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The Retrotranslocation Protein Derlin-1 Binds Peptide:*N*-Glycanase to the Endoplasmic Reticulum

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The deglycosylating enzyme, peptide:*N*-glycanase, acts on misfolded N-linked glycoproteins dislocated from the endoplasmic reticulum (ER) to the cytosol. Deglycosylation has been demonstrated to occur at the ER membrane and in the cytosol. However, the mechanism of PNGase association with the ER membrane was unclear, because PNGase lacked the necessary signal to facilitate its incorporation in the ER membrane, nor was it known to bind to an integral ER protein. Using HeLa cells, we have identified a membrane protein that associates with PNGase, thereby bringing it in close proximity to the ER and providing accessibility to dislocating glycoproteins. This protein, Derlin-1, has recently been shown to mediate retrotranslocation of misfolded glycoproteins. In this study we demonstrate that Derlin-1 interacts with the N-terminal domain of PNGase via its cytosolic C-terminus. Moreover, we find PNGase distributed in two populations; ER-associated and free in the cytosol, which suggests the deglycosylation process can proceed at either site depending on the glycoprotein substrate.

INTRODUCTION

In eukaryotes proteins are synthesized on membrane-bound ribosomes and undergo a folding process in the lumen of the endoplasmic reticulum (ER; Ellgaard and Helenius, 2001, 2003). Misfolded or incompletely assembled multisubunit glycoproteins are recognized by the endoplasmic reticulum-associated degradation (ERAD) pathway regulated largely by their N-linked polymannose oligosaccharides. In this quality control system, lectin-like molecular chaperones, calnexin and calreticulin, recognize the $Glc_1Man_9GlcNAc_2$ oligosaccharide and assist in folding of newly synthesized glycoproteins. Lectin interaction with $Glc_1Man_9GlcNAc_2$ glycans after trimming with ER alpha-glucosidases, and alpha-mannosidases sorts out persistently unfolded glycoproteins for N-deglycosylation and degradation by the proteasome (Cresswell and Hughes, 1997; Kopito, 1997; Brodsky and McCracken, 1999; Plemper and Wolf, 1999; Romisch, 1999; Spiro, 2004).

The mannose trimming of *N*-linked glycans plays an important role in the ERAD of glycoproteins (Spiro, 2004). In both yeast and human cells, it is reported that the misfolded glycoproteins in the ER are degraded through ERAD only after the glycan is trimmed to the Man8B form, while misfolded proteins stay within the ER when they retain the Man9 form of oligosaccharides. Accordingly, it was assumed that there existed a Man8-binding lectin, later identified as EDEM, that recognizes misfolded glycoproteins in the Man8B form in the ER and directs them to the ERAD pathway (Hosokawa *et al.*, 2001). The misfolded glycoproteins exit the ER via a multiprotein complex referred to as a retrotranslocon or dislocon (Plemper *et al.*, 1997; Bebok *et al.*, 1998; de Virgilio *et al.*, 1998; Plemper *et al.*, 1999; Tsai *et al.*, 2002). In alternative models both PNGase and proteasomes may be either free in the cytosol or ER membrane imbedded or attached (Spiro, 2004).

A well-studied model for the ERAD pathway utilizes the human cytomegalovirus, which affects the MHC class I antigen presentation. The virally encoded proteins US2 and US11 act on the misfolded MHC class I heavy chains (HC) and catalyze their exit from ER to the cytosol. Earlier work exploiting this system demonstrated a retrotranslocation channel consisting of Sec61 complex that catalyzed the US2-mediated dislocation of MHC class I HC (Wiertz *et al.*, 1996; Ye *et al.*, 2001). Very recently Sec61 channel has also been demonstrated to be a membrane receptor for the proteasome (Kalies *et al.*, 2005). Recently, another mechanism for dislocation has been described, comprising of an ER membrane protein Derlin-1, which dislocates MHC class I HC in conjunction with p97, Ufd1, and Np14 (Lilley and Ploegh, 2004; Ye *et al.*, 2004). The action of Derlin-1 is believed to be specific for US11-dependent class I HC dislocation, as it is neither found to interact with US2 nor catalyze US2-dependent class I HC dislocation. (Lilley and Ploegh, 2004).

After exit from the ER lumen, HCs encounter the deglycosylating peptide:*N*-glycanase (PNGase; Suzuki *et al.*, 2000) and consequently are deglycosylated at the ER membrane or in the cytosol (Misaghi *et al.*, 2004). Although deglycosylation is not an absolute requirement, it is thought to facilitate proteasomal degradation by providing increased accessibility to the proteasome pore by removal of the bulky glycan chains from misfolded glycoproteins (Hirsch *et al.*, 2003). Therefore, it seems that PNGase can function upstream of the proteasome and play an important role in turnover of misfolded N-linked glycoproteins. Reports on the cellular localization of PNGase are conflicting. PNGase has been reported to localize in the cytosol (Suzuki *et al.*, 1998), ER

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surface (Suzuki *et al.*, 1997), and the lumen of the ER (Weng and Spiro, 1997). Previous studies showed that glycosylated proteins or deglycosylated intermediates are predominantly associated with the microsomes rather than with the cytosol (Kitzmuller *et al.*, 2003). The observation that PNGase is found in the cytosol and associated with the ER (Katiyar *et al.*, 2004) can be explained by the presence of alternative ERAD routes, with deglycosylation either coupled to or independent of retrotranslocation (Misaghi *et al.*, 2004). However, PNGase lacks any signal that would facilitate its association with the ER membrane and allow it to act coretrotranslocationally. Moreover, a possible membrane-integrated receptor for PNGase has yet to be identified. A recent finding that Derlin-1 mediates translocation of HCs to the cytosol prompted us to explore the possible interaction of PNGase with Derlin-1 because HCs are known to be substrates for PNGase (Wiertz *et al.*, 1996; Shamu *et al.*, 1999, 2001; Blom *et al.*, 2004).

Derlin-1 is a 22-kDa ER membrane protein consisting of four transmembrane domains with both the amino and carboxy termini in the cytosol (Lilley and Ploegh, 2004; Ye *et al.*, 2004). Derlin-1 interacts with a cytosolic AAA ATPase, p97 *via* VIMP (VCP-interacting membrane protein; Ye *et al.*, 2004). p97 is proposed to aid in extracting the misfolded glycoproteins from the ER to the cytosol for degradation by the proteasome. Our objective was to study the possible interaction between PNGase and Derlin-1. We used the techniques of coimmunoprecipitation, gel-filtration, and GST binding assays. Full-length PNGase and Derlin-1 were found to coimmunoprecipitate. Moreover, the N-terminal domain of PNGase and the C-terminus of Derlin-1 were required for the interaction of the two proteins. GST-binding assays confirmed the direct interaction of PNGase with Derlin-1. We have also shown that PNGase is present in two forms, membrane-associated and free in the cytosol. The membrane-associated PNGase coelutes with Derlin-1 upon gel filtration. Taken together, all of these findings suggest that the PNGase associated with the ER via Derlin-1 catalyzes the deglycosylation of a subset of glycoprotein substrates in a coretrotranslocational manner.

MATERIALS AND METHODS

Plasmid Constructs

Human Derlin-1 in a pEGFP vector was a gift from Dr. Hidde Ploegh (Harvard Medical School, Boston, MA). PCR reactions were performed to generate various fragments (Δ187–251, Δ1–16, Δ1–154, Δ1–187, Δ44–251) having 5′ *Bam*HI and 3′ *Eco*RI′ site. Full-length Derlin-1 was also cloned into pcDNA 4.0 vector containing a *Myc*-tag at the C-terminus using 5′ *Eco*RI and 3′ *Xho*I sites. To synthesize mPNG1-GFP fusion plasmid for expression in mammalian cell culture, the mPNG1 plasmid was amplified by PCR to introduce a 5′ *Eco*RI and 3′ *Sal*I site. After digestion and purification, full-length mPNG1 was subcloned into a pEGFP-C1 plasmid (CLONTECH, Palo Alto, CA) to generate a C-terminal GFP fusion. Truncation constructs of mPNG1 (ΔMC, ΔC, ΔNC, ΔN, ΔMN; see Figure 5A) were also subcloned into pEGFP-C1 vector. Full-length mPNG1 and truncations (ΔMC, ΔC, ΔNC, ΔN, ΔMN) were also subcloned into a pcDNA3.0 vector with an HA-tag at the C-terminus. The HA-tag (HA_3) was introduced in pcDNA3.0 using 5′ *Not*I and 3′ *Xho*I sites. Truncation constructs obtained after PCR reactions using 5′ *Eco*RI and 3′ *Not*I were ligated into pcDNA3.0 containing a HA-tag at the C-terminus.

Histidine-tagged mPNG1 (mPNG1-His_6) and ΔMC-mPNG1-His_6 constructs for *Escherichia coli* expression in pET-28a(+) were made as described previously (Park *et al.*, 2001). A GST-Derlin-1-C fusion plasmid was constructed to have the C-terminal 64 amino acids of Derlin-1 fused to glutathione *S*-transferase (GST) at its N-terminus in order to carry out in vitro GST pulldown assays.

Mammalian Cell Culture and Transfection

HeLa (R19) cell monolayers were maintained in DMEM (GIBCO-BRL, Rockville MD) supplemented with 10% bovine calf serum (BCS) and 100 μg/ml each of penicillin and streptomycin (GIBCO-BRL) at 37°C. Transfections of plasmid-DNAs were performed by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in DMEM supplemented with 10% BCS without antibiotics according to the manufacturer's protocol.

Immunoprecipitation and Immunoblotting

Immunoprecipitations were performed with 2×10^7 cells. Twenty-four hours after transfection cells were harvested, washed with phosphate-buffered saline (PBS; GIBCO), and lysed in digitonin buffer (1% digitonin, 25 mM Tris.HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$ with 1 mM phenylmethylsulfonyl fluoride [PMSF] and protease inhibitor cocktail; Pierce, Rockford, IL) for 1 h at 4°C. The cell lysate was centrifuged for 15 min at 14,000 rpm. The supernatant obtained was incubated with desired antibody for 15 min at room temperature followed by the addition of protein A/G agarose beads as required and incubated overnight at 4°C. The beads were washed three times in buffer containing 0.2% digitonin, 10 mM Tris.HCl, pH 7.4, 150 mM NaCl and 5 mM EDTA. Immune complexes on beads were eluted with 2× SDS/PAGE sample buffer and analyzed by SDS-PAGE followed by immunoblotting. Most of the antibodies used in this study were obtained from commercial sources. Mouse monoclonal anti-GFP and anti-HA antibodies were purchased from CLONTECH, mouse monoclonal anti-calnexin, mouse monoclonal anti-α-tubulin, and rabbit polyclonal anti-GFP were obtained from Abcam (Cambridge, MA) and rabbit polyclonal anti-HA, anti-His, and anti-Myc, mouse monoclonal anti-GST and anti-Myc were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-calnexin was a gift from Dr. D. Brown (State University of New York at Stony Brook, NY). For immunoprecipitation antibodies were used at a dilution of 1:500. For immunoblotting 1:3000 dilution was used for primary as well as for HRP-conjugated secondary antibodies.

Immunofluorescence

Cells were fixed in 3% paraformaldehyde for 20 min at room temperature and permeabilized in 0.5% Triton X-100 on ice for 7 min. Cells were washed in PBS plus 0.5% NGS and were incubated with the primary antibodies as indicated (HA 1:200 dilution; calnexin 1:100 dilution). AF-488- or AF-555-conjugated secondary antibodies were used as required. Nuclear DNA was stained with 4′,6-diaminidino-2-phenyindole (DAPI). Cells were analyzed using confocal microscope (Zeiss, Thornwood, NY) to visualize the proteins under study.

Overexpression and Purification of Constructs

mPNG1-$(His)_6$ and ΔMC-mPNG1-$(His)_6$ constructs were transformed into *E. coli* BL21(DE3)pLysS cells. The cells were grown in LB media at 37°C and expression was induced by adding 1 mM isopropyl β-D-thiogalactoside (IPTG) when the cells reached an absorbance (A_{600}) of 0.6–0.8. After 3 h of induction at 30°C the cells were harvested and resuspended in lysis buffer containing 1× PBS, 1% Triton X-100, 10 mM β-mercaptoethanol (βMe), and 1 mM PMSF followed by sonication on ice using a Branson sonicator. The cell extract was centrifuged and supernatant was allowed to pass through chelating Sepharose column obtained from Amersham Biosciences (Piscataway, NJ) charged with Ni^{++}. Subsequent purification was performed according to the manufacturer's protocol. Purity of the eluted fractions was analyzed by SDS-PAGE.

GST-Derlin-1-C was transformed in *E. coli* DH5α cells and expression was induced by addition of 0.1 mM IPTG to the culture. After induction at 30°C for 3 h cells were lysed in buffer containing 1× PBS, 1% Triton X-100, 10 mM βMe, and 1 mM PMSF. The lysate was centrifuged at 14,000 rpm and the supernatant was added to glutathione agarose beads. After a 2-h incubation at 4°C, the beads were then washed three times with the lysis buffer. The bound protein was then eluted in buffer containing 20 mM reduced glutathione (GSH) in 50 mM Tris-HCl, pH 8.0. The same elution conditions were used to purify GST alone. Pure protein was analyzed on SDS-PAGE. Eluted proteins were subjected to buffer exchange (1× PBS, 10 mM βMe, and 1 mM PMSF) using a Centricon from Millipore (Billerica, MA).

Gel Filtration

Mammalian cell lysate was prepared in digitonin lysis buffer (1% digitonin, 25 mM Tris.HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$ with 1 mM PMSF and protease inhibitor cocktail). The total cell lysate was loaded on an analytical size Superdex 200 HR 10/30 (Amersham Pharmacia) size exclusion chromatography column (preequilibrated with digitonin lysis buffer) and 0.6-ml fractions were collected. Eluted proteins in fractions were subjected to immunoprecipitation using monoclonal anti-HA antibody followed by immunoblotting with polyclonal anti-HA and polyclonal anti-GFP antibodies. Gel filtration was also performed to detect interaction between ΔMC-mPNG1-His_6 and GST-Derlin-1-C. ΔMC-mPNG1-His_6 and GST-Derlin-1-C (1× PBS, 1% Triton X-100, 10 mM βMe, and 1 mM PMSF) were mixed at a 1:1 M ratio and incubated for 2 h at room temperature. Samples were analyzed on Superdex 200 HR 10/30 as described above. Eluted proteins in fractions were resolved on SDS-PAGE followed by immunoblotting with the indicated antibodies.

In vitro Binding Assay

GST alone or GST-Derlin-1-C fusion protein extracts (1 ml) were incubated with 30 μl (bed volume) of GSH-agarose beads for 1 h in binding buffer (1× PBS, 1% Triton X-100, 10 mM βMe, and 1 mM PMSF) at room temperature, washed five times with binding buffer, and incubated with full-length mPng1p-His_6 or ΔMC-mPng1p-His_6 in binding buffer for 1 h at RT. Beads were washed five times in binding buffer followed by elution of the bound proteins with SDS-PAGE sample buffer. Eluted proteins were resolved on SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with polyclonal anti-His or monoclonal anti-GST antibody.

Subcellular Fractionation and PNGase Activity Assay

Subcellular fractionation was performed on a sucrose gradient with 5×10^7 HeLa cells after lysing in lysis buffer as described earlier (Baker *et al.,* 1990). The ER membranes sedimenting at the interphase of 1.2 and 1.5 M sucrose were collected, washed, pelleted, and resuspended in 1 ml digitonin lysis buffer (containing 1% digitonin, 25 mM Tris.HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$ with 1 mM PMSF and protease inhibitor cocktail) for 1 h at 4°C and subjected to immunoprecipitation with anti-HA and anti-GFP antibodies to detect the distribution of PNGase and Derlin-1 in HeLa cells. Immunoprecipitation was also performed on the microsome-free cytosolic fraction and total cell lysate obtained from 5×10^7 cells in digitonin lysis buffer (1 ml). Immune complexes were recovered from digitonin lysates using Protein A/G agarose beads and were washed in 0.2% digitonin in 10 mM Tris.HCl, pH 7.4, 150 mM NaCl, and 5 mM EDTA. Immune complexes were resolved on SDS-PAGE, transferred to nitrocellulose, and probed with the specified antibody. Subcellular fractions were also subjected to Western blotting with anti-calnexin (mouse monoclonal) as an ER marker and anti-α-tubulin (mouse monoclonal) as a cytosolic marker.

The total cell lysate (10 μL), microsome free cytosol (10 μL) and ER membranes (10 μL) were assayed for PNGase activity using a fetuin-derived asialoglycopeptide I ($([^{14}C]CH3)_2$Leu-Asn($GlcNAc_5Man_3$-Gal_3)-Asp-Ser-Arg) as substrate as previously described (Suzuki *et al.,* 1994, 1998). Radioactivity was monitored on a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and quantitated by using ImageQuant (version 1.2).

RESULTS

A Membrane Receptor for PNGase

Several previous studies have reported that at least a portion of PNGase is associated with the ER based on biochemical analysis as well as enzyme assays (see *Introduction*). PNGase has been shown to participate in the deglycosylation of MHC class 1 HCs and a recent report revealed that deglycosylation of HCs occurred in association with the ER membrane as well as in the cytosolic fractions (Lilley and Ploegh, 2004). A retrotranslocation channel formed by an ER transmembrane protein, Derlin-1, is believed to be involved in the dislocation of MHC class I HCs from the ER to the cytosol. Deglycosylation of HCs that are still associated with the ER membrane fraction raised the possibility that the deglycosylation was mediated by membrane associated PNGase. However, PNGase lacks the signals expected to be necessary for its association with the ER.

To identify a possible ER membrane-associated receptor protein for PNGase we studied human Derlin-1 (hDerlin-1), an ER membrane protein that contains four transmembrane segments, with both its N- and C-termini in the cytosol (Figure 1A; Lilley and Ploegh, 2004; Ye *et al.,* 2004). We used the mouse homolog of PNGase (mPNGase) because mouse and human homologues of PNGase are highly conserved proteins (Suzuki *et al.,* 2000). Henceforth, hDerlin-1 and mPNGase are referred as Derlin-1 and PNGase, respectively. To analyze a possible interaction between PNGase and Derlin-1, HeLa cells, cotransfected with pcDNA 3.0 vector and Derlin-1-GFP (lane 1), pEGFP vector alone and PNGase-HA (lane 2), or PNGase-HA and Derlin-1-GFP (lane 3), were lysed in 1% digitonin buffer and the total cell lysate was subjected to immunoprecipitation with monoclonal anti-HA antibody or polyclonal anti-GFP antibody. The immunoprecipitation was subjected to SDS/PAGE followed by immunoblotting using antibodies indicated in Figure 1B. The results in Figure 1B, lane 3, show that PNGase and Derlin-1 coprecipitate. No precipitation was observed with each of the vectors alone (lane 1 and 2). In a previous report GFP-tagged Derlin-1 was used as a dominant negative construct based on the fact that the addition of a folded domain to a flexible cytoplasmic tail of Derlin-1 would interfere with interactions on the cytoplasmic face of ER and consequently would block dislocation of HCs from the ER to the cytosol (Lilley and Ploegh, 2004). To rule out the possibility of any nonspecific interaction of PNGase with Derlin-1-GFP on the cytoplasmic face of ER, we used another construct of Derlin-1 containing a Myc-tag at the C-terminus (Derlin-1-Myc) and GFP-tagged PNGase (PNGase-GFP). As shown in Figure 1C, lane 3, Derlin-1-Myc and PNGase-GFP were found to interact with each other as detected by coimmunoprecipitation and immunoblotting (Figure 1C, lane 3). Therefore, the epitope tag did not interfere with immunoprecipitation. The pcDNA 4.0 vector alone and pEGFP vector alone did not coprecipitate with PNGase-GFP and Derlin-1-Myc, respectively (Figure 1C, lanes 1 and 2). To confirm that PNGase and Derlin-1 interact within the cells before lysis and not during the coimmunoprecipitation procedure, PNGase-HA and Derlin-1-GFP were separately expressed in HeLa cells. Cells expressing PNGase-HA were lysed and mixed with a lysate from cells expressing Derlin-1-GFP. The mixed lysate was subjected to overnight immunoprecipitation with either mono-HA or mono-GFP antibodies using the same conditions as previously described. Immunoblots with polyclonal anti-HA and anti-GFP antibodies demonstrated that PNGase-HA and Derlin-1-GFP did not coimmunoprecipitate when both the proteins were expressed separately and mixed together after cell lysis (Supplementary Figure 1). Thus postlysis interactions between PNGase-HA and Derlin-1-GFP did not account for the coimmunoprecipitation results.

To further rule out the possibility of a nonspecific interaction between PNGase and an ER membrane protein, we tested for interaction between PNGase and the ER membrane protein calnexin. As shown in Figure 1D, PNGase was unable to coimmunoprecipitate calnexin. Together, these data suggest that Derlin-1 specifically interacts with PNGase and forms a complex with it in close proximity to the ER.

Derlin-1 Interacts with PNGase via Its Cytosolic Domain at the C-terminus

As shown in Figure 1A, both the N- and C-termini of Derlin-1 are in the cytosol (Lilley and Ploegh, 2004), we therefore expected that the association of PNGase would occur either through the cytosolic N- or C-terminus of Derlin-1. To determine the domain of Derlin-1 responsible for mediating interaction with PNGase a variety of Derlin-1 truncation constructs were prepared (Supplementary Figure 2). The expression and localization of these was studied by immunofluorescence microscopy. As shown in Supplementary Figure 3, A–D, all constructs except Derlin-1-GFPΔ187–251 were mislocalized in HeLa cells; no colocalization was observed with PNGase-HA. Derlin-1-GFPΔ187–251 showed a perinuclear localization, similar to the wild type. However no colocalization was observed with PNGase-HA (Supplementary Figure 3E), suggesting that cytosolic C-terminus of Derlin-1 is required to bring PNGase in close proximity to the ER. On Western blot analysis we found that Derlin-1-GFPΔ187–251 was expressed to the same level as full-length Derlin-1 (Figure 2B, lane 4, right panel), whereas the expression of the other constructs could not be detected (unpublished data). We therefore used this construct for our subsequent studies.

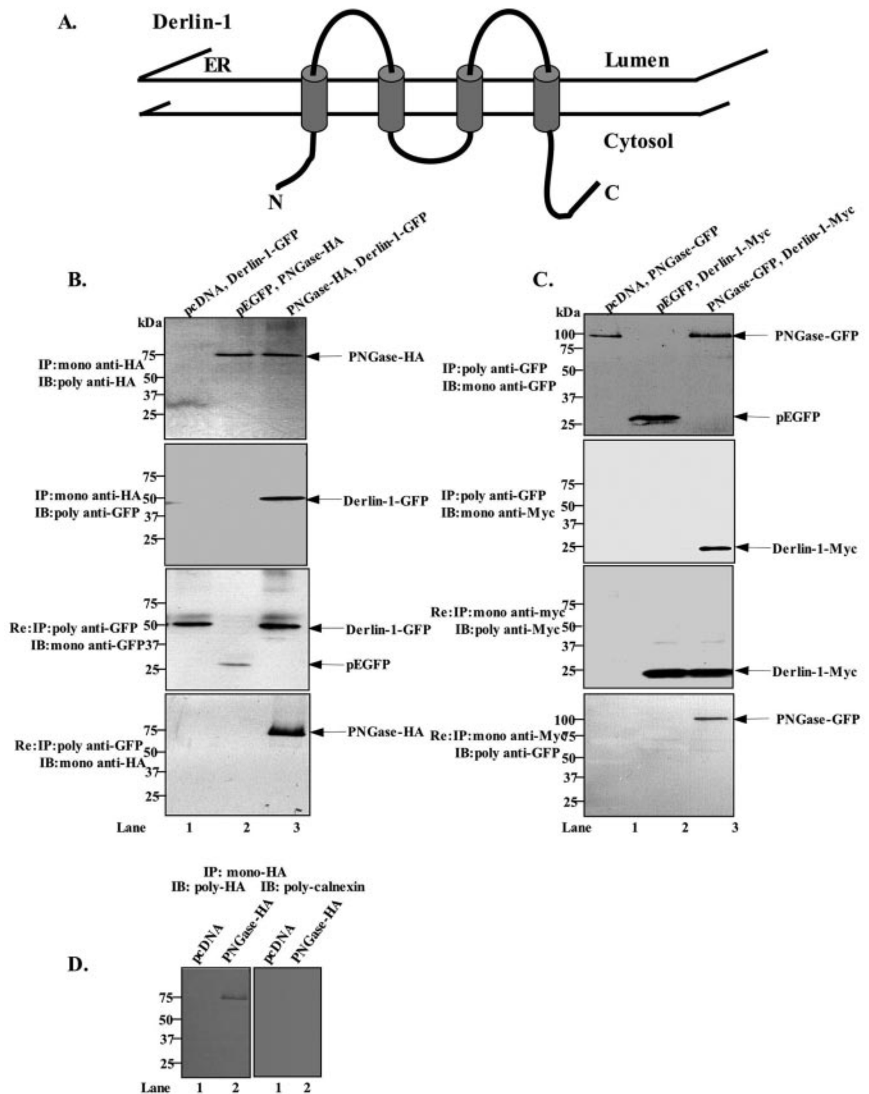

Figure 1. Association of PNGase with Derlin-1 in vivo. (A) Topology of Derlin-1 containing four transmembrane domains with both the N- and C-termini in the cytosol. (B) Interaction of PNGase with Derlin-1 analyzed by coimmunoprecipitation. HeLa cells were cotransfected with pcDNA vector and Derlin-1-GFP (lane 1), pEGFP and PNGase-HA (lane 2), or PNGase-HA and Derlin-1-GFP (lane 3) and immunoprecipitated with monoclonal anti-HA antibody. Reverse immunoprecipitation was also performed with polyclonal anti-GFP antibody. Immune complexes obtained were resolved on SDS-PAGE and immunoblotted with antibodies as indicated. (C) Epitope tag has no effect on the interaction of PNGase with Derlin-1. HeLa cells were cotransfected with pcDNA vector and PNGase-GFP (lane 1), pEGFP and Derlin-1-Myc (lane 2), or PNGase-GFP and Derlin-1-Myc (lane 3) and immunoprecipitated with monoclonal anti-Myc antibody. Reverse immunoprecipitation was carried out with polyclonal anti-GFP antibody. Immune complexes obtained were resolved on SDS-PAGE and immunoblotted with antibodies as indicated. (D) Calnexin, an ER membrane protein, did not coprecipitate with PNGase. HeLa cells transfected with pcDNA vector alone (lane 1) and PNGase-HA (lane 2) were immunoprecipitated with monoclonal anti-HA antibody, resolved on SDS-PAGE, and immunoblotted with polyclonal anti-HA and polyclonal anti-calnexin antibodies as indicated.

HeLa cells cotransfected with the pcDNA 3.0 vector alone and Derlin-1-GFP (Figure 2B, lane 1, left and right panels), pEGFP and PNGase-HA (Figure 2B, lane 2, left and right panels), PNGase-HA and Derlin-1-GFP (Figure 2B, lane 3, left and right panels) or PNGase-HA and Derlin-1-Δ187–251GFP (Figure 2B, lane 4, left and right panels) were lysed in digitonin buffer and subjected to immunoprecipitation with monoclonal anti-HA antibody (left panel) or to reverse immunoprecipitation with polyclonal anti-GFP antibody (right panel). The immune complex was recovered on Protein A/G agarose beads, after elution with SDS-PAGE sample buffer, and then analyzed by SDS/PAGE. Immunoblotting revealed the coprecipitation of full-length PNGase-HA and Derlin-1-GFP (Figure 2B, lane 3, left and right panels). Neither the pEGFP nor pcDNA 3.0 vectors alone coprecipitated with either PNG1-HA or Derlin-1-GFP, respectively (Figure 2B, lanes 1 and 2, left and right panels). No coprecipitation of PNG1-HA was observed with Derlin-Δ187–251GFP (Figure 2B, lane 4, left and right panels). This result provides evidence that the C-terminus of Derlin-1 is essential for interaction with PNGase.

Localization of PNGase and Derlin-1

In an earlier study examining the distribution of endogenous PNGase we observed extensive colocalization of PNGase with calnexin, a well-known ER marker (Katiyar *et al.,* 2004). Overexpressed full-length PNGase-HA and Derlin-1-GFP were found to be colocalized with each other around the ER (Figure 3A), although the presence of cytoplasmic PNGase was also observed. The localization pattern of over expressed PNGase in this study is therefore consistent to that observed for the endogenous protein (Katiyar *et al.,* 2004). As shown in Figure 3, B and C, overexpressed PNGase-HA and Derlin-1-GFP were also found to colocalize with the ER membrane protein, calnexin, included as an ER marker. Previously it has been demonstrated that Derlin-1 colocalized with calnexin (Lilley and Ploegh, 2004). The pEGFP vector alone (control) did not show any colocalization with PNGase (Figure 3D; high-resolution black and white images for the localization studies are shown in Supplementary Figure 4).

A Small Fraction of PNGase Associates with the ER

Next we undertook to confirm the interaction of PNGase with Derlin-1 using gel filtration. HeLa cells were cotransfected with

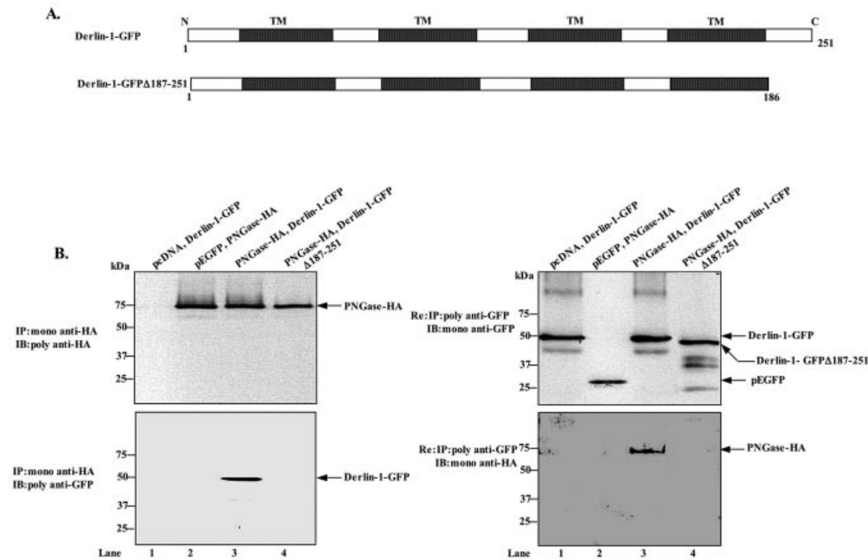

Figure 2. The cytosolic C-terminus of Derlin-1 interacts with PNGase. (A) Constructs of Derlin-1-GFP used in this study. (B) Co-immunoprecipitation of PNGase-HA with Derlin-1-GFP: HeLa cell lysate coexpressing pcDNA vector and Derlin-1-GFP (lane 1), pEGFP vector and PNGase-HA (lane 2), PNGase-HA and Derlin-1-GFP (lane 3), or PNGase-HA and Derlin-1-GFPΔ187–251 (lane 4) were immunoprecipitated with monoclonal anti-HA antibody (left panel) or reverse immunoprecipitation with polyclonal anti-GFP antibody (right panel). Immune complexes were resolved on SDS-PAGE, and immunoblotting was performed with antibodies as indicated.

PNGase-HA and Derlin-1-GFP followed by extraction of proteins in digitonin buffer. The total cell lysate was then loaded on a Superdex 200 HR 10/30 size exclusion chromatography column that was preequilibrated with digitonin buffer (Park *et al.,* 2001). Individual fractions collected were then immunoprecipitated with monoclonal anti-HA in order to concentrate PNGase-HA and to detect if any Derlin-1-GFP was interacting with it. This immunoprecipitate was analyzed by SDS/PAGE and immunoblotting with polyclonal anti-HA and polyclonal anti-GFP to detect PNGase HA and Derlin-1 GFP, respectively. PNGase and Derlin-1 were present in fractions 15–21 (Figure 4A). The profile revealed the coelution and coprecipitation of a small fraction of PNGase with the bulk of the Derlin-1. Most of the PNGase was present in fractions 22–29, which contained no detectable Derlin-1. Thus, these data suggest that not only are there two populations of PNGase, membrane-associated and free, but also that both Derlin-1 and PNGase are associated with each other.

Next we examined the distribution of PNGase-HA and Derlin-1-GFP in HeLa cells by subcellular fractionation. Subcellular fractionation was performed on 5×10^7 cells as described in experimental procedures. The same volume of digitonin buffer was used for each fraction in order to compare the distribution of PNGase and Derlin-1 in the membrane free cytosol and the ER membrane fractions. The total cell lysate obtained from 5×10^7 cells was used to estimate the total protein amount. All fractions, namely, the total cell lysate, membrane free cytosol or ER membrane fraction, were immunoprecipitated with anti-HA or anti-GFP followed by SDS/PAGE and immunoblotting. The same fractions were analyzed to detect calnexin as a marker for the ER and α-tubulin as a marker for cytosol. The results in Figure 4B, lane 1, shows that Derlin-1 and PNGase along with the subcellular markers calnexin and tubulin were present in the total cell lysate, as expected. The bulk of PNGase was found in the membrane free cytosolic fraction (Figure 4B, top left panel, lane 2); much less PNGase was detected in the membrane fraction (Figure 4B, top left panel, lane 3). In contrast, almost all of the Derlin-1 was present in the membrane fraction (Figure 4B, bottom left panel, lane 3) with virtually none in the membrane free cytosol (Figure 4B, bottom left panel, lane 2). Calnexin showed the same distribution as Derlin-1, with all of it in the ER membrane fraction (Figure 4B, top right panel, lane 3) and none in the cytosolic fraction (Figure 4B, top right panel, lane 2). Tubulin as a marker for cytosol was also analyzed in all three fractions as shown in Figure 4B, bottom right panel. As expected, no tubulin was detected in the ER membrane fraction, excluding the possibility of cytosolic contamination.

Because we immunologically established the presence of PNGase in the membrane free cytosol and ER membrane fraction, we then determined the level of enzyme activity of PNGase in both fractions. As shown in Figure 4C, PNGase activity (using ^{14}C-labeled glycopeptide as a substrate) was detected in total cell lysate, membrane free cytosol, and ER membrane fraction. Maximum activity was found in the total cell lysate (Figure 4C, lane 2) followed by the cytosolic fraction (Figure 4C, lane 3), whereas the lowest level of activity was detected in ER membrane fraction (Figure 4C, lane 4). As observed in lane 1, no PNGase activity was detected in the cell extract expressing pcDNA 3.0 vector alone. Endogenous PNGase activity could not be detected in cells expressing pcDNA 3.0 vector alone under the experimental conditions used because only 10 μl of 1.0 ml was used in the assay. Additionally, no PNGase activity was detected in a mutant PNGase where a catalytic cysteine is mutated to alanine (Katiyar *et al.,* 2004), included as a negative control. Thus the data obtained from subcellular fractionation and PNGase activity assay were found to be consistent.

The N-terminus of PNGase Interacts with Derlin-1

To determine the domain of PNGase interacting with Derlin-1, we evaluated a collection of PNGase constructs containing different domains with GFP-tag at the C-terminus as shown in Figure 5A. The N-terminus contains a highly conserved PUB (Peptide:*N*-glycanase/UBA or UBX-containing protein domains; Suzuki *et al.,* 2001a, 2001b) or PUG (Peptide:*N*-glycanase and other putative nuclear UBA or UBX domains; Doerks *et al.,* 2002) domain implicated in protein-protein interactions. The functional significance of the C-terminal extension of PNGase in higher eukaryotes, as compared with yeast, is not yet known. As shown in Figure 5A, six constructs of PNGase with different domain boundaries, N-terminus alone (ΔMC), N-terminus and M-domain (ΔC), M-domain alone (ΔNC), M-domain and C-termini (ΔN) as well as C-terminus alone (ΔNM), were prepared so as to

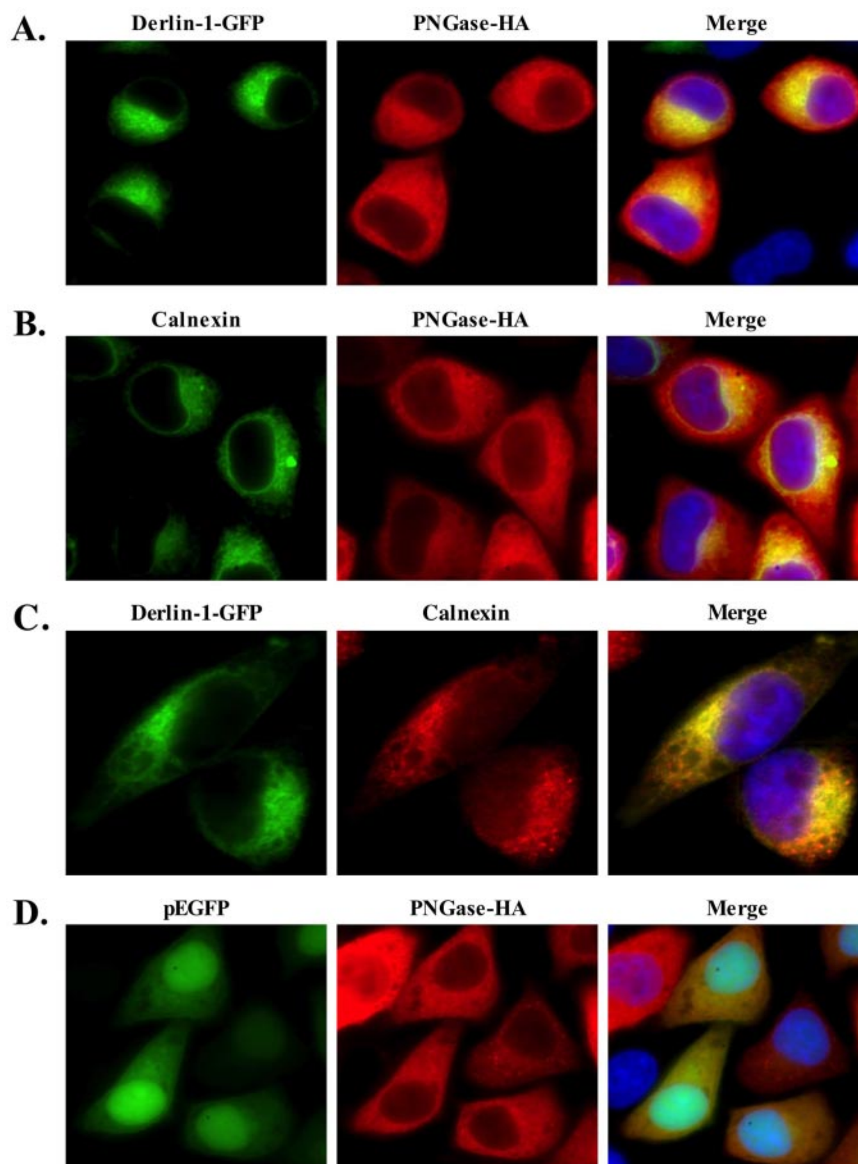

Figure 3. PNGase colocalizes with Derlin-1. HeLa cells were expressed with the plasmids as indicated, stained with the corresponding antibodies, and visualized by fluorescent microscopy. (A) Colocalization of PNGase-HA with Derlin-1: Derlin-1 staining (green), PNGase-HA staining (red), merge of PNGase-HA and Derlin-1 (yellow shows the colocalization of two proteins). (B) Colocalization of PNGase-HA with calnexin: calnexin staining (green), PNGase-HA staining (red), merge of PNGase-HA and calnexin (yellow shows the colocalization of two proteins). (C) Colocalization of Derlin-1 with calnexin: Derlin-1 staining (green), calnexin staining (red), merge of Derlin-1 and calnexin (yellow shows the colocalization of two proteins). (D) Colocalization of pEGFP vector alone with PNGase-HA: pEGFP staining (green), PNGase-HA staining (red), merge of pEGFP vector alone and PNGase-HA. DAPI (blue) shows nuclear staining.

determine which domain of PNGase was capable of interacting with Derlin-1.

The constructs were coexpressed with Derlin-1-Myc in HeLa cells. Cells were lysed in digitonin buffer and coimmunoprecipitation in the total cell extract was performed with monoclonal anti-Myc antibody as well as reverse co-immunoprecipitation with polyclonal anti-GFP antibody. Samples were subjected to SDS/PAGE followed by immunoblotting. As shown in Figure 5B, HeLa cells coexpressing pcDNA 4.0 vector and PNGase-GFP showed no interaction (Figure 5B, lane 1), which served as a negative control. This was further confirmed when no coprecipitation was observed by coexpression of pEGFP vector alone and Derlin-1-Myc (Figure 5B, lane 2). As expected using anti-Myc-antibody, full-length PNGase-GFP coimmunoprecipitated with Derlin-1-Myc; the same was observed in the reverse-immunoprecipitation with anti-GFP antibody (Figure 5B, lane 3). Given that PNGase has a PUB/PUG domain at the N-terminus, we suspected that it might be responsible for the interaction with Derlin-1. This was indeed the case, because the N-terminus containing construct ΔMCPNGase-GFP coprecipitated with Derlin-1-Myc in both immunoprecipitation with anti-Myc antibody (Figure 5B, lane 4) and the reverse immunoprecipitation with anti-GFP antibody (Figure 5B, lane 4). The PNGase-GFP construct with both the N- and M-domains also was found to interact with Derlin-1-Myc (Figure 5B, lane 5). On the contrary, PNGase-GFP constructs lacking the N-terminal segments (ΔNC, ΔN, and ΔNM) failed to interact with Derlin-1-Myc (Figure 5B, lanes 6–8). Similar results were obtained with PNGase constructs containing HA-tag at the C-terminus and GFP-tagged Derlin-1 (unpublished data).

In Vitro GST-binding Assay and Gel Filtration Confirm the Interaction of the C-terminal Domain of Derlin-1 with the N-terminal Domain of PNGase

Although the interaction of PNGase with Derlin-1 in total cell lysate of HeLa cells was analyzed in coimmunoprecipitation experiments, these results do not exclude the possibility of an indirect interaction of PNGase with Derlin-1 via another partner. To test for direct interaction between PNGase and Derlin-1, we used a GST-binding assay. A

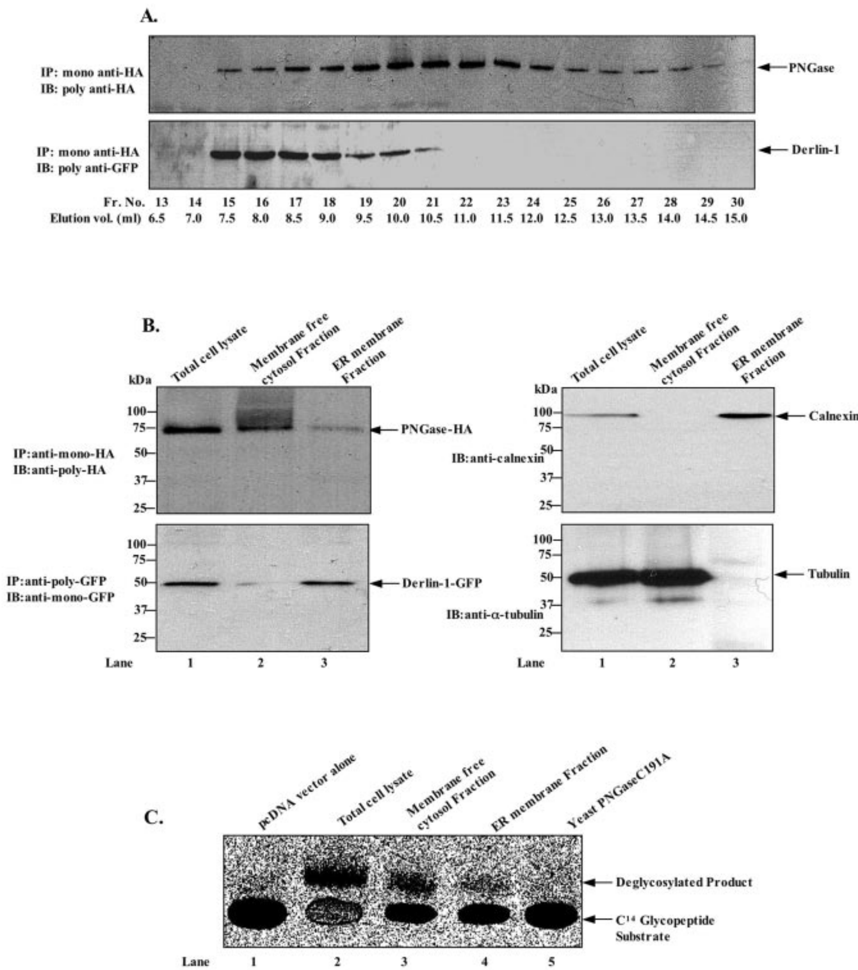

Figure 4. A small fraction of membrane-bound PNGase associates with Derlin-1. (A) HeLa cells coexpressing PNGase-HA and Derlin-1-GFP were lysed in 1% digitonin buffer, and the resulting cell lysate was subjected to Superdex 200 HR10/30 analytical size exclusion chromatography column preequilibrated with 1% digitonin buffer. Fractions obtained were subjected to coimmunoprecipitation with anti-HA (monoclonal) to detect interaction between PNGase and Derlin-1. The immunoprecipitates, bound on protein A/G agarose beads, were eluted in 2× SDS-PAGE sample buffer resolved on SDS-PAGE, and analyzed by immunoblotting with polyclonal anti-HA or monclonal anti-GFP. A minor fraction of PNGase coelutes with the bulk of Derlin-1 (fractions 15–20) followed by the elution of free PNGase (fractions 22–29). (B) Distribution of PNGase in HeLa cells. Subcellular fractionation was performed as described in *Materials and Methods.* Shown are HeLa cell lysate (lane 1), membrane free cytosolic fraction (lane 2), and ER membrane fraction (lane 3). Equal volumes of all the fractions were subjected to immunoprecipitation with monoclonal anti-HA (for PNGase-HA) and polyclonal anti-GFP (for Derlin-1-GFP) followed by SDS/PAGE and immunoblotting with antibodies as indicated. Anti-Calnexin antibody and anti-α-tubulin antibody were used as markers for ER and cytosol, respectively (top and bottom right panels, respectively). (C) PNGase enzyme activity in subcellular fractions. Activity assay for PNGase was performed using 10 μl of each fraction as described in *Materials and Methods.* Shown are pcDNA vector alone (lane 1), total cell lysate from HeLa cells (lane 2), membrane free cytosolic fraction (lane 3), ER membrane fraction (lane 4), and yeast PNGase mutant C191A (lane 5) used as a negative control.

fusion protein containing GST and the cytosolic domain of Derlin-1 (GST-Derlin-1-C) was bound on glutathione beads. Purified recombinant full-length PNGase-His (PNGase-His) or PNGase-His with the N-terminus alone (ΔMCPNGase-His) were incubated with GST-Derlin-1-C bound on GSH agarose beads. Purified PNGase-His and ΔMCPNGase-His are shown in Figure 6A, lanes 1 and 4. PNGase-His and ΔMCPNGase-His did not bind to GST alone (Figure 6A, lanes 2 and 5), whereas PNGase-His and ΔMCPNGase-His bound to GST-Derlin-1-C (Figure 6A, lanes 3 and 6). Thus, the in vitro GST-binding assay confirms the N-terminus of PNGase indeed binds directly to the cytosolic C-terminus of Derlin-1.

To exclude any possibility of artifacts in the GST-binding assay, we used gel filtration to confirm a direct physical interaction between ΔMCPNGase and GST-Derlin-1-C. For gel filtration, ΔMCPNGase and GST-Derlin-1-C proteins were purified to electrophoretic homogeneity as described in *Materials and Methods.* ΔMCPNGase and GST-Derlin-1-C, mixed at a 1:1 M ratio, eluted as a single peak on a size exclusion chromatography column. The elution volume and mass of peak components (ΔMCPNGase and GST-Derlin-1-C) was found to be consistent with the marker proteins analyzed on the same column and under the same experimental conditions. When the corresponding fractions were analyzed by SDS/PAGE and immunoblotted with anti-His or anti-GST, both proteins were detected in the same fractions indicating the formation of a complex (Figure 6B). For comparison, each of the proteins was analyzed individually on the chromatography column to determine their elution patterns. When these peaks were analyzed by the same method, the molecular weight of GST-Derlin-1-C (Figure 6C) was experimentally determined to be 30 kDa on SDS-PAGE analysis, and its elution volume was found consistent with the marker protein (carbonic anhydrase, 29 kDa: Elution volume 17.2 ml). However, the molecular weight of ΔMCPNGase (Figure 6D) was determined to be 20 kDa based on SDS-PAGE analysis but it showed an apparent molecular weight of 12.4 kDa (marker protein cytochrome c,12.4 kDa eluted at the same volume). This apparent molecular weight is significantly lower than the molecular weight assessed by SDS-PAGE. This discrepancy may be attributed to the compact size of this protein attained after folding. In any case, the complex containing both of the proteins eluted first, followed by the elution of GST-Derlin-1-C and ΔMCPNGase-His, as expected based on the size of complex and each of the proteins. These results further established that the N-terminus of PNGase alone can efficiently bind to the C-terminus of Derlin-1.

DISCUSSION

As discussed previously, PNGase has been reported to be localized in the cytosol (Suzuki *et al.*, 1998) and also reported to be associated with the ER membrane (Suzuki *et al.*, 1997; Katiyar *et al.*, 2004). Conflicting evidence also suggested

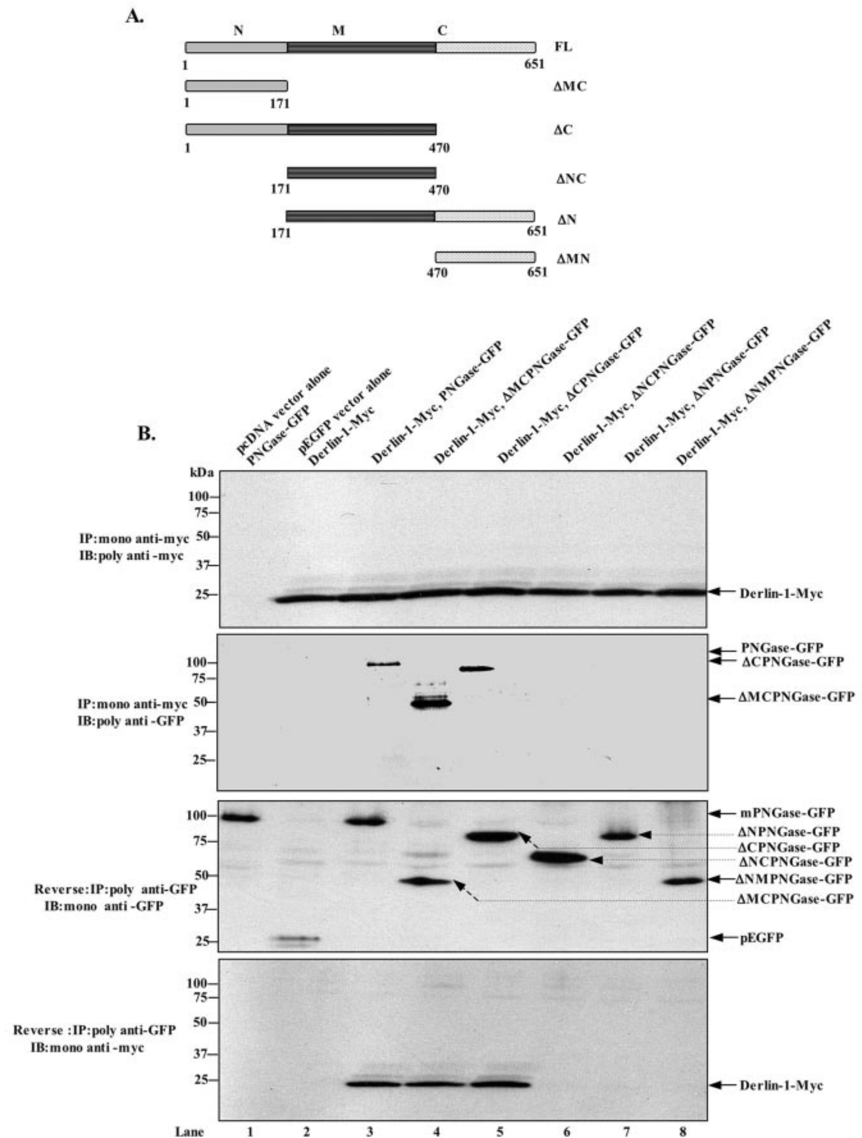

Figure 5. The N-terminus of PNGase interacts with Derlin-1. (A) Deletion constructs of PNGase used in this study. (B) HeLa cell lysates obtained from cells coexpressing pcDNA vector alone and PNGase-GFP (lane 1), pEGFP vector alone and Derlin-1 Myc (lane 2), Derlin-1-Myc and PNGase-GFP (lane 3), Derlin-1-Myc and ΔMCPNGase-GFP (lane 4), Derlin-1-Myc and ΔCPNGase-GFP (lane 5), Derlin-1-Myc and ΔNCPNGase-GFP (lane 6), Derlin-1-Myc and ΔNPNGase-GFP (lane 7), and Derlin-1-Myc and ΔNMPNGase-GFP (lane 8) were subjected to immunoprecipitation with monoclonal anti-Myc antibody. Reverse immunoprecipitation was performed using polyclonal anti-GFP antibody. Immune complexes bound on protein A/G agarose beads were eluted with 2× SDS-PAGE sample buffer resolved on SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies as indicated.

localization in the ER lumen (Weng and Spiro, 1997). PNGase in higher eukaryotes acts on a diverse range of misfolded glycoprotein substrates, namely immunoglobulin (Ig) subunits (Mancini *et al.*, 2000), MHC class I heavy chains (Kamhi-Nesher *et al.*, 2001), a ribophorin I variant (Kitzmuller *et al.*, 2003), cog thyroglobulin mutant (Tokunaga *et al.*, 2000), cystic fibrosis transmembrane regulator (Xiong *et al.*, 1999), and T-cell receptor (TCR) α-subunits (Yu *et al.*, 1997).

In a study using a novel PNGase inhibitor (Z-VAD(OMe)-fmk), Misaghi *et al.* (2004) found that two well-characterized substrates of PNGase, TCRα and MHC class I heavy chains (HCs), showed different cellular sites of deglycosylation. In the absence of the PNGase inhibitor the deglycosylated form of HCs were observed in the cytosol as well as in the ER membrane fraction. In contrast, all HCs remained glycosylated in the presence of PNGase inhibitor, regardless of their subcellular distribution. This indicates that the process of dislocation of HCs is not dependent on deglycosylation. The finding that deglycosylated HCs remain associated with the ER membrane fraction in the absence of PNGase inhibitor, suggests a mechanism in which HCs encounter PNGase at the ER membrane. During HC dislocation PNGase removes N-linked glycans before the release of the polypeptides into the cytosol for subsequent degradation by the proteasome. In fact, in the studies of Wiertz *et al.* (1996) only the deglycosylated forms of MHC class I molecules were noted to be associated with the Sec61 complex of the ER membrane, indicating that glycoproteins in this channel must also encounter *N*-glycanase. Another example of the ER membrane-localized deglycosylation is the protein ovalbumin in which site specific N-deglycosylation was attributed to an ER-situated *N*-glycanase (Suzuki *et al.*, 1997). In contrast to HCs, TCRα apparently utilizes a different dislocation machinery for degradation (Misaghi *et al.*, 2004). Fully glycosylated TCRα were found in the membrane fraction in the presence of proteasome inhibitors suggesting that TCRα dislocation is coupled to proteolytic degradation.

We earlier reported evidence that endogenous PNGase could be detected associated with the ER in a variety of cell lines including Hela (Katiyar *et al.*, 2004). In the current study we have carried out over expression of epitope-labeled PNGase and confirmed our earlier observations as well as identified the protein that mediates PNGase binding to the ER. The fact that MHC class I HCs are PNGase

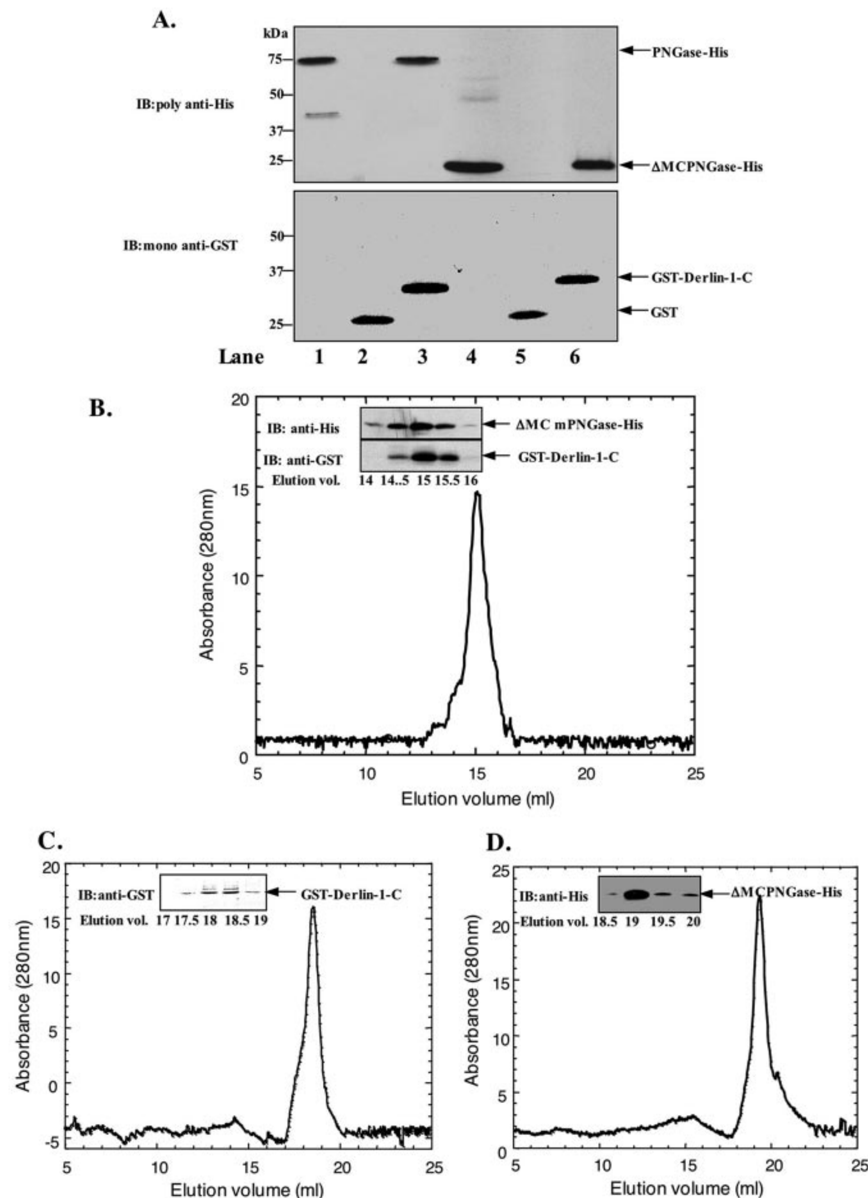

Figure 6. The interaction of N-terminus of PNGase with the cytosolic C-terminus of Derlin-1 was confirmed by in vitro GST-binding assay (A) and gel filtration (B–D). (A) GST or GST-Derlin-1-C expressed in *E. coli* DH5α were purified and allowed to bind on GSH-agarose beads. *E. coli* BL21(DE3) cell lysate expressing full-length PNGase-$(His)_6$ and ΔMCPNGase-$(His)_6$ were used for in vitro GST-binding assay followed by SDS-PAGE and immunoblotting with anti-His and anti-GST antibodies. Shown are PNGase-$(His)_6$ in cell lysate (lane 1), GSH beads incubated with GST alone and PNGase-$(His)_6$ (lane 2), GSH beads carrying GST-Derlin-1-C and PNGase-$(His)_6$ (lane 3), cell lysate ΔMCPNGase-$(His)_6$ (lane 4), GSH beads incubated with GST alone and ΔMCPNGase-$(His)_6$ (lane 5), and GSH beads incubated with GST-Derlin-1-C and ΔMCPNGase-$(His)_6$ (lane 6). (B) Size exclusion chromatography (Superdex 200 HR 10/30) profile after mixing ΔMCPNGase-His and GST-Derlin-1-C in a 1:1 M ratio. The inset shows immunoblots of the peak fractions with anti-His and anti-GST antibodies, respectively. The presence of both proteins in a single peak indicates formation of a complex. (C) Elution profile of free GST-Derlin-1-C. Inset shows the immunoblot of peak fractions with anti-GST antibody. (D) Elution profile of free ΔMCPNGase-His. Inset shows the immunoblot of peak fractions with anti-His antibody.

substrates and that deglycosylated HCs are detected in the ER membrane associated with Derlin-1 (Lilley and Ploegh, 2004) prompted us to explore the possible interaction between Derlin-1 and PNGase. Initially we carried out immunoprecipitation experiments to detect interaction between Derlin-1 and PNGase. Derlin-1 and PNGase were found to coimmunoprecipitate regardless of the epitope tag. Derlin-1 contains four transmembrane domains and the N- and C-termini are located in the cytosol (Lilley and Ploegh, 2004; Ye *et al.*, 2004). Therefore, we predicted that the observed interaction of PNGase with Derlin-1 could occur at the N- or C-terminus. Coimmunoprecipitation with the deletion constructs of Derlin-1 revealed that the Derlin-1 lacking its C-terminus cytosolic domain failed to coprecipitate with PNGase. Next we showed that the cytosolic C-terminus of Derlin-1 interacted directly with PNGase. Several experimental approaches established that it was the N-terminus of PNGase that bound to Derlin-1. The most conserved region of PNGase, the M-domain, which contains the active site residues (Katiyar *et al.*, 2002), does not interact with Derlin-1, suggesting that extension of N-terminal PNGase in higher eukaryotes (which is absent in yeast PNGase) may facilitate deglycosylation of misfolded glycoprotein substrates by association of the deglycosylating enzyme with the ER membrane. Thus, it seems likely that the cytosolic C-terminus of Derlin-1 interacts with the N-terminus of PNGase and thus brings the deglycosylating enzyme close enough to the ER membrane to catalyze the deglycosylation process on the membrane of certain misfolded glycoprotein substrates that are retrotranslocated from the ER lumen to the cytosol via Derlin-1. Consistent with this idea are the findings that 1) the total cell lysate of HeLa cells when subjected to gel filtration showed coelution of Derlin-1 with a small fraction of the total PNGase and 2) subcellular fractionation studies confirmed this observation. Despite the low abundance of PNGase at the ER membrane, its level is sufficient to coprecipitate almost all of the Derlin-1 (unpublished data).

On the basis of all of our results we propose the model shown in Figure 7, in which PNGase as well as being free in the cytosol is associated with the ER membrane. Retrotrans-

Figure 7. Model of PNGase mediated deglycosylation of misfolded glycoproteins retrotranslocated via channel formed by Derlin-1 (MHC class I HCs is used as a model substrate; there may be other glycoprotein substrates that utilize this pathway). As shown initially, a progressive series of protein-protein interactions occur. Derlin-1 interacts with the HCs and targets these to the cytosol for degradation by the proteasome. As shown, the N-terminus of PNGase interacts with the cytosolic C-terminus of Derlin-1 and consequently membrane-bound PNGase facilitates deglycosylation of the HCs in a coretrotranslocational manner, CHO indicates the carbohydrate chains. In this model we propose that the degradation of misfolded glycoprotein substrates occurs in close vicinity of the ER. On deglycosylation, HR23B acts as a receptor for deglycosylated substrates (Katiyar *et al.,* 2004) and targets them to the proteasome for degradation by virtue of its interaction with the S_4 subunit. HR23B is known to be present close to the ER and its C-terminus has been shown to interact with PNGase (Suzuki *et al.,* 2001; Katiyar *et al.,* 2004). (Other Derlin-1 interacting partners p97 and VIMP [Ye *et al.,* 2004] are not shown in this model.) A retrotranslocation channel formed by Sec61 complex is also shown in this model that may be involved in the translocation of a subset of misfolded protein substrates. Recently, the Sec61 channel has been demonstrated to be a membrane receptor for the proteasome (Kalies *et al.,* 2005), suggesting that the degradation of misfolded proteins does indeed occur in close vicinity to the ER. Whether the retrotranslocation channel formed by Derlin-1 also directly interacts with the proteasome remains to be determined.

location of misfolded proteins may utilize the Sec61 translocon, which has been shown to bind the proteasome (Kalies *et al.,* 2005). Alternatively, certain glycoproteins such as HC may utilize another pathway. In this case, the HCs bind transiently to Derlin-1 and are dislocated across the ER into the cytosol. It has previously been shown that deglycosylated glycoprotein binds to HR23B (Katiyar *et al.,* 2004). As illustrated in this study, PNGase via its N-terminus interacts with the C-terminus of Derlin-1, thereby bringing it in close proximity to the ER membrane. The N-terminus and the middle domain of PNGase has already been shown to be functional in binding PNGase to HR23B (Katiyar *et al.,* 2004), which in turn interacts with the S_4 subunit at the base of the proteasome lid. Because the majority of PNGase is in the cytosol, interaction of PNGase with Derlin-1 at the ER membrane may be required for the deglycosylation of a subset of misfolded glycoprotein substrates, such as the HCs. Glycoproteins like TCRα, on the other hand, may be deglycosylated by the free cytosolic PNGase enzyme.

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