS to remove unbound primary antibody, followed by incubation with the secondary antibody (goat anti-rabbit IgG conjugated to Alexa 568, from Molecular Probes; or swine anti-goat IgG conjugated to FITC, from Caltag) for 60 min. The coverslips were mounted in PBS on glass slides and viewed as described above.

Immunoblot analysis

Cells were lysed in 1 % SDS (200 μl per dish). The lysates (10 μl) were mixed with 2 × Laemmli sample buffer (10 μl), and analysed by SDS/PAGE (12 % gel), followed by immunoblot assay using the ECL® reagents (Amersham Pharmacia) and antibodies specifically against prenylin (our goat antiserum), FLAG (monoclonal from Sigma) and Myc (monoclonal from Sigma) as indicated. The results were quantified by using a densitometer (Molecular Dynamics).

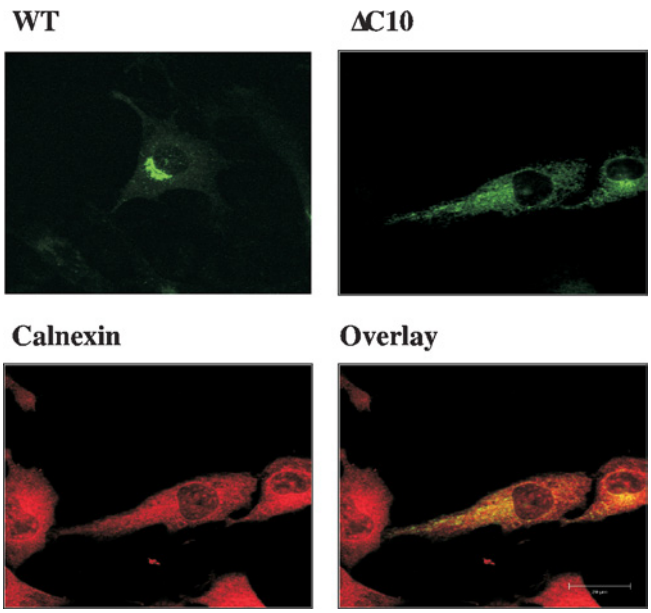

Figure 1 Truncation of C-terminal 10 residues results in retention of prenylin in the ER

WT prenylin and the C-terminal truncated mutant (ΔC10) were expressed as GFP-fusion proteins in BHK cells, followed by processing for confocal fluorescence microscopy. The upper two panels show one of the 16 confocal sections for WT and ΔC10. The cells expressing prenylin$^{\Delta C10}$ were also immunostained with an anti-calnexin antibody. The lower two panels show the ER localization of calnexin in red and its co-localization with prenylin$^{\Delta C10}$ in yellow (overlay). Scale bar, 20 μm.

Cross-linking and immunoprecipitation

BHK cells expressing FLAG–prenylin and Myc–prenylin, or various prenylin mutants as indicated, were incubated with the membrane-permeant cross-linking reagent DSS (disuccinyl suberate; 0.1 mM) (Pierce Biotechnology) or BMH (bismaleimidohexane; 0.1 mM) (Pierce Biotechnology) for 30 min, and quenched with 10 mM lysine or cysteine respectively. Cells were then lysed in 1 % SDS (200 μl per dish) and the lysates were either immunoprecipitated [16] with an anti-FLAG monoclonal antibody (Sigma) or directly analysed by immunoblot analysis with the anti-prenylin antibody. The immunoprecipitated proteins were analysed by SDS/PAGE (12 % gel), followed by immunoblot analysis with anti-FLAG and anti-Myc antibodies.

RESULTS

Di-acidic motif is not required for ER export of prenylin

Truncation of the C-terminal 10 amino acid residues (aa 176 to 185) of prenylin abolished its ability to exit ER (Figure 1). This was demonstrated by expressing the WT (wild-type) and C-terminal truncation mutant (prenylin$^{\Delta C10}$) as GFP-fusion proteins in BHK cells and determining their intracellular localization by confocal fluorescence microscopy. Whereas the WT prenylin showed the half-moon-shaped perinuclear pattern typical of Golgi localization (Figure 1, WT), which had been previously confirmed to co-localize with the Golgi marker Man II [3], the ΔC10 mutant was retained in the ER, as shown by its dispersed, reticular

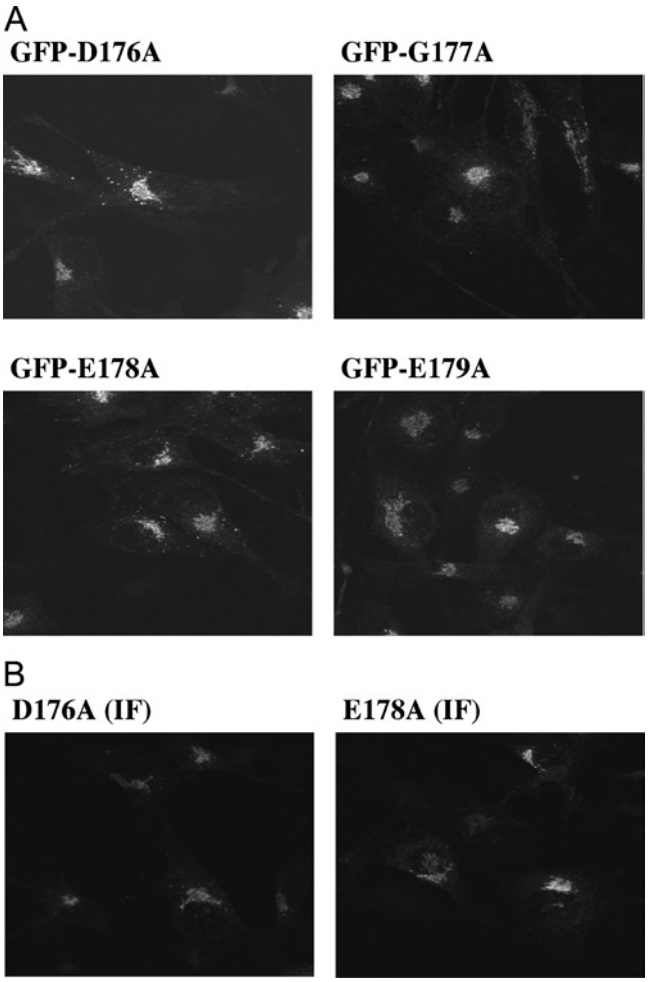

Figure 2 Di-acidic motif (Asp^{176}-Gly-Glu) is not required for ER export of prenylin

(**A**) The alanine substitution mutants D176A, G177A, E178A and E179A were expressed as GFP-fusion proteins in BHK cells, followed by processing for confocal fluorescence microscopy. Confocal images of cells expressing each of the indicated mutants are shown. Note that they all show the same Golgi localization pattern as the WT prenylin (see also Figure 1). (**B**) The D176A and E178A mutants were expressed without the GFP tag via the pcDNA3 vector, and their intracellular localization was determined by indirect immunofluorescence (IF) microscopy. Confocal images of cells expressing the mutants, as indicated, are shown.

localization pattern throughout the cytoplasm (Figure 1, ΔC10) and its co-localization with the ER membrane protein calnexin (Figure 1, overlay). To investigate if the C-terminal 10 residues contain an ER export signal, we initially focused on the di-acidic motif Asp^{176}-Gly-Glu in this region and changed each of the three residues to alanine. In addition, we also mutated the next residue Glu^{179} to alanine. The resulting mutants [D176A (Asp^{176} → Ala), G177A, E178A, and E179A] were expressed as GFP-fusion proteins in BHK cells by recombinant Sindbis viruses, and their intracellular localization was determined by confocal fluorescence microscopy. At steady state, all four mutants showed the same perinuclear Golgi localization pattern as WT prenylin (Figure 2A). The GFP tag did not have any effect on the transport and localization of prenylin [3]. Indeed, when the indicated mutants were expressed without the GFP tag and via a different expression vector (pcDNA3), they exhibited the same Golgi localization pattern, as shown by immunofluorescence microscopy using the prenylin-specific goat antiserum (Figure 2B). Thus the GFP

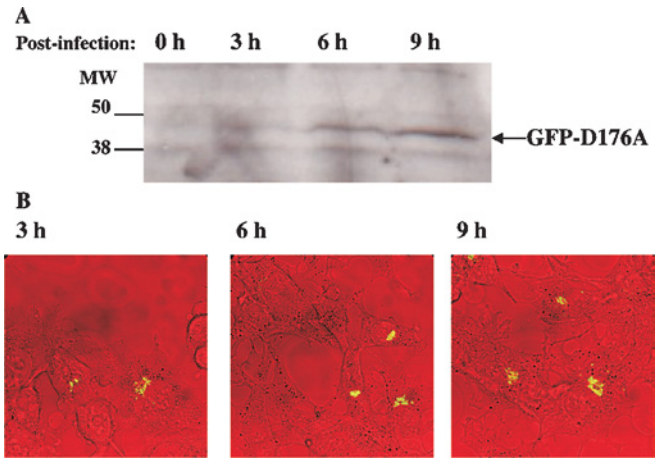

Figure 3 Golgi localization of the prenylin^{D176A} mutant is not affected by protein expression level

(**A**) Expression levels of GFP–prenylin^{D176A} in BHK cells at the indicated times post-infection were analysed by immunoblotting with the goat antiserum for prenylin. MW, molecular-mass markers (in kDa). (**B**) Confocal sections of BHK cells expressing GFP–prenylin^{D176A} at the indicated times post-infection. The fluorescence image of GFP–prenylin^{D176A} has been overlaid on to the transmission light image of the cell to reveal the relative position of GFP–prenylin^{D176A} in the cell.

tag and the choice of expression vector had no effect on the intracellular localization of these prenylin proteins.

We further determined if the level of protein expression could affect the intracellular localization of the D176A mutant. In this case, GFP–prenylin^{D176A} was expressed in BHK cells via the Sindbis virus vector. At 0, 3, 6 and 9 h post-infection, the level of GFP–prenylin^{D176A} expression was determined by immunoblot analysis (Figure 3A), and at the same time the intracellular localization of GFP–prenylin^{D176A} was determined by confocal fluorescence microscopy (Figure 3B). At 3 h post-infection, GFP–prenylin^{D176A} expression was already detectable, but was at a low level; the expression increased up to 5-fold with time (Figure 3A). During this period, GFP–prenylin^{D176A} was consistently localized to the Golgi complex, as demonstrated by its concentrated perinuclear localization pattern (Figure 3B).

Mutations of the C-terminal Val^{185} of prenylin can abolish its ER export

Since none of the mutations at or near the di-acidic motif had effect on the ER export of prenylin, we made additional mutations by substituting alanine for each of the six remaining C-terminal residues. The six prenylin mutants were expressed as GFP-fusion proteins in BHK cells, and their intracellular localization was determined by confocal fluorescence microscopy. Only one mutant (V185A) showed a partial block in ER export, as shown by the appearance of a reticular ER localization pattern (Figure 4A). The other mutants were indistinguishable from the WT prenylin (Figure 1) in that they were predominantly localized to the Golgi complex at steady state (Figure 4A). The difference in localization was not due to different expression levels, since all these mutants were expressed at similar levels in the cell, as determined by immunoblot analysis (Figure 4B).

The prenylin^{V185A} mutant had the C-terminal Val^{185} replaced with alanine. The fact that this mutation partially retained prenylin in the ER suggested the importance of the C-terminal residue in the ER export of prenylin. Thus the C-terminal Val^{185} was further investigated by substitution with all 18 other amino acids. The

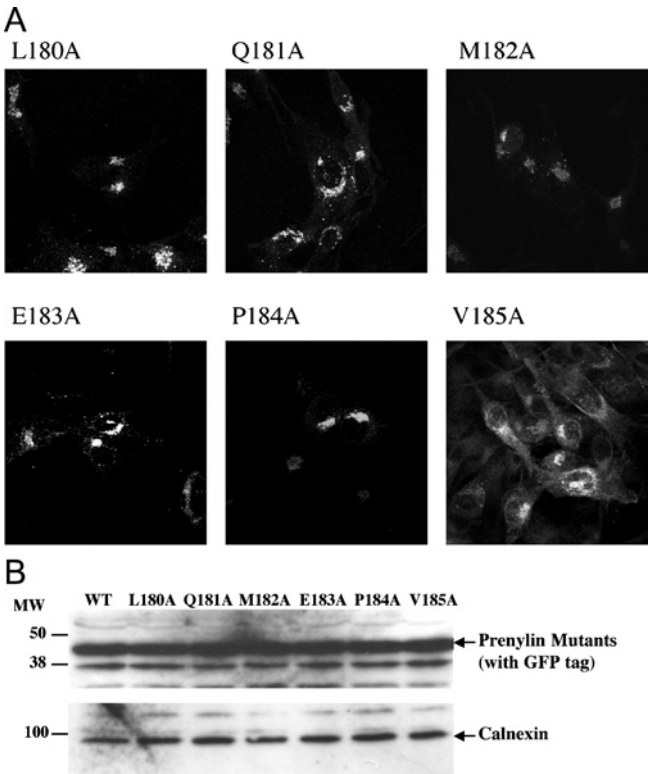

Figure 4 C-terminal Val[185] is important for ER export of prenylin

The alanine substitution mutants L180A, Q181A, M182A, E183A, P184A and V185A were expressed as GFP-fusion proteins in BHK cells, followed by processing for confocal fluorescence microscopy. (**A**) Confocal images of cells expressing each of the mutants as indicated are shown. Note that only the V185A mutant exhibits significant ER retention, as shown by its dispersed reticular pattern throughout the cytoplasm, similar to the ΔC10 mutant (see also Figure 1). (**B**) Immunoblot analysis of expression levels of WT and the indicated mutants, as well as the endogenous protein control (calnexin).

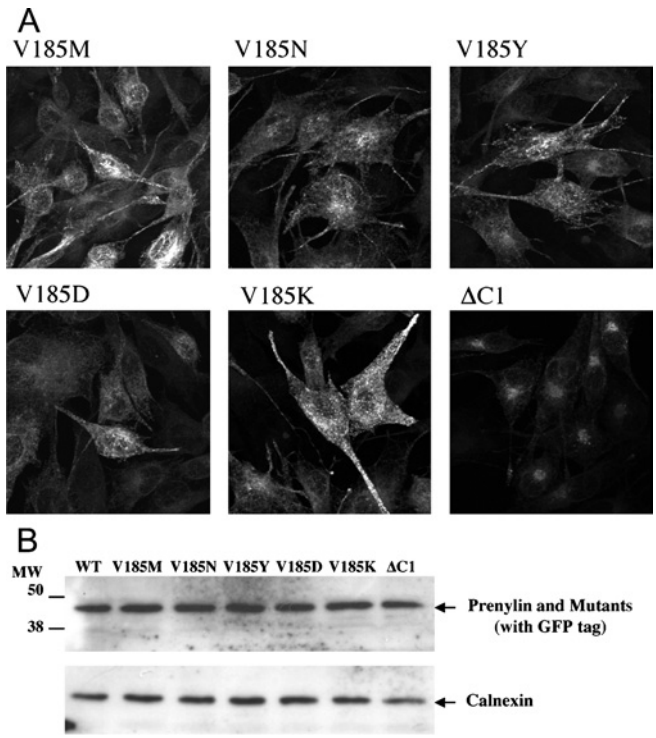

Figure 5 Most substitutions at the C-terminal Val[185] result in blocking of prenylin ER export

In addition to prenylin^{V185A}, we generated 18 other substitution mutants at Val[185] and expressed them as GFP-fusion proteins in BHK cells, followed by processing for confocal fluorescence microscopy. (**A**) Confocal images for five of these mutants, including V185M, V185N, V185Y, V185D and V185K, as well as one truncation mutant with Val[185] deleted (ΔC1), are shown. The data for other mutants are summarized in Table 1. (**B**) Immunoblot analysis of expression levels of WT and the indicated mutants, as well as the endogenous protein control (calnexin).

resulting substitution mutants were expressed as GFP-fusion proteins and their intracellular localization was determined by confocal fluorescence microscopy. All the mutants, except V185R, were defective in ER export (Figure 5A), even though they were expressed at the same level as the WT protein (Figure 5B). In fact, 14 of the 18 mutants completely lost the ability to exit the ER, as shown by their reticular ER localization pattern throughout the cytoplasm without detectable perinuclear Golgi localization (Figure 5A and Table 1). Three other mutants (V185I, V185C and V185P) were partially blocked in ER export, similarly to the V185A mutant (summarized in Table 1). These mutants exhibited a largely ER localization pattern with detectable Golgi accumulation. Interestingly, the V185R mutant showed a normal Golgi localization pattern, similar to the WT protein (Figure 6). In Figure 6, we compared further these three types of Val[185] mutants, represented by V185K, V185C and V185R, in terms of their co-localization pattern with the Golgi marker Man II.

We further determined if lowering the expression level could correct the defect of these mutants in ER export. In this case, we examined one of the defective mutants (V185K) in a similar experiment described above for GFP–prenylin^{D176A} (see Figure 3). GFP–prenylin^{V185K} was expressed in BHK cells via the Sindbis virus vector. At 0, 3, 6 and 9 h post-infection, the level of GFP–prenylin^{V185K} expression was determined by immunoblot analysis (Figure 7A), while, at the same time, the intracellular localization of GFP–prenylin^{V185K} was determined by confocal fluorescence

Table 1 Effect of substitution mutations at Val[185] on ER export

This Table summarizes the confocal microscopy data on the intracellular localization of the Val[185] substitution mutants. The localization patterns are generally classified into three groups with WT, ΔC10 and V185A as criteria for none (No), complete (Yes), and partial ER retention (Partial) (see also Figure 6).

Prenylin mutants	ER retention
WT	No
V185M	Yes
V185N	Yes
V185Y	Yes
V185D	Yes
V185K	Yes
V185H	Yes
V185E	Yes
V185Q	Yes
V185T	Yes
V185S	Yes
V185L	Yes
V185W	Yes
V185F	Yes
V185G	Yes
V185C	Partial
V185I	Partial
V185P	Partial
V185A	Partial
V185R	No

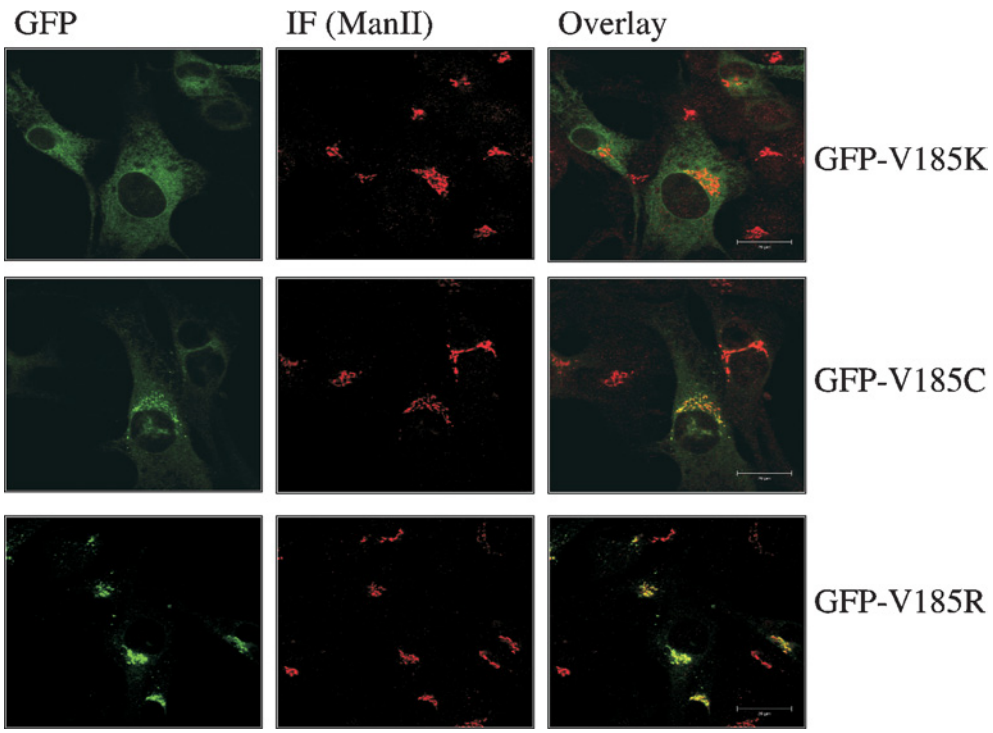

Figure 6 V185K, V185C and V185R represent three types of mutants with complete, partial and no block in ER export

BHK cells expressing GFP–prenylin^{V185K}, GFP–prenylin^{V185C} and GFP–prenylin^{V185R} were fixed and immunostained with the rabbit antiserum for the Golgi marker Man II and goat anti-rabbit IgG conjugated to Alexa 568 (Molecular Probes). Shown are confocal images of the GFP-fusion proteins (GFP), the immunofluorescence localization of Man II in the same cells [IF(ManII)] and the overlay of the green and the red images (Overlay). Scale bar, 20 μm.

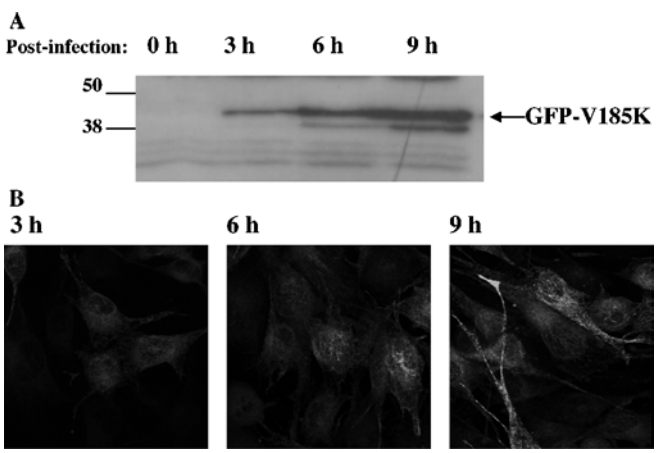

Figure 7 ER localization of the V185K mutant is not affected by protein expression level

(**A**) Expression levels of GFP–prenylin^{V185K} in BHK cells at the indicated times post-infection analysed by immunoblotting with the goat antiserum against prenylin. Molecular-mass marker values (kDa) are shown on the left-hand side. (**B**) Confocal images of BHK cells expressing GFP–prenylin^{V185K} at the indicated times post-infection are shown.

microscopy (Figure 7B). At 3 h post-infection, GFP–prenylin^{V185K} was expressed at a low level; the expression increased up to 5-fold with time (Figure 7A). During this period, GFP–prenylin^{V185K} was consistently retained in the ER, as shown by its dispersed reticular localization pattern (Figure 7B), suggesting that its defect in ER export is due to the mutation itself, rather than the level of protein expression.

Two possibilities existed regarding the important role of Val^{185} in the ER export of prenylin: either it serves as part of an ER export signal or it is important in forming a competent conformation for ER export. To distinguish between these two possibilities, we made the prenylinΔC1 mutant by deleting the C-terminal Val^{185} residue and expressed it as a GFP-fusion protein in BHK cells. Interestingly, the prenylinΔC1 mutant was only partially blocked in ER-to-Golgi transport, and its intracellular distribution was similar to that of prenylin^{V185A} (Figure 4A), with detectable perinuclear Golgi localization (Figure 5A). Again the protein level was similar to that of other mutants (Figure 5B). Thus deletion of the C-terminal Val^{185} had a less negative effect on ER-to-Golgi transport than most of the substitution mutations (Figure 5A and Table 1), suggesting that Val^{185} itself is not part of an ER export signal.

C-terminal Val^{185} important for proper dimer conformation required for ER export

We observed that a small portion of prenylin molecules migrated more slowly on SDS/PAGE, and appeared to be the size of a dimer (approx. 40 kDa) if the samples were not boiled before SDS/PAGE. This observation was consistent with a previous report that immunoblot analysis of Triton X-100 tissue extracts with a rabbit antiserum monospecific for prenylin identified a band about the size of a dimer [4]. However, these observations did not establish that the larger form was actually a prenylin dimer. Its physiological relevance was also unclear, since it was likely to be formed after cell lysis, rather than in intact cells.

To determine if prenylin indeed forms dimers in the cell, we constructed two epitope-tagged prenylins: FLAG–prenylin and Myc–prenylin, and co-expressed them in BHK cells. The cells

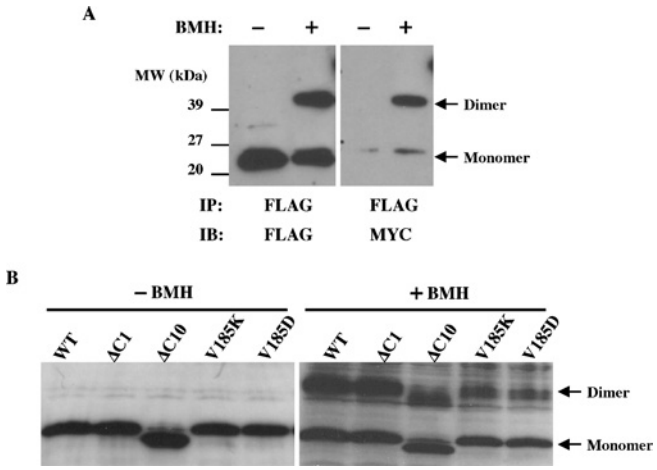

Figure 8 ER export-defective mutants are defective in forming a proper dimer conformation in the cell

(**A**) BHK cells co-expressing FLAG–prenylin and Myc–prenylin were either treated (+) or not treated (−) with the cross-linking agent BMH before cell lysis. Cell lysates were then immunoprecipitated (IP) with the anti-FLAG antibody, followed by immunoblotting (IB) with either the anti-FLAG or anti-Myc antibody as indicated. The prenylin monomer and dimer positions are indicated. Molecular mass marker values (kDa) are indicated on the left-hand side. (**B**) BHK cells expressing WT prenylin or the indicated mutants were either treated (+ BMH) or not treated (− BMH) with the cross-linking agent BMH before cell lysis. Cell lysates were then analysed by SDS/PAGE, followed by immunoblot assay with the goat antiserum for prenylin. The prenylin monomer and dimer positions are indicated.

were treated with membrane-permeant cross-linking reagents DSS or BMH. Cell lysates were then immunoprecipitated with the anti-FLAG antibody, followed by immunoblot analysis with either the anti-FLAG or the anti-Myc antibody (Figure 8A). Although DSS, an amino-group-reactive reagent, had no effect (results not shown), BMH, which reacts with sulphydryl groups, effectively cross-linked prenylin molecules into dimers, which migrated at approximately the same position as the 40-kDa band mentioned above, and were recognized by both anti-Myc and anti-FLAG antibodies (Figure 8A). BMH, as a non-cleavable cross-linker, led to the formation of prenylin dimers that were stable in SDS/PAGE. In the absence of BMH, the prenylin molecules ran only as 20 kDa monomers (Figure 8A). Following immunoprecipitation with anti-FLAG antibody, Myc–prenylin was detected mostly at the dimer position, indicating it had been cross-linked with FLAG–prenylin (Figure 8A). The small amount of monomeric Myc–prenylin detected was likely due to free non-cross-linked Myc–prenylin molecules in the lysates, which were bound to FLAG–prenylin during the immunoprecipitation process and became monomeric upon boiling in SDS. These results indicated that prenylin molecules indeed formed homodimers in the cell.

We further examined prenylin$^{\Delta C1}$ and several mutants defective in ER export (prenylin$^{\Delta C10}$, prenylin^{V185K} and prenylin^{V185D}) in the cross-linking experiments. Interestingly, whereas prenylin$^{\Delta C1}$ was efficiently cross-linked into a dimer similarly to the WT prenylin with 70 % in the dimer form, all the export-defective mutants showed dramatic reduction in forming this dimer with only 10–20 % in the dimer form (Figure 8B), suggesting conformational differences between export-competent and export-defective prenylin proteins. Thus the inability of ΔC10 and Val^{185} mutants to exit the ER correlated with their defect in forming this export-competent dimer conformation. We also observed that the export-defective mutants became less stable upon BMH treatment (results not shown), further suggesting their conformational differences with the WT protein. This observation can explain the lower levels of prenylin$^{\Delta C10}$, prenylin^{V185K} and prenylin^{V185D} in Figure 8(B), although it is not yet clear at present what caused the instability.

DISCUSSION

Prenylin is a member of a newly discovered family of proteins that interact with the prenyl group of Ras-related small GTPases, as detected in yeast two-hybrid and biochemical binding assays [1–4]. In addition to the Golgi-localized prenylin, the only other member of this family identified thus far (prenylated Rab acceptor 2) is localized in the ER [5]. There is also a family of yeast homologues (Yip and Yif) [2,17,18]. The specific intracellular localization may dictate their interaction with distinct sets of prenylated proteins and thus are functionally important. We have investigated the mechanism of how prenylin is exported out of ER and transported to the Golgi complex.

Prenylin contains two long internal hydrophobic domains, with each passing through the membrane twice, and adopts a membrane topology with both N- and C-terminal domains in the cytoplasm [7]. We have previously shown that the entire N-terminal domain (78 residues) can be deleted without any effect on its ER export and Golgi localization [3]. In the present study, we have demonstrated the importance of the C-terminal domain in prenylin localization, and further identified the ultimate C-terminal Val^{185} as critical for the ER export of prenylin. All substitution mutations at Val^{185}, except V185R, reduce the ability of prenylin to exit ER either completely or partially, indicating the stringent requirement for the short-branched side chain of Val at the ultimate C-terminus of prenylin. Although this C-terminal Val^{185} could serve as an ER export signal for prenylin, as suggested for several proteins [19–21], deletion of Val^{185} has less negative effect on the ER export of prenylin than many Val^{185} substitution mutants, suggesting that Val^{185} may not be part of an ER export signal. Interestingly, only arginine can functionally replace the Val^{185} for efficient ER export, suggesting that in the microenvironment of prenylin and its interaction with the membrane, arginine behaves like a hydrophobic valine residue. Other mutations at Val^{185} appear to have disrupted the competent conformation required for prenylin ER export. This explanation is further supported by our demonstration that prenylin molecules form homodimers in the cell. This dimer conformation can be identified in the cell by the cell-permeant cross-linker BMH. The ΔC10 and Val^{185} mutants that fail to exit the ER are defective in forming such a dimer. The fact that the amino-group-reactive DSS does not work in the cross-linking reaction suggests that there are no properly positioned amino groups at the prenylin dimer interface.

Prenylin is a small 20 kDa membrane protein with N- and C-terminal cytoplasmic domains. Our truncation and alanine-scanning mutagenesis studies do not show a recognizable ER export signal in either of these two domains. The results are consistent with the idea that prenylin molecules with a proper dimer conformation may follow the bulk-flow to exit ER, although we cannot rule out the possibility that prenylin could be exported from the ER by partnering with other proteins that do contain ER export signal(s). A well-known ER export signal is the di-acidic motif (Asp-Xaa-Glu) originally found in the vesicular-stomatitis virus glycoprotein [9,10]. The C-terminal domain of prenylin actually contains such a di-acidic motif (Asp^{176}-Gly-Glu), but our results clearly show that it is dispensable for prenylin ER export. The D176A and E178A mutants exhibit the same Golgi localization pattern as the WT prenylin. We have reproduced these results twice with two independent clones for each mutant. These results contrast with a previous report, which suggested that the D176A and E178A mutants are retained in the ER [5].

We cannot directly compare the microscopy data, since in the previous report the original data were not shown, but simply summarized in a table [5]. However, we are able to confirm our results by showing that the D176A and E178A mutants, when expressed without the GFP tag and from a different vector, still localize to the Golgi complex. We further show that the difference in localization is not due to the difference in expression level by demonstrating that the intracellular localization of both types of prenylin mutants (D176A and V185K), which are localized to Golgi and ER respectively, is not affected by their expression level in the cell. While the discrepancy remains to be resolved, another possibility could be the difference between mouse (present study) compared with rat [5] homologues. In this regard, there are two changes in the primary sequence, Gly^{13} (mouse) versus Val^{13} (rat) in the N-terminal domain and Met^{172} (mouse) versus Ile^{172} (rat) in the C-terminal domain [3,4].

Recent studies have suggested that prenylin and other members of the family may play a role in vesicular transport along the exocytic pathway [18,22] and between late endosomes and the *trans*-Golgi network [6]. In an *in vitro* ER-to-Golgi transport assay, antibodies against the yeast Yip1p and Yif1p proteins can block the fusion between ER-derived vesicles and the Golgi membrane [18]. In mammalian cells, prenylin, a homologue of the yeast Yip3p, may promote the dissociation of GDP-dissociation-inhibitor–Rab complexes for a subset of Rab proteins, including Rab9 that facilitates the late endosome-to *trans*-Golgi network transport [6]. Over-expression of several prenylin mutants has been shown to inhibit the vesicular-stomatitis virus glycoprotein transport to the cell surface [22]. Some of these mutants are defective in ER-to-Golgi transport and are retained in the ER, suggesting that proper Golgi localization and beyond may be critical for prenylin function.

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