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Cleavage of a Multispanning Membrane Protein by an Intramembrane Serine Protease[†]

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ABSTRACT: All intramembrane proteases are known to cleave membrane proteins with a single transmembrane helix. Such cleavages often release anchored soluble domains, which play a role in physiologically important inter- and intracellular processes. However, in many cases the physiological roles/substrates of intramembrane proteases are not known. It is interesting that no multispanning substrates were identified so far, despite the fact that intramembrane proteases have promiscuous substrate recognition and cleavage capabilities. Here we determined whether, in a synthetic experimental system, intramembrane proteases have the capability to interact with and cleave multispanning membrane proteins. We utilized the *Escherichia coli* rhomboid GlpG, an intramembrane serine protease, and truncated versions of the *E. coli* multidrug transporter MdfA as model multispanning membrane proteins. On the basis of *in vivo* and *in vitro* studies on the association of GlpG with various MdfA constructs and their cleavage, we conclude that GlpG is able to recognize and cleave truncated forms of MdfA but not the intact protein. We propose that GlpG has the capacity to act on unfolded multispanning membrane proteins, thus providing an incentive for investigating possible physiological consequences.

Cleavage of integral membrane proteins inside the membrane milieu is a rapidly emerging biological concept with major implications in several inter- and intracellular pathways (*1*−*4*). Intramembrane proteolysis is catalyzed by integral membrane proteases that belong to several families (*5*), of which serine proteases (termed rhomboids) are relatively well studied also structurally (*6*). Interestingly, although rhomboids exist in almost every living cell (*7*), only several have been characterized and shown to perform different functions and cleave different substrates in different species and organelles (*8*). The only fully documented common theme of their function is that rhomboids cleave inside integral membrane proteins with a single transmembrane helix (TM),[1] either in a type I configuration ($N_{out}-C_{in}$) (*9*) or in a type II ($N_{in}-C_{out}$) (*10*). Neither the substrates nor their cleavage sites have major consensus characteristics, and accordingly, rhomboids from different species are sometimes able to cleave the same substrates or synthetic model substrates (*11*−*13*). Indeed, several studies support the notion that the substrate cleavage site contains helix-destabilizing amino acid residues, which enable the scissile bond to access the protease active residues (*14*, *15*). However, this is not always the case both with rhomboids (*16*) and with other intramembrane proteases (*17*) (reviewed in ref *18*). Therefore, the overall emerging evidence strongly suggests that rhomboids are promiscuous in their substrate recognition and cleavage capabilities, and this raises the question how nonsubstrate membrane proteins avoid unintentional cleavage.

We studied the rhomboid protease GlpG from *Escherichia coli* as a model for intramembrane serine proteases. Although its physiological role is unknown, previous work demonstrated that GlpG can cleave model type I membrane proteins (*13*, *15*). Here we examined the theoretical possibility that GlpG might be able to cleave multispanning (type III) membrane proteins by utilizing a synthetic experimental system. Notably, according to several of the crystal structures of *E. coli* GlpG (*19*−*21*), and the assumption that substrates approach the active site in between TM2 and TM5, the putative entrance region would accommodate a maximum of one TM if these TMs move apart. Therefore, if the protease cleaves type III membrane proteins, they should be loosely folded. Our results showed that, indeed, GlpG was able to interact with and cleave disrupted versions of a multispanning membrane protein both *in vivo* and *in vitro*. As hypothesized, only aberrant (truncated) forms were substrates whereas the intact membrane protein did not interact with the protease and was resistant to cleavage.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. *E. coli* Top10 (Invitrogen) was used for the propagation and preparation of various plasmid constructs. The kanamycin resistance gene was removed from *E. coli* K12 BW25113*glp::kan* (Keio collection) (*22*), and the resulting strain, *E. coli* K12 BW25113Δ*glpG*, was used for *in vivo* cleavage experiments and expression of the various substrates and full-length MdfA. Wild-type- and GlpG(S201A)-6H were expressed and purified from *E. coli* C43(DE3) (*23*). Plasmids encoding GlpG derivatives, various MdfA-PhoA hybrids, and MdfA-6H used in this study are listed in Suppporting Information Table S1. All plasmids were constructed using synthetic deoxyoligonucleotides (not shown) and PCR.

In Vivo Cleavage Experiments. For studying cleavage of MdfA-PhoA hybrids by chromosomally encoded GlpG, *E. coli* BW25113Δ*glpG* or BW25113 was transformed with plasmids encoding MdfA-PhoA hybrids. For studying cleavage by

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[1]Abbreviations: GlpG, an *Escherichia coli* intramembrane serine protease; TM, transmembrane helix; PhoA, alkaline phosphatase; DDM, *n*-dodecyl β-D-maltopyranoside; NTA, nitrilotriacetic acid.

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plasmid-encoded GlpG, *E. coli* BW25113Δ*glpG* harboring either pCV3-*tacP-GlpG-6H* or pCV3-*tacP-GlpG*(*S201A*)-*6H* was transformed with compatible plasmids encoding MdfA-PhoA fusions. To characterize the GlpG transmembrane core domain, plasmids encoding GlpG derivatives were transformed with pT75-*araP-L114-PhoA*. In all cases, the transformants were grown overnight at 37 °C in LB medium supplemented with ampicillin (200 μg/mL) and with or without kanamycin (30 μg/mL) for plasmid-encoded or chromosomally encoded GlpG, respectively. Cells were diluted in fresh medium supplemented also with isopropyl β-D-thiogalactoside (IPTG) for plasmid-encoded GlpG and grown to OD_{600} ~0.9. The cultures were then induced with 0.2% arabinose for 1 h and harvested, and membranes were purified as described previously (*24*).

[^{35}*S*]*Methionine Labeling and Immunoprecipitation*. *E. coli* BW25113Δ*glpG* harboring either pCV3-*tacP-GlpG-6H* or pCV3-*tacP-GlpG*(*S201A*)-*6H* was transformed with plasmid encoding L114-PhoA and grown overnight at 37 °C in M9 containing 0.4% glycerol, 1 mM $MgSO_4$, thiamin (1 μg/mL), ampicillin (200 μg/mL), kanamycin (30 μg/mL), and all amino acids (15 μg/mL) except for methionine and cysteine. Cells were diluted into fresh medium supplemented also with IPTG for induction of GlpG and grown to OD_{600} of 0.5. The cultures were then induced with 0.2% arabinose for 30 min. In pulse-labeling analysis, aliquots (0.5 mL) were labeled with 20 μCi of [^{35}S]methionine (1000 Ci/mmol; Izotop) for various times, whereas in pulse-chase experiments aliquots were labeled for 3 min and an excess of unlabeled memthionine was added (final concentration, 20 mM). Samples were withdrawn at different times, and proteins were immunoprecipitated as described previously (*25*) using antibodies to alkaline phosphatase (PhoA). Immunoprecipitated material was separated by 12% SDS−PAGE and subjected to autoradiography.

Protein Expression and Purification for in Vitro Studies. Expression and purification of wild-type GlpG-6H or GlpG-(S201A)-6H, the substrates L114-PhoA-6H, G172-PhoA-6H, L114-6H, and G172-6H, and full-length MdfA-6H and MdfA-PhoA-6H as controls were conducted as follows. Cells were grown at 37 °C in LB medium supplemented with kanamycin (30 μg/mL) for wild-type GlpG-6H and GlpG(S201A)-6H expression or with ampicillin (200 μg/mL) for substrates and full-length hybrids expression. Cells were grown until midlog phase and induced with either 0.5 mM IPTG or 0.2% arabinose for expression of GlpG or MdfA constructs, respectively. Cells were shifted to 16 °C, harvested after 14 h, and disrupted by French pressure cell (Aminco) using high pressure for routine cell disruption. Unbroken cells and cell debris were removed by centrifugation at 10000*g* for 10 min at 4 °C. Subsequently, membrane fractions were isolated by ultracentrifugation (100000*g* for 1 h at 4 °C) and solubilized by 1% *n*-dodecyl β-D-maltopyranoside (DDM) (Anatrace) in 50 mM HEPES (pH 7.5), 500 mM NaCl, and 10% glycerol (buffer A). Following clarification of the sample by ultracentrifugation, solubilized proteins were incubated with preequilibrated nickel nitrilotriacetic acid beads (Ni-NTA; QIAGEN) in buffer A supplemented with 15 mM imidazole and 0.05% DDM for 3 h at 4 °C with agitation for protein binding. The beads were washed first with 10 column volumes (CV) of buffer A supplemented with 0.05% DDM and 15 or 30 mM imidazole for substrates and full-length hybrids or GlpG-6H and GlpG(S201A)-6H, respectively, and then with 10 CV of buffer A supplemented with 0.05% DDM and 30 or 45 mM imidazole for substrates and full-length hybrids or GlpG-6H and GlpG(S201A)-6H, respectively. Bound proteins were eluted using 1 CV of buffer A supplemented with 400 mM imidazole and 0.05% DDM. Purified proteins were then dialyzed in 50 mM HEPES (pH 7.5), 120 mM NaCl, 10% glycerol, and 0.05% DDM (~10 h), and substrates were concentrated (using Vivaspin 10 kDa cutoff) to ~1 mg/mL.

Protein Expression and Purification for N-Terminal Sequencing. For expression and purification of the *in vivo* generated cleavage products, cells harboring plasmids encoding wild-type GlpG and each substrate were grown at 37 °C in LB supplemented with kanamycin (30 μg/mL), ampicillin (200 μg/mL), and IPTG (0.5 mM) until midlog phase and induced with 0.2% arabinose for 1.5 h. Cells were harvested, and cleaved products were purified as described above.

Copurification Studies (*from in Vivo Samples*). *E. coli* BW25113Δ*glpG* harboring plasmids encoding GlpG(S201A) (as control), GlpG(S201A)-6H, or wild-type GlpG-6H together with plasmids encoding either L114- or G172-PhoA were grown at 37 °C in LB supplemented with ampicillin (200 μg/mL), kanamycin (30 μg/mL), and IPTG (0.5 mM) to OD_{600} of 1.5. The cultures were then induced with 0.2% arabinose for 1.5 h, harvested, and disrupted by sonication. Protein purification for the copurification experiment was performed as described above, except for minor changes: Buffer A was replaced with 50 mM HEPES (pH 8.0), 250 mM NaCl, and 10% glycerol (buffer B), and Ni-NTA beads were loaded with equal amounts of total membrane-solubilized proteins. Western blot analysis was used to examine the presence of substrates in the eluted fractions.

Coimmunoprecipitation Studies (*from in Vivo Samples and in Vitro*). Solubilized proteins (see previous paragraph) were incubated with protein A−Sepharose (Sigma-Aldrich) cross-linked to rabbit anti-alkaline phosphatase antibodies (Rockland) by dimethyl pimelimidate·2HCl (DMP) (Pierce) at 4 °C for 3 h with agitation. Immunocomplexes were collected and washed twice with buffer B supplemented with 0.05% DDM and then twice with buffer C (50 mM HEPES, pH 8.0, 500 mM NaCl, 10% glycerol), also supplemented with 0.05% DDM. Finally, immunocomplexes were dissolved in SDS sample buffer and analyzed by Western blotting. For the *in vitro* coimmunoprecipitation experiments, G172- and MdfA-PhoA-6H and GlpG-(S201A)-6H were purified as described above using Ni-NTA. Increasing concentrations of purified G172- or MdfA-PhoA-6H (0.07−3 μM) were incubated with constant amounts (1.5 μM) of GlpG(S201A)-6H at 25 °C for 20 min. Samples were immunoprecipitated using anti-alkaline phosphatase antibodies as described above and analyzed by Western blotting.

N-Terminal Amino Acid Sequence Analysis. For identification of GlpG-cleavage sites, purified *in vivo* and *in vitro* generated cleavage products were subjected to SDS−PAGE, electroblotted to a polyvinylidene fluoride (PVDF) membrane, and sequenced by Protein Sequencer Procise 491 (Applied Biosystem).

In Vitro Cleavage Experiments. *In vitro* cleavage reactions were performed as described previously (*26*). Briefly, purified protease (wild type or GlpG(S201A)) (~1 μg) and each substrate (~1 μg) were mixed in a final volume of 20 μL in 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% DDM and incubated at 37 °C. Aliquots were taken at different time points and subjected to SDS−PAGE and Coomassie brilliant blue staining.

Western Blotting. Membrane fractions (10−15 μg of protein) were subjected to 12−17% SDS−PAGE. Proteins were electroblotted to nitrocellulose membranes and probed with

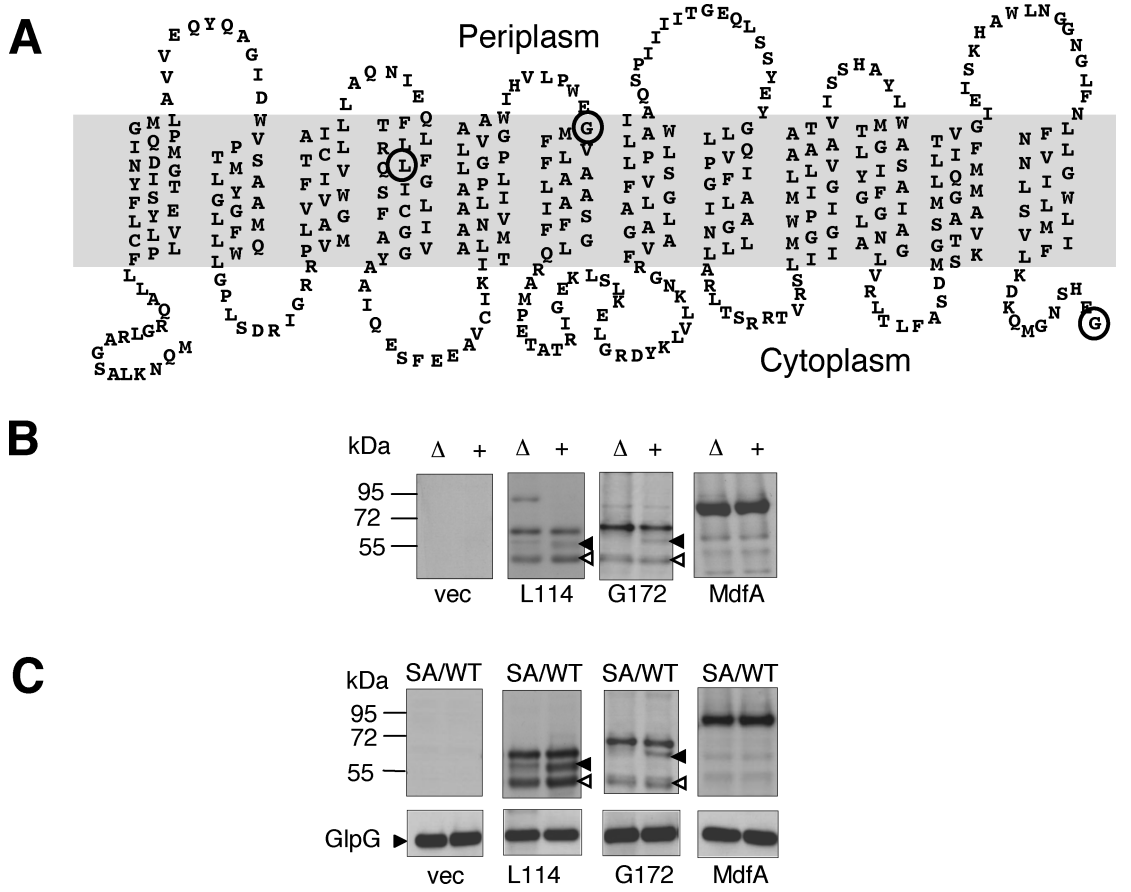

FIGURE 1: Cleavage of MdfA-PhoA hybrids by GlpG *in vivo*. (A) Secondary-structure model of MdfA (*31*). Residues at the MdfA-PhoA fusion joints of the selected hybrids are circled. (B) Hybrids were expressed in *E. coli* BW25113Δ*glpG* (Δ) and wild-type (+) cells. Membrane proteins were examined by Western blotting using antibodies against alkaline phosphatase. Putative cleavage products are indicated by filled arrowheads. Released alkaline phosphatase is indicated by empty arrowheads. (C) MdfA hybrids were coexpressed from plasmids with either GlpG-6H (WT) or GlpG(S201A)-6H (SA) in *E. coli* BW25113Δ*glpG*. MdfA hybrids were examined as in (B). The expression of GlpG-6H and GlpG(S201A)-6H was visualized using India HisProbe-horseradish peroxidase (lower panels).

rabbit anti-alkaline phosphatase antibodies (for detection of PhoA hybrids) or HisProbe-HRP-conjugated goat anti-rabbit immunoglobulin antibodies (for 6H-tagged proteins).

RESULTS

GlpG-Mediated Cleavage of Full-Length and Truncated Forms of MdfA in Vivo. Initially, we examined the possibility that GlpG might be able to cleave type III membrane proteins *in vivo* by coexpressing the protease and the tested membrane proteins. As a model substrate, we utilized MdfA, a 12-TM multidrug transporter from *E. coli* (*27*). In addition to the wild-type transporter, several aberrant (C-terminally truncated) MdfA-PhoA hybrids were constructed, composed of decreasing numbers of TMs. These hybrids contain a C-terminally fused signal-peptide-less alkaline phosphatase (PhoA) as a tag, as described previously (*28*, *29*) (Figure 1A). The GlpG-mediated cleavage of the MdfA-PhoA hybrids was initially evaluated *in vivo* in wild-type *E. coli* and its Δ*glpG* strain as a negative control, and three hybrids were selected for detailed analysis (L114-PhoA, G172-PhoA, and MdfA-PhoA). As shown in Figure 1B (black arrowheads), specific differences were observed with hybrids L114- and G172-PhoA, where a fast migrating band was detected by anti-PhoA antibodies only in samples from wild-type cells, perhaps representing cleaved products. Notably, free PhoA is also clearly detected in samples from both *E. coli* strains (open arrowheads) (see also ref *29*). This phenomenon suggests that an additional, GlpG-unrelated cleavage between MdfA and PhoA occurs *in vivo* (see later). To examine further whether cleavage of L114- and G172-PhoA hybrids is dependent on functional GlpG, we coexpressed the hybrids with plasmid-borne GlpG-6H or its inactive mutant having an alanine substitution for the protease active site serine (S201A) in the Δ*glpG* strain. As shown in Figure 1C, a lower molecular weight band is observed with L114- and G172-PhoA in cells expressing wild-type GlpG-6H. In contrast, no such band is observed in cells expressing the inactive mutant GlpG(S201A)-6H. Importantly, no specific proteolytic fragments were observed with the full-length MdfA protein.

The Catalytic Core of GlpG Is Sufficient for Cleavage of L114-PhoA. Previous studies have established that the long soluble N-terminal domain of GlpG (Figure 2A) is not required for cleavage of type I integral membrane substrates (*20*, *30*). Here we tested whether the membrane-embedded enzymatic core of GlpG is sufficient also for cleavage of the multispanning substrate L114-PhoA *in vivo*. To this end, several 6-histidine-tagged N-terminally truncated GlpG constructs were coexpressed with the substrate, and the membranes were examined by Western blotting with anti-PhoA antibodies (for the substrate) and HisProbe (for the GlpG constructs). The results clearly demonstrate that all of the GlpG constructs, except for the S201A mutant, are able to release the same low molecular weight product (Figure 2B), suggesting that the N-terminal domain is not required for the observed cleavage of multispanning membrane protein constructs.

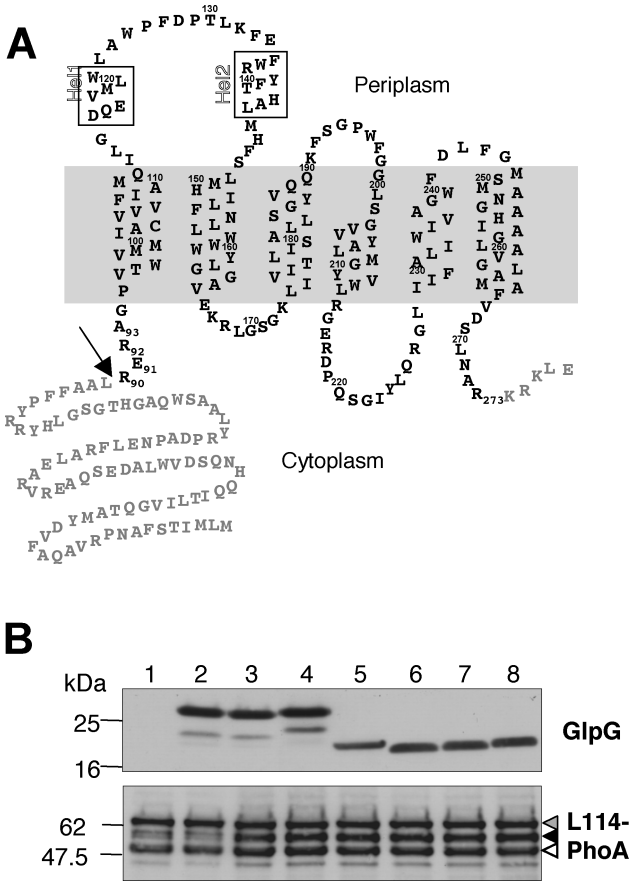

FIGURE 2: Cleavage of L114-PhoA by the transmembrane catalytic core of GlpG. (A) Secondary structure of GlpG. The genetically engineered transmembrane core of GlpG is shown by bold letters (residues 90−273). (B) Membranes were prepared from *E. coli* BW25113Δ*glpG* harboring a plasmid encoding L114-PhoA together with either of the following plasmids: empty vector (lane 1), GlpG-(S201A)-6H (lane 2) as negative controls, GlpG-6H (lane 3), 6H-GlpG (lane 4), 6H-GlpGΔ89 (lane 5), 6H-GlpG (93−273) (lane 6), 6H-GlpG (92−273) (lane 7), and 6H-GlpG (90−273) (lane 8). Membranes proteins were analyzed by Western blotting using India HisProbe-horseradish peroxidase (upper panel) and anti-PhoA antibodies (lower panel). Arrowheads indicate the following: gray, full-length hybrid; black, cleavage product; white, alkaline phosphatase.

Characterization of the GlpG-Mediated Cleavage of L114-PhoA in Vivo. To follow the cleavage reaction *in vivo*, *E. coli* Δ*glpG* cells simultaneously expressing L114-PhoA and wild-type GlpG or GlpG(S201A) were pulse-labeled with [^{35}S]methionine. After 3 min of labeling, an excess of unlabeled methionine was added, and the incubation was continued for up to 3 h (Figure 3A). Samples were withdrawn at the indicated times, and L114-PhoA was immunoprecipitated with anti-PhoA antibodies. Figure 3A shows that the intensity of the radioactive band corresponding to full-length L114-PhoA gradually decreased both with the S201A mutant and with wild-type GlpG, whereas the intensity of the cleaved product (full arrowhead), observed only with wild-type GlpG (Figure 3A, right panel), was increased with time. Free PhoA (open arrowhead) was also increased with time, both with GlpG(S201A) and with wild-type GlpG, indicating, as proposed earlier, that this cleavage is GlpG independent. Since the PhoA moiety is fused to the C-terminus, the results support the notion that GlpG cleaves the tested hybrids at an N-terminal site. The cleavage *in vivo* is rather slow as a small amount of the full-length hybrid is apparent even after 180 min. This is not surprising, as previous studies have already

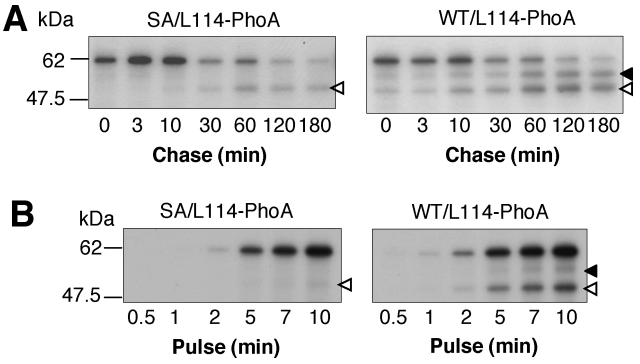

FIGURE 3: Characterization of the cleavage of L114-PhoA by GlpG *in vivo*. (A) Pulse and chase with L114-PhoA. *E. coli* BW25113Δ*glpG* expressing L114-PhoA and either GlpG(S201A) (SA, left panel) or wild-type GlpG (WT, right panel) were labeled for 3 min by [^{35}S]methionine and chased with excess of unlabeled methionine. Samples were taken at the indicated time points, immunoprecipitated using anti-alkaline phosphatase antibodies, and examined by SDS−PAGE and autoradiography. Cleavage product is indicated by filled arrowheads and the released alkaline phosphatase by open arrowheads. (B) Pulse labeling with L114-PhoA. *E. coli* BW25113Δ*glpG* expressing L114-PhoA and either GlpG(S201A) (SA, left panel) or wild-type GlpG (WT, right panel) were labeled by [^{35}S]methionine for different times and treated as in (A).

shown that rhomboid-mediated cleavage kinetics is relatively slow (*13*).

To examine the activity of GlpG toward the L114-PhoA hybrid further, we performed pulse-labeling experiments that allow analyses of both the synthesis of L114-PhoA and its cleavage. *E. coli* Δ*glpG* cells simultaneously expressing L114-PhoA and wild-type GlpG or GlpG(S201A) were pulse-labeled with [^{35}S]methionine for short times (0.5−10 min) and immunoprecipitated with anti-PhoA antibodies. Figure 3B shows that in both strains the intensity of the radioactive bands corresponding to the full-length L114-PhoA or to the GlpG-independent cleaved PhoA (open arrowheads) increased with time. However, a GlpG-dependent cleavage product (black arrowhead) was observed only in cells expressing wild-type GlpG, and its intensity also increased with time. Importantly, no other bands were detected even after 10 min, thus lending support to the notion that GlpG alone is responsible for L114-PhoA cleavage. These results strongly support the proposal that GlpG directly cleaves the type III fusion proteins L114- and G172-PhoA *in vivo* at an N-terminal site.

Identification of the in Vivo GlpG-Mediated Cleavage Sites in L114 and G172. Identification of the exact cleavage site required tagging of the hybrids with 6-histidines and their purification from cells expressing GlpG (Figure 4A,B). As shown, both with L114- and with G172-PhoA-6H, three proteins were purified. The upper band represents the full-length hybrid (gray arrowheads), the lower band is PhoA-6H (open arrowheads), and in between is the GlpG-cleaved product (left panels in Figure 4A,B, black arrowheads). The purified cleavage products were sequenced, and in both cases the same N-terminal residues were identified (GIDWVP), indicating that GlpG cleaves these proteins at the loop connecting TM1 and TM2 of MdfA, between A49 and G50, three residues away from the membrane according to the topology model (Figure 4C). However, it is noteworthy that there is no precise structural information about MdfA and, consequently, it is impossible to determine the exact location of the cleavage with respect to the membrane. The presented topology is based on the results of gene fusion

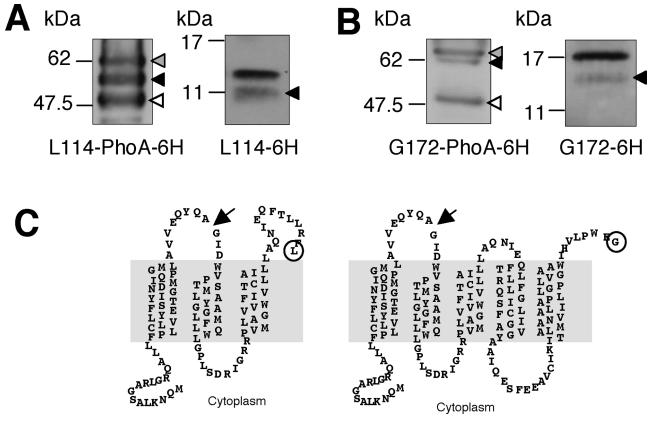

FIGURE 4: Identification of the *in vivo* cleavage sites. L114-PhoA-6H and G172-PhoA-6H [left panels in (A) and (B), respectively] as well as L114-6H and G172-6H [right panels in (A) and (B), respectively] were purified from cells coexpressing wild-type GlpG. Samples (1 μg) were analyzed by Western blotting using India HisProbe-horseradish peroxidase. (C) Purified cleavage products were analyzed by N-terminal sequencing, and the cleavage sites are indicated (arrows) in L114 (left panel) and G172 (right panel).

studies (*28*, *29*) and homology modeling (*31*). In any case, GlpG-mediated cleavage of substrates at a membrane proximal site is not surprising as it was observed in the past((*13*) (for review see ref *6*). In addition to the N-terminal sequencing of the cleaved substrates we also sequenced the N-terminus of the released PhoA moiety. The identified sequence is FLASD, demonstrating a cleavage site inside a hydrophilic sequence in between L114 and PhoA: LAQNIEQFTLL**R**^**F**LASDSYT. This cleavage site does not resemble other known sites in rhomboid substrates, which are usually formed by small residues (e.g., glycine, alanine). To test whether the GlpG-independent proteolytic release of PhoA influenced the GlpG-mediated cleavage, new substrates without PhoA were constructed in which L114 and G172 were tagged only by 6 histidines. The new proteins were coexpressed with GlpG (or its S201A mutant; data not shown) and purified from solubilized membranes. As shown in Figure 4 (right panels in A and B), in both cases, a fast migrating cleavage product was observed and purified. Both the full-length constructs and their putative proteolytic products were analyzed by N-terminal sequencing. The results confirmed that the upper bands represent the full-length hybrids and that the N-terminal sequences of the lower bands were identical to those of the cleaved PhoA hybrids (GIDWVP). Therefore, we concluded that the GlpG-mediated cleavage of the hybrids is not dependent on the C-terminally fused PhoA.

GlpG Interacts with the Truncated Versions of MdfA. An additional approach to studying substrate recognition by GlpG is copurification and coimmunoprecipitation experiments. We utilized DDM-solubilized membranes isolated from cells coexpressing L114- or G172-PhoA hybrids and either GlpG(S201A) with no 6 histidines (as a control), GlpG(S201A)-6H, or wild-type GlpG-6H. In the copurification experiments, Ni-NTA beads (for purifying the 6H-tagged GlpG or its mutant) were loaded with equal amounts of total membrane proteins, and the presence of L114- and G172-PhoA in the eluted fraction was examined by Western blotting with anti-PhoA antibodies. As shown in Figure 5A,B (right panels), both L114-PhoA and G172-PhoA were copurified with either GlpG(S201A)-6H or wild-type GlpG-6H. Remarkably, however, the amount of L114- and G172-PhoA copurified with GlpG(S201A)-6H was much higher than the amount copurified with wild-type GlpG-6H or untagged GlpG-(S201A), indicating a stable or prolonged interaction between the S201A mutant and the substrates. In all cases small amounts of the cleaved products were also copurified with wild-type GlpG-6H, as might have been expected if the cleavage or the release of the product or both are slow. In addition, the results show that small amounts of the released PhoA also appear in the copurified material in all cases, indicating that the preparation contains small amounts of unrelated proteolytic impurities. To allow more stringent conditions, we performed the reciprocal experiment by coimmunoprecipitation. DDM-solubilized membrane proteins, also isolated from cells coexpressing L114- or G172-PhoA hybrids and either GlpG(S201A) (as a control), GlpG(S201A)-6H, or wild-type GlpG-6H, were subjected to immunoprecipitation with anti-PhoA beads (for the immunoprecipitation of the PhoA-tagged substrates). Solubilized membranes and immunocomplexes were analyzed using Western blotting analysis. As shown in Figure 5C,D (right panels), both GlpG(S201A)-6H and wild-type GlpG-6H were coprecipitated with either L114- or G172-PhoA. However, both substrates coprecipitated higher amounts of the mutant GlpG(S201A)-6H compared to the native GlpG-6H, again indicating prolonged association between the S201A mutant and the substrates. Taken together, our results strongly support the concept that GlpG interacts with the tested type III substrates.

Our studies showed that GlpG does not cleave full-length MdfA (Figure 1B,C), suggesting that the two proteins do not interact with each other. To test this possibility, we compared the coimmunoprecipitation of purified GlpG(S201A)-6H by purified MdfA-PhoA-6H versus the substrate G172-PhoA-6H, using anti-PhoA antibodies. Increasing amounts of G172-PhoA-6H or MdfA-PhoA-6H were incubated without (not shown) or with constant amounts of GlpG(S201A)-6H and excess of anti-PhoA beads, and the coprecipitated material was analyzed. As shown in Figure 5E, the amount of GlpG(S201A)-6H coimmunoprecipitated with the substrate G172-PhoA-6H (left panel) was substantially higher than that of MdfA-PhoA-6H (right panel). It is likely that the minor amount of coimmunoprecipitated GlpG-(S201A)-6H represents traces of the protein found in the same DDM micelles of MdfA-PhoA-6H. These results suggest that GlpG interacts with the substrate and not with the intact MdfA.

Cleavage of L114 and G172 by GlpG in Detergent Solution. To investigate further the functional interaction between GlpG and the type III membrane substrates, we conducted an *in vitro* assay with purified components. Both wild-type and GlpG(S201A)-6H were purified by Ni-NTA chromatography in the presence of the detergent DDM (Figure 6A). In addition, the 6-His-tagged substrates L114 and G172 (Figure 6B, C, left panels) and the PhoA hybrids (Figure 6D,E, left panels), as well as the full-length MdfA-6H and MdfA-PhoA-6H (not shown), were overexpressed in *E. coli* Δ*glpG* cells and purified. As shown, both with L114- and with G172-PhoA-6H, two related proteins were purified. The upper band represents the full-length hybrid (Figure 6D,E, left panels, full triangles), whereas the lower band is PhoA-6H (open triangles), indicating once again that PhoA cleavage is GlpG independent. In the absence of PhoA (L114-6H and G172-6H), a single major protein was purified in both cases (Figure 6B,C, left panels). Following the purification of the enzymes and substrates, wild-type or GlpG(S201A)-6H was mixed with each substrate or with full-length MdfA, in the presence of DDM at 37 °C for various times. Clearly, with the truncated versions of MdfA, fast migrating cleaved products

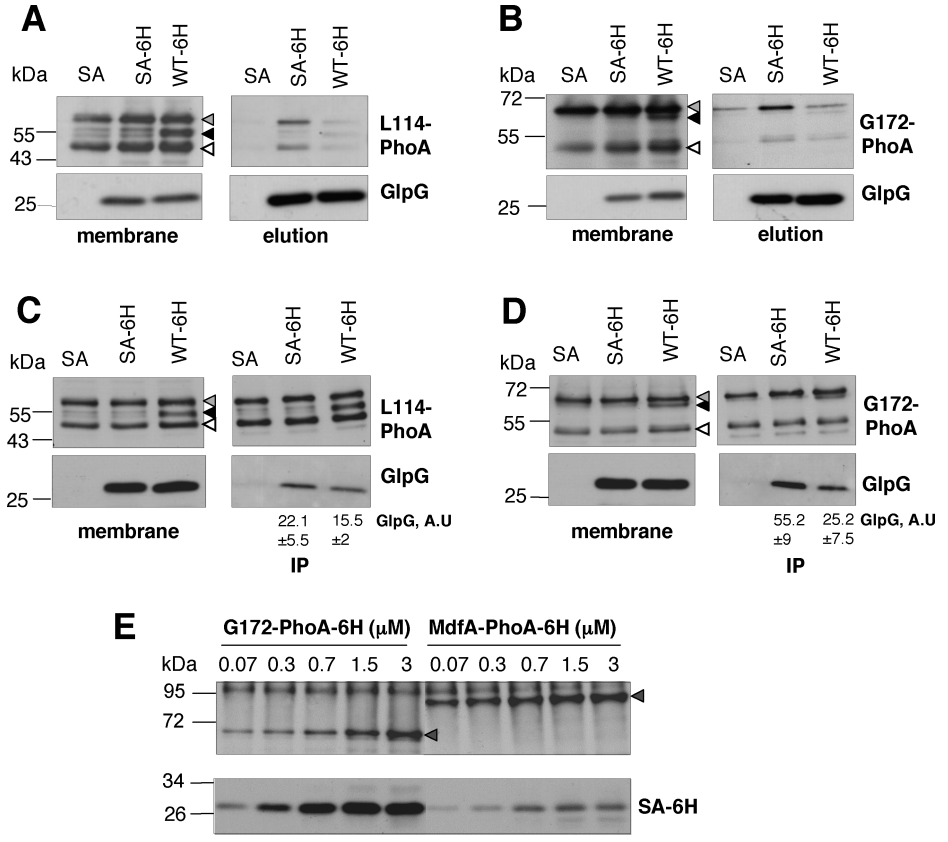

FIGURE 5: Copurification and coimmunoprecipitation of L114-PhoA, G172-PhoA, and MdfA-PhoA with GlpG and GlpG(S201A). GlpG(S201A) (SA) as control, GlpG(S201A)-6H (SA-6H), or wild-type GlpG-6H (WT-6H) were expressed with either L114-PhoA (A, C) or G172-PhoA (B, D). Membranes were isolated and solubilized using 1% DDM. (A, B) Samples containing equal amounts of proteins were incubated with Ni-NTA bead, and proteins were purified. (C, D) Samples were immunoprecipitated using anti-alkaline phosphatase antibodies. (E) *In vitro* coimmunoprecipitation of GlpG(S201A)-6H with G172-PhoA-6H or MdfA-PhoA-6H. G172-PhoA-6H, MdfA-PhoA-6H, and SA-6H were purified separately using Ni-NTA. G172-PhoA-6H or MdfA-PhoA-6H was incubated without (not shown) or with GlpG(S201A)-6H (1.5 μM) at 25 °C for 20 min. Samples were immunoprecipitated using anti-alkaline phosphatase antibodies. Solubilized membrane proteins (15 μg), elution fractions (20 μL) (A, B), or immunoprecipitated material (20 μL) (C−E) were analyzed by Western blotting using antibodies against alkaline phosphatase (upper panels) or India HisProbe-horseradish peroxidase (lower panels). The amount of coimmunoprecipitated GlpG was quantified and is given in arbitrary units (A.U.) in (C) and (D). Arrowheads indicate the following: gray, full-length hybrid; black, cleavage product; white, alkaline phosphatase.

were formed in the presence of GlpG-6H but not with GlpG(S201A)-6H (Figure 6B−E, right panels), and the intensity of the lower bands in L114- and G172-6H increased with time (Figure 6B,C, right panels). These results indicate that the cleavage requires active GlpG. In contrast, as examined by SDS−PAGE and N-terminal sequencing, the full-length proteins MdfA-PhoA-6H (not shown) and MdfA-6H (Figure 6F) were not cleaved under the same conditions.

Surprisingly, Figure 6 also shows that the *in vitro* generated cleavage products are larger than the cleavage products obtained *in vivo*. Therefore, we analyzed their N-terminal sequences, which showed identical cleavage sites in all the substrates. However, as predicted from the migration in SDS−PAGE, the position of the *in vitro* GlpG-catalyzed cleavage is different from that obtained *in vivo* and is located only 15 amino acids away from the N-termini of the proteins (LLFPLC, Figure 6G). These results suggest that, *in vitro*, the substrates enter the active site of GlpG in an inverted topology compared to the situation *in vivo*, further illustrating the promiscuous capabilities of GlpG. Also, these results suggest that the mode of interaction between the protease and the substrates is greatly influenced by the medium (native membranes versus detergent solutions) and underscore the potential risk of drawing mechanistic conclusions solely based on detergent-solubilized membrane proteins. In any case, we conclude that, also *in vitro*, GlpG is able to interact with and cleave the tested type III membrane proteins.

DISCUSSION

GlpG represents a large group of intramembrane serine proteases called rhomboids, of which only several were shown to have specific but different roles in cells and organelles (*8*). In all known physiologically relevant cases rhomboids were shown to cleave substrates with a single TM, but no consensus sequence was identified in the cleavage site, and the emerging view is that rhomboids are rather promiscuous. This notion is supported by observations that rhomboids of certain origins can cleave substrates from different species (*9*, *12*, *14*). This property of the proteases thus raises several questions as to how they recognize specific substrates, how other membrane proteins avoid unintentional cleavages, and whether these proteases are also involved in general intramembrane quality control pathways. Several modes of regulation could potentially prevent cleavage of non-substrate membrane proteins, such as regulated localization (*32*, *33*) or direct interaction of regulators with the protease or the substrate (*34*). Another possibility is that membrane proteins are simply inaccessible to the protease if they are tightly folded or associated with partners. This may also explain why rhomboids

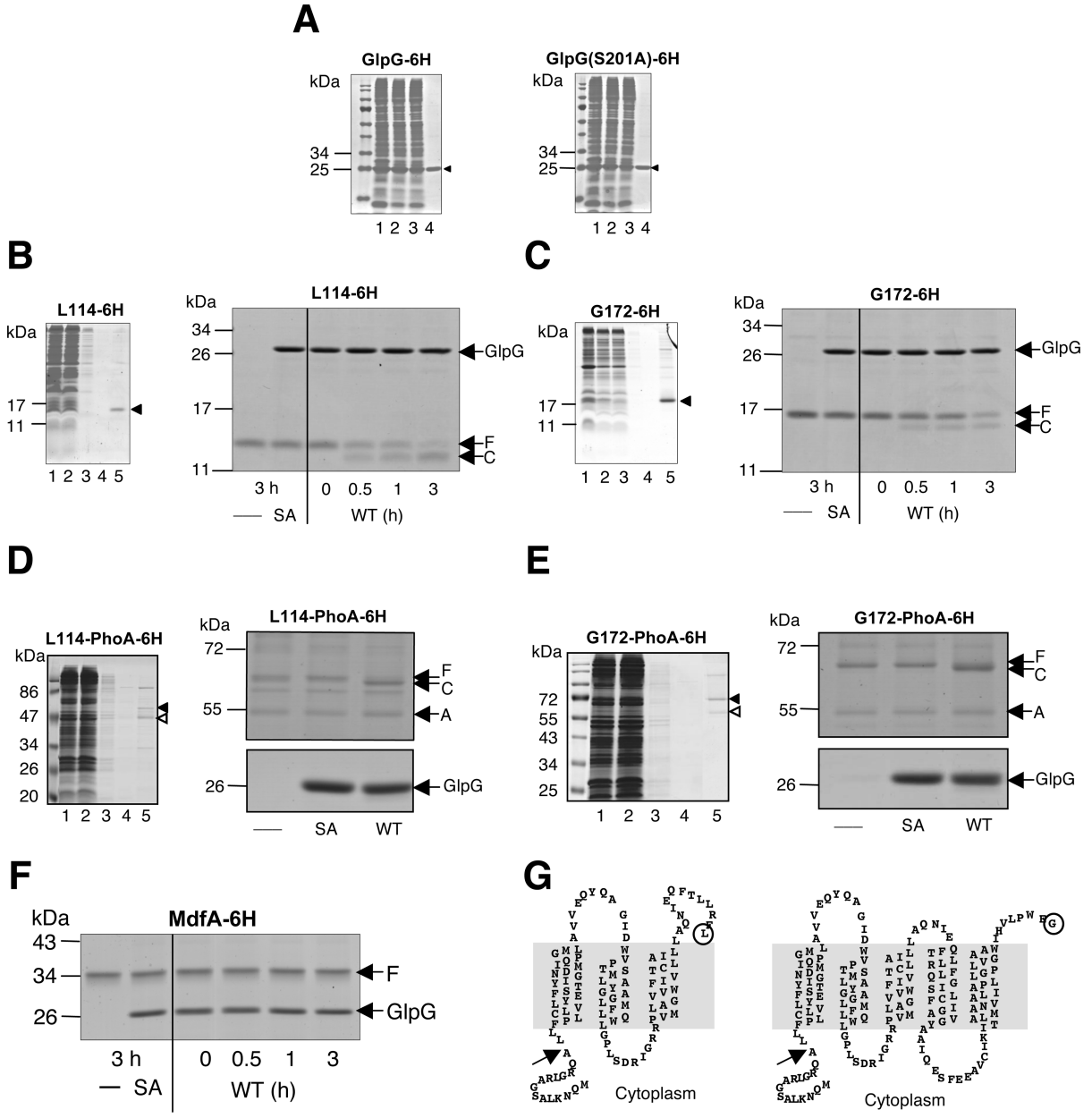

FIGURE 6: Cleavage of purified L114 and G172 by GlpG *in vitro*. (A) Purification of wild-type GlpG-6H (left panel) and GlpG(S201A)-6H (right panel) by Ni-NTA chromatography. Lane 1, membranes; lane 2, solubilized membranes after ultracentrifugation; lane 3, flow-through; lane 4, elution. (B−E) Purification (left panel) and *in vitro* cleavage (right panel) of L114-6H (B), G172-6H (C), L114-PhoA-6H (D), and G172-PhoA-6H (E). (B−E) Left panels: lane 1, membranes; lane 2, flow-through; lanes 3 and 4, wash; lane 5, elution. Samples were normalized according to the total amount of protein in the membrane fractions. (F) Incubation of purified MdfA-6H with purified GlpG(S201A)-6H or wild-type GlpG as indicated. (B−E, right panels) and (F): Purified substrates [or MdfA-6H in (F)] (1 μg) were mixed with wild-type GlpG-6H (1 μg, WT), GlpG(S201A)-6H (1 μg, SA), or buffer alone (−) and incubated at 37 °C for 3 h or as indicated. Samples were analyzed by SDS−PAGE and Coomassie brilliant blue staining. F and C indicate full-length substrates and cleaved products, respectively, and A indicates alkaline phosphatase. (G) Positions of GlpG-catalyzed cleavages were identified by N-terminal sequencing and are indicated by arrows.

were shown to cleave proteins with a single transmembrane helix and that other intramembrane proteases require release of single transmembrane helices by site 1 cleavage (S1P) (*35*).

In the present report, we show that, in theory, GlpG is able to cleave multispanning membrane proteins (type III). Interestingly, although several truncated forms of MdfA were cleaved, GlpG did not interact or cleave the full-length protein neither *in vivo* nor *in vitro*. Moreover, truncated constructs that have an intact N-terminal half of the protein were also resistant to cleavage by GlpG *in vivo* (data not shown), suggesting that these constructs are inaccessible to the protease. This notion is consistent with structural data on proteins in this superfamily (MFS), which showed that they are composed of N- and C-terminal structurally autonomous domains (*36*). We therefore favor the possibility that the cleaved substrates must be loosely packed (or unfolded) (Figure 7). This notion is strongly supported by the observation that GlpG is coimmunoprecipitated *in vitro* with the truncated substrates but not with the full-length MdfA (Figure 5E).

The *in vivo* cleavage of the truncated MdfA constructs was identified at the periplasmic side of the membrane. This orientation of cleavage fits previous results obtained with all rhomboid substrates and correlates with the location of the GlpG active site with respect to the membrane (for review, see refs *6* and *37*). It is interesting that, *in vivo*, the MdfA constructs were cleaved by

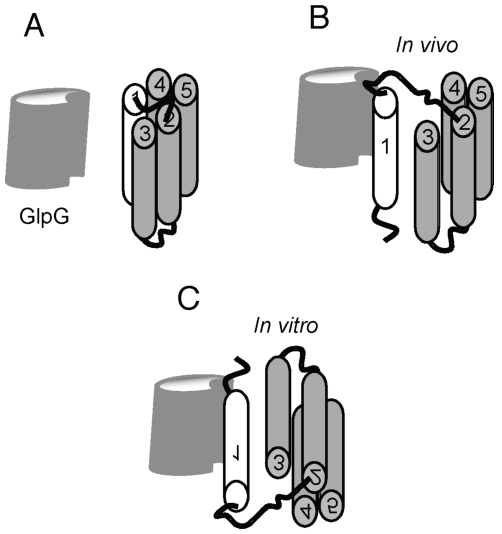

FIGURE 7: Hypothetical model for cleavage of truncated MdfA by GlpG in the membrane (A and B) and in detergent solution (C). (A) Folded membrane proteins are sterically excluded from the GlpG active site. (B) Unfolded membrane proteins might "release" single TMs (white) that gain accessibility to the protease substrate-binding pocket (*21*). (C) Cleavage in detergent solution, where the substrate probably enters the GlpG active site in an inverted topology.

GlpG at a membrane adjacent site that is amazingly similar to that identified in a model substrate containing TM2 of another MFS protein, LacY (*13*), despite the fact that there is no sequence similarity between LacY and MdfA in this region. It is possible, however, that both proteins share similar structural properties in the loop connecting TM1 and TM2 that enable functional interaction with GlpG. Moreover, it was shown that GlpG has a strong preference for small side chains such as those of alanine, serine, or glycine at the substrate cleavage site (*15*) as shown here for the cleavage site in the MdfA constructs and previously for other rhomboid substrates (*6*).

Cleavage of the truncated MdfA constructs *in vitro* in DDM solution occurred at a different location. According to the 3D model of MdfA, both cleavage sites (*in vivo* and *in vitro*) are located three or two residues away from the membrane (Figures 4C and 6G); however, the orientations of the cleavage sites with respect to the membrane are different. Whereas cleavage *in vivo* occurs in a periplasmic loop, the substrates are cleaved in their cytoplasmic N-terminal tail *in vitro*, implying that in detergent micelles the substrates enter the active site of GlpG in an inverted topology. The observation that GlpG is able to interact in opposite orientations with the truncated MdfA constructs exemplifies further its promiscuous nature.

As shown *in vitro* (Figure 6) and seemingly also *in vivo* (Figure 3B, right panel), GlpG-mediated proteolysis is extremely slow compared to soluble proteases. This phenomenon is known from previous *in vitro* studies of rhomboids' function (Figure 4 in ref *13*, Method section in ref *12*, Figure 4 in ref *11*). The reason for this slow kinetics is not readily clear but is likely to reflect either slow association with the substrate or the slow release of the product, processes that could be inherently different inside the membrane or in detergent micelles compared to aqueous solution. Structural data on the substrate−GlpG complex, which are not yet available, might help in better understanding of the slow kinetics.

In conclusion, the results presented here revealed that, in addition to their identified specific substrates, rhomboids have the capacity to cleave aberrant type III constructs of a membrane protein and therefore might participate in membrane protein recycling as proposed previously (*5*, *38*, *39*). This hypothesis will be studied further with cells defective in membrane protein biogenesis.

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SUPPORTING INFORMATION AVAILABLE

Table S1: Plasmids encoding GlpG derivatives, MdfA-PhoA hybrids, and MdfA-6H constructs. This material is available free of charge via the Internet at http://pubs.acs.org.

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