

Tyrosine phosphorylation of p97 regulates transitional endoplasmic reticulum assembly *in vitro*

Christine Lavoie*[†], Eric Chevet*[†], Line Roy*, Nicholas K. Tonks[‡], Ali Fazel*, Barry I. Posner[§], Jacques Paiement[¶], and John J. M. Bergeron*^{||}

*Department of Anatomy and Cell Biology, McGill University, Montreal, QC, H3A 2B2, Canada; [†]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724; [§]Department of Medicine, McGill University, Montreal, QC, Canada; and [¶]Département de Pathologie et Biologie Cellulaire, Faculté de Médecine, Université de Montréal, Montréal, QC, H3C 3J7, Canada

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The ATPase associated with different cellular activities family member p97, associated p47, and the t-SNARE syntaxin 5 are necessary for the cell-free reconstitution of transitional endoplasmic reticulum (tER) from starting low-density microsomes. Here, we report that membrane-associated tyrosine kinase and protein-tyrosine phosphatase (PTPase) activities regulate tER assembly by stabilizing (PTPase) or destabilizing (tyrosine kinase) p97 association with membranes. Incubation with the PTPase inhibitor bpV-(phen) inhibited tER assembly coincident with the enhanced tyrosine phosphorylation of endogenous p97 and its release from membranes. By contrast, the tyrosine kinase inhibitor, genistein, promoted tER formation and prevented p97 dissociation from membranes while increasing p97 association with the t-SNARE syntaxin 5. Purification of the endogenous tyrosine kinase activity from low-density microsomes led to the identification of JAK-2, whereas PTPH1 was identified as the relevant PTPase. The p97 tyrosine phosphorylation state is proposed to coordinate the assembly of the tER as a regulatory step of the early secretory pathway.

The endoplasmic reticulum (ER)-Golgi transitional elements consist essentially of two types. The first type, composed of vesicular tubular clusters, has been described as a pre-Golgi intermediate, which is physically distinct from the ER, and which acts as a transport vehicle for protein delivery to the Golgi complex (1–6, 38). The second type, composed of a network of smooth tubules in continuity with the rough ER, is known to vary widely within different cell types. It has been described as a compartment functioning as an exit site for biosynthetic material shuttling between the ER and the Golgi complex or the ER and the intermediate compartment. The latter type of intermediate compartment includes the budding compartment of mouse hepatitis virus (7), the site of accumulation of the E1 glycoprotein of the rubella virus (8), and the site of accumulation of vesicular stomatitis virus glycoproteins in cells injected with anti- β -coatamer protein (9). Transitional ER (tER) membrane networks consisting of anastomosing smooth tubules continuous with rough membrane cisternae have been reconstituted *in vitro* (10–12) and were shown to have similar morphological and biochemical characteristics to the transitional element described above.

The ATPase associated with different cellular activities ATPase p97 was shown to regulate membrane fusion and assembly of tER *in vitro* (12). In addition, p97 was observed to dissociate from the ER membranes during tER assembly (12). The size of the tER membrane networks assembled in this cell-free system was constant (10). This suggested an additional regulatory step, wherein modulation of the number of rounds of fusion limited the size of the tER compartment. P97 membrane association was suspected as the regulatory step with phosphorylation of p97 as the trigger.

In both the budding yeast *Saccharomyces cerevisiae* and mammalian WISH cells, cdc48p/p97 tyrosine phosphorylation (13) coincides with its relocalization from the ER to the nucleus (yeast) or to the centrosome (mammalian WISH cells). Tyrosine phosphorylation of p97 in T cells has been observed as a consequence of p60^{lyn} overexpression as well as treatment of these cells with protein-tyrosine phosphatase (PTPase) inhibitors (14). In addition, in activated B lymphocytes, p97 becomes tyrosine phosphorylated after treatment with the PTPase inhibitor, sodium vanadate (15). Moreover, p97 is the major substrate of the protein-tyrosine phosphatase PTPH1 (16), and overexpression of PTPH1 in NIH 3T3 cells leads to p97 dephosphorylation coinciding with an inhibition of cell-cycle progression and the accumulation of cells throughout the cell cycle (16).

Here, we demonstrate that a cycle of tyrosine phosphorylation and dephosphorylation of p97 regulates p97 association with the t-SNARE syntaxin 5 and subsequent membrane fusion in a cell-free system, which reconstitutes tER formation. We also identify the candidate tyrosine kinase and phosphotyrosine phosphatase as JAK-2 and PTPH-1, respectively.

Experimental Procedures

Materials. Anti-p97 was a kind gift of R. Hendricks (EMBL, Germany). Purified rat p97 was generously provided by J. Lanoix and T. Nilsson (EMBL, Germany). Anti-Bip and anti-JAK-2 were kindly given by L. Hendershot and J. Ihle, respectively (St. Jude Children's Research Hospital, Memphis, TN). Anti-syntaxin 5 antibodies were kindly provided by W. Hong (Institute of Molecular and Cell Biology, Singapore). Anti-PTPH1 antibodies and GST-PTPH1 constructs were described previously (16).

Analytical Fractionation and *In Vitro* Reconstitution of Transitional ER.

Subfractions enriched in rough and smooth vesicles were obtained from a step-gradient of sucrose used to separate rough microsomes, low-density microsomes (LDM), and Golgi derivatives from total microsomes (10–12). Low-density microsomes were separated by isopycnic density gradient centrifugation on linear sucrose gradients (d1.05–1.25). Fractions were collected from the top of the gradient, and equal volumes of each fraction were analyzed for their content of PTPH1, JAK-2, Bip, and p97

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Abbreviations: ER, endoplasmic reticulum; tER, transitional endoplasmic reticulum; LDM, low-density microsomes; PTPase, protein-tyrosine phosphatase.

[†]These authors contributed equally to this work.

^{||}To whom reprint requests should be addressed. E-mail: Bergeron@med.mcgill.ca.

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by immunoblot analysis followed by ECL revelation and visualized by radioautography using Kodak X-Omat AR film. Quantitation was done by scanning densitometry. Results were normalized according to the method of Beaufay *et al.* (17). Reconstitution of tER was done in 0.25 ml containing 150 μ g of microsomal protein LDM/100 mM Tris-HCl, pH 7.4/5 mM MgCl₂/1 mM GTP/2 mM ATP/0.1 mM DTT/0.02 mM PMSF/0.09 μ g ml⁻¹ leupeptin/50 mM sucrose. This mixture was incubated at 37°C up to 240 min.

Morphological Analysis. After incubation, membranes were fixed using 2.5% glutaraldehyde and recovered onto Millipore membranes by the random filtration technique (18) and processed for electron microscopy. Estimates of the lengths of embedded and sectioned rough and smooth membranes in the membrane networks were obtained from EM micrographs by morphometry using the membrane intersection counting procedure (19). The electron micrographs were fastened onto a measuring tablet (Graphic Master, Numonics, Landsdale, PA), and the Sigma-Scan measurement system (Jandel, San Rafael, CA) was used to digitize morphometric data. The analyzed areas correspond to the surface area of embedded and sectioned membrane pellicule calculated on electron micrograph at low magnification ($\times 550$). Five micrographs were counted for each sample in each of the three independent experiments.

Protein Purification. Low-density microsomes from rat liver (600 μ g to 5 mg) were solubilized in 3 to 15 ml of 30 mM Tris-HCl, pH 8.0/150 mM NaCl/1.5% CHAPS/1 mM PMSF/100 μ M bpV(phen) (20, 21)/1 μ g/ml leupeptin/5 kallikrein-inhibiting units/ml aprotinin (buffer A) for 30 min. Lysate was centrifuged for 30 min at 100,000 $\times g_{av}$, and the supernatant was incubated with ATP-agarose beads (Sigma). Beads were then extensively washed with buffer A, and bound proteins were eluted by a step gradient (from 150 mM NaCl to 900 mM NaCl). Each fraction was then tested for the presence of kinase activity by incubation with 0.5 mg/ml poly Glu-Tyr in 30 mM Tris-HCl, pH 8.0/150 mM NaCl (final concentration)/10 mM MgCl₂/1 μ Ci [γ -³²P]ATP. After reaction, substrates were spotted onto 3MM filter paper and precipitated in 5% trichloroacetic acid, 1% pyrophosphate. Filters were then washed extensively, and radioactivity was measured by scintillation counting. Fractions containing the highest specific activity were concentrated and either *in vitro* autophosphorylated in 20 mM Tris-HCl, pH 8.0/10 mM MgCl₂/5 mM MnCl₂/1 μ Ci [γ -³²P]ATP for 20 min at 30°C, then resolved by SDS/PAGE and radioautographed on X-Omat AR films, or resolved by SDS/PAGE followed by immunoblotting with anti-JAK-2 antibodies.

Pull-Down, Immunoprecipitation, *in Vitro* Phosphorylation. Cytosol or LDM were incubated as described above in the presence or in the absence of 100 μ M bpV(phen) and 100 μ Ci [γ -³²P]ATP. Phosphorylated samples were then solubilized in 25 volumes of 50 mM Hepes, pH 7.5/5 mM EDTA/1% Triton X-100/150 mM NaCl/10 mM sodium phosphate/0.5 mM DTT/50 mM NaF/5 mM iodoacetic acid, centrifuged at 100,000 $\times g$ for 20 min, and then incubated in the presence of glutathione-Sepharose beads carrying 15 μ g of GST-PTPH1 D811A mutant for 4 h at 4°C. Beads were then pelleted and washed four times with the same buffer. Proteins pulled-down were resolved by SDS/PAGE and either immunoblotted with anti-p97 or anti-GST antibodies and revealed by enhanced chemiluminescence or directly radioautographed to X-Omat AR films. JAK-2 or nonimmune serum immunoprecipitates from LDM lysate (as described above) were *in vitro* phosphorylated in 20 mM Tris-HCl, pH 8.0/5 mM MgCl₂ in the presence of 100 μ Ci [γ -³²P]ATP. Phosphorylated material was either directly resolved by SDS-PAGE or released with 1%

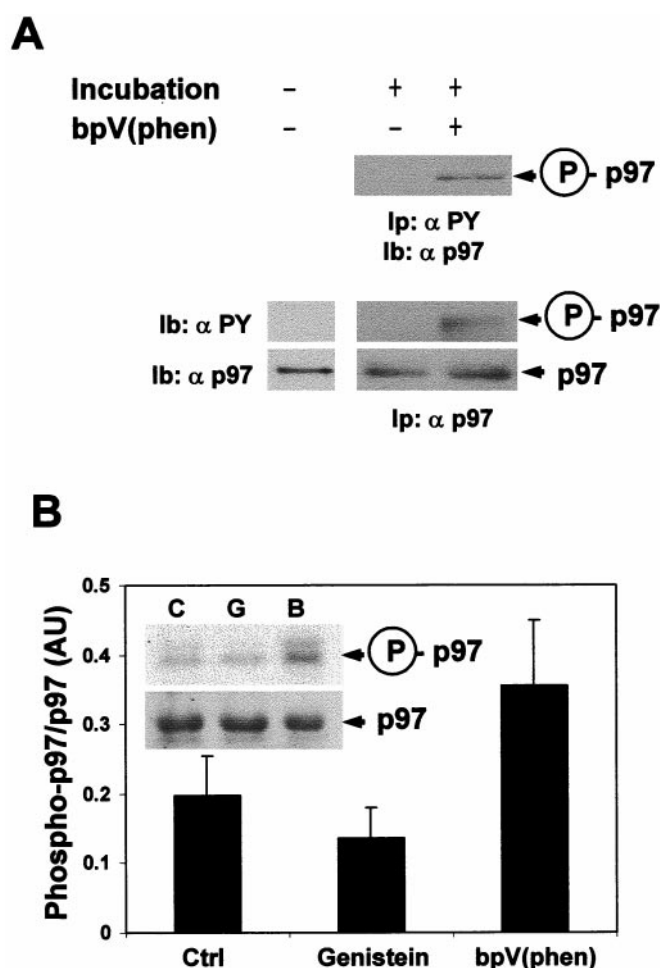


Fig. 1. Tyrosine phosphorylation of p97. (A) LDM were incubated or not using tER assembly conditions as previously described (10–12) in the presence or absence of 100 μ M bpV(phen). Solubilized membranes were immunoprecipitated either with antiphosphotyrosine antibodies (Ip: α PY, top gel) or with anti-p97 antibodies (Ip: α p97, middle and lower gels) followed by immunoblotting using anti-p97 (Ib: α p97, top and bottom gels) or anti-phosphotyrosine antibodies (Ib: α PY, middle gel) and revealed by chemiluminescence (ECL). A representative experiment is shown ($n = 4$) with similar results observed each time. (B) LDM were incubated in the same medium described above but containing 100 μ Ci [γ -³²P]ATP and the absence or presence of either 100 μ M bpV(phen) or 200 μ M genistein. After p97 immunoprecipitation, the proteins in the immunoprecipitate were separated by SDS/PAGE and transferred onto nitrocellulose membrane. The membrane was subjected to radioautographic analysis using X-Omat AR film (top gel), then probed with anti-p97 and revealed by ECL (bottom gel). Densitometric analysis of the x-ray films was performed, and the representation is the mean of three independent experiments \pm SD for the three incubation conditions.

SDS 50 mM Tris-HCl, pH 8.0, for 10 min at 95°C and sequentially immunoprecipitated with anti-p97 or anti-JAK-2 antibodies.

Results

We used a cell-free system to assess the relevance of tyrosine phosphorylation of p97 in membrane fusion. This assay reconstitutes the assembly of 1.5- μ m-diameter transitional ER networks from a starting preparation of low-density microsomes (0.08–0.12- μ m diameter). The assay requires ATP hydrolysis by p97 and the t-SNARE syntaxin 5 for membrane fusion (12).

Tyrosine phosphorylation of endogenous p97 was demonstrated using conditions that promote assembly of tER (12). LDM (1.17 g cc⁻¹) from rat liver were incubated in the presence of Mg²⁺ GTP, Mg²⁺ ATP, and the tyrosine phosphatase inhib-

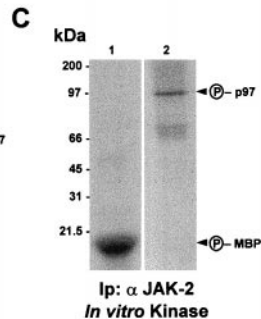
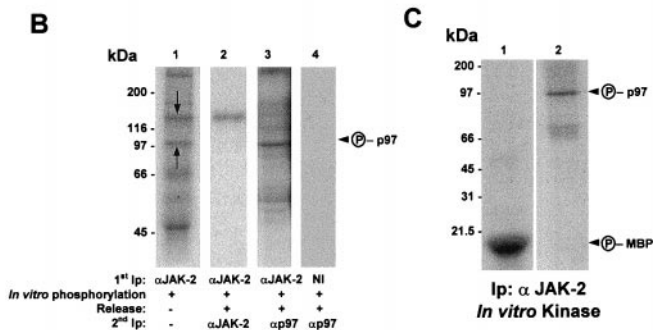
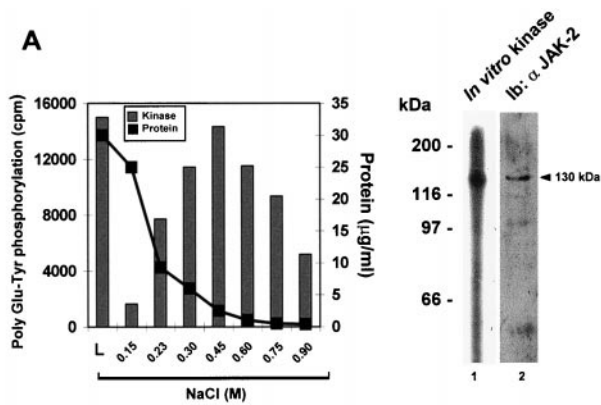


Fig. 2. Purification of tyrosine kinase activities from LDM. (A) Low-density microsomes from rat liver (600 μ g to 5 mg) solubilized were incubated with ATP-agarose beads. Eluted fractions were then tested for the presence of kinase activity by incubation with 0.5 mg/ml poly Glu-Tyr, and radioactivity was measured by scintillation counting. Protein concentration was measured for each fraction and is reported on the graph. L represents 5% of total protein lysate chromatographed on ATP-agarose. Fractions containing the highest specific activity were concentrated and either *in vitro* autophosphorylated (Right, lane 1) or directly resolved by SDS/PAGE followed by immunoblotting with anti-JAK-2 antibodies (Right, lane 2). (B) *In vitro* phosphorylation of JAK-2 immunoprecipitate from LDM (lane 1, overnight exposure) or *in vitro* phosphorylation of JAK-2 immunoprecipitate from LDM, followed by release and sequential immunoprecipitation with anti-JAK-2 antibodies (lane 2, overnight exposure) or anti-p97 antibodies (lane 3, 40-h exposure). In lane 4, a nonimmune serum (NI) was used in the primary immunoprecipitation, and an anti-p97 was used for the secondary immunoprecipitation (overnight exposure). (C) *In vitro* phosphorylation of 10 μ g of myelin basic protein (MBP, lane 1) or purified p97 (lane 2) by JAK-2 immunocomplex from LDM. A representative experiment is shown ($n = 2$).

itor bpV(phen) (20, 21). Immunoblot and immunoprecipitation analysis revealed the tyrosine phosphorylation of p97 (Fig. 1A). With an *in vitro* phosphorylation assay using intact membranes, the same inhibitor enhanced the incorporation of 32 P from [γ - 32 P]ATP into endogenous p97 (1.8-fold stimulation; Fig. 1B, $P < 0.001$). Although less marked, the tyrosine kinase inhibitor, genistein, reproducibly inhibited 32 P incorporation into endogenous p97 (1.4-fold inhibition; Fig. 1B, $P < 0.009$). Hence, these membranes contained endogenous kinase(s) and phosphatase(s) responsible for phosphorylation and dephosphorylation of p97 on tyrosine residue(s).

The identity of candidate kinase(s) responsible for the tyrosine phosphorylation of p97 in LDM was investigated by ATP-agarose chromatography of LDM lysates. Fractions were eluted by an NaCl step gradient and tested for tyrosine kinase activity with poly Glu-Tyr as substrate (Fig. 2A, graph). The fractions extracted with 0.45 and 0.6 M NaCl, which contained the highest tyrosine kinase specific activity (Fig. 2A, graph),

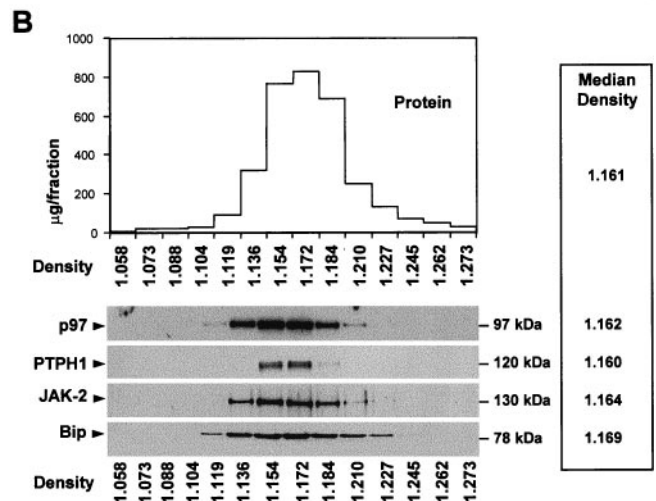
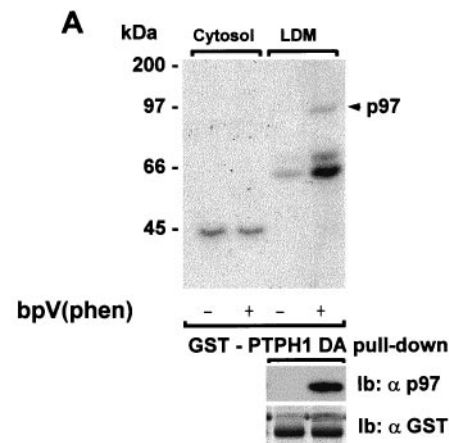


Fig. 3. Identification of p97 tyrosine phosphatase from low-density microsomes. (A) Cytosol or LDM were incubated as described above in the presence or in the absence of 100 μ M bpV(phen) and 100 μ M [γ - 32 P]ATP. Lysates were then incubated in the presence of glutathione-Sepharose beads carrying 15 μ g of GST-PTPH1 D811A mutant for 4 h at 4°C. Proteins pulled-down were resolved by SDS/PAGE and either immunoblotted with anti-p97 or anti-GST antibodies and revealed by enhanced chemiluminescence (Middle and Bottom) or directly radioautographed to X-Omat AR films (Top). A representative experiment is shown ($n = 3$). (B) Analytical fractionation by isopycnic density gradient centrifugation of LDM. Each fraction was tested for its content p97, PTPH1, JAK-2, and Bip by immunoblotting. Quantitation was done as described under *Experimental Procedures*.

were pooled, concentrated, dialyzed, and then analyzed by SDS/PAGE after *in vitro* autophosphorylation with [γ - 32 P]ATP. Only one major autophosphorylated band with a mobility corresponding approximately to 130 kDa was detected (Fig. 2A, Right, lane 1, arrowhead).

After screening with different antibodies raised against protein tyrosine kinases with a comparable molecular mass, immunoblotting revealed JAK-2 in the purified fraction from LDM (Fig. 2A, Right, lane 2). After JAK-2 immunoprecipitation from solubilized LDM and *in vitro* phosphorylation with [γ - 32 P]ATP, seven radiolabeled polypeptides were observed in the immunoprecipitates, which were not in a nonimmune immunoprecipitate (Fig. 2B, lane 4). One corresponded to the mobility of JAK-2 (downward pointing arrow, Fig. 2B, lane 1) and one to that of p97 (upward pointing arrow). The identity of the phosphoproteins migrating at 130 and 97 kDa as JAK-2 and p97, respectively, was confirmed by sequential immunoprecipitations (Fig. 2B, lanes 2 and 3, respectively). JAK-2 associated phosphoproteins were

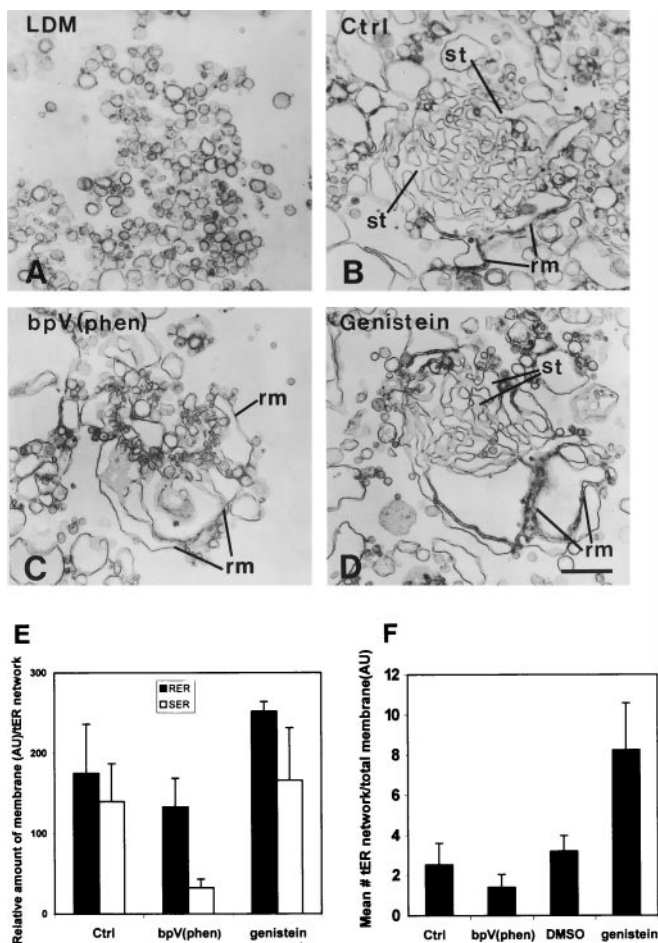


Fig. 4. Effect of bpV(phen) and genistein on transitional ER assembly. LDM (A) were incubated using assembly conditions as previously described (B) in the presence of 100 μ M bpV(phen) (C) or 350 μ M genistein (D) for 180 min at 37°C. After the incubation, membranes were fixed and processed for electron microscopy. rm, rough membrane; st, smooth tubules. Scale bars represent 0.5 μ m. (E) Morphometric studies of the amount of rough endoplasmic reticulum and smooth endoplasmic reticulum membranes in tER networks reconstituted in presence or absence of 100 μ M bpV(phen) or 350 μ M genistein. Average values (\pm SD) are shown from three separate experiments. RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum. (F) Amount of tER assembly per total membrane reconstituted in presence or absence of 100 μ M bpV(phen) or 350 μ M genistein. Average values (\pm SD) are shown from three separate experiments. The mean number of networks was not significantly different when compared between control and DMSO ($P > 0.05$, $n = 21$) but was significantly different when compared between control and bpV(phen) ($P < 0.005$, $n = 27$) or DMSO and genistein ($P < 0.001$, $n = 20$). The amount of tER networks counted was as follows: control, 112 networks on 13 micrographs; bpV(phen), 50 networks on 14 micrographs; DMSO, 90 networks on 8 micrographs; and genistein, 219 networks on 12 micrographs. Results were expressed as average number of tER networks per 200 μ m² of membrane pellicules.

released by SDS at 95°C followed by immunoprecipitation with anti-JAK-2 or anti-p97 antibodies. This revealed single phosphoproteins at 130 and 97 kDa, respectively (Fig. 2B, lanes 2 and 3). When JAK-2 immunoprecipitates were immunoblotted with anti-p97 antibodies, the same band at 97 kDa was visualized (data not shown). The JAK-2 immunoprecipitate from LDM phosphorylated the exogenous substrate myelin basic protein (Fig. 2C, lane 1) as well as p97 purified from rat liver cytosol (Fig. 2C, lane 2). Taken together, these results identify JAK-2 as an endogenous kinase of LDM that associates with and phosphorylates endogenous p97.

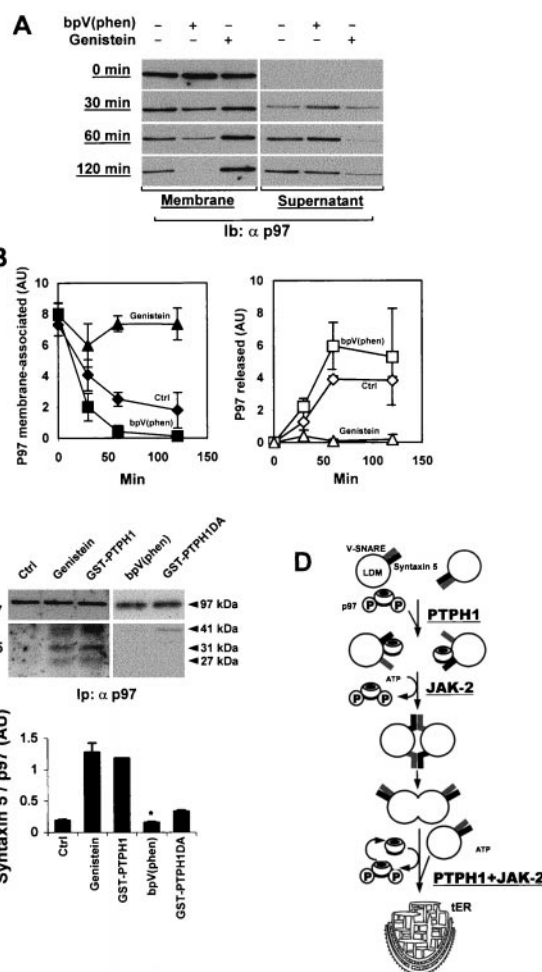


Fig. 5. (A) Association of p97 to the membranes is regulated by its tyrosine phosphorylation. LDM were incubated as previously described (10–12) in the presence or absence of 100 μ M bpV(phen) or 350 μ M genistein for different periods. The samples were then placed on ice and received 1 ml each of 0.25 M sucrose containing 3 mM imidazole. The samples were then centrifuged at 100,000 $\times g$ for 30 min. The membranes were then dissolved directly in Laemmli buffer. The proteins in the supernatant fractions were concentrated by trichloroacetic acid precipitation. Proteins were then separated by SDS/PAGE, followed by immunoblotting with anti-p97 antibodies. (B) Densitometric analysis of the above immunoblots. Each value is the mean of three independent experiments \pm SD. Closed symbols stand for membrane-associated p97 and open symbols for p97 released from the membranes, respectively, \blacklozenge , \blacktriangle ; \blacklozenge , \blacktriangle , genistein; \blacksquare , \square , bpV(phen). (C) LDM (600 μ g) were first incubated 45 min on ice as previously described, except that Hepes was replaced by 6 mM imidazole, pH 7.4; this incubation buffer contained or not 350 μ M genistein or 30 μ g of GST-PTPH1 or GST-PTPH1D811A or 100 μ M bpV(phen). Microsomes were then solubilized in the same buffer containing 1.5% CHAPS. After spinning in a microfuge for 15 min at maximal speed, lysates were immunoprecipitated with anti-p97 antibodies. Immunoprecipitates were then resolved by SDS/PAGE, transferred onto nitrocellulose, and immunoblotted with either anti-syntaxin 5 antibodies or anti-p97 antibodies and revealed by ECL. A representative experiment is shown (control, genistein, bpV(phen), and GST-PTPH1DA, $n = 3$; GST-PTPH1, $n = 2$). Quantitation was made by scanning densitometry of the x-ray films and represents the mean \pm SD (when $n = 3$); * indicates significant values $P < 0.04$. For syntaxin 5, the intensities corresponding to the three bands visualized were added. (D) Representative scheme of the sequence of events regulating p97 association with LDM.

Recently, p97 has also been identified as the major substrate for the PTPase PTPH1 using a substrate-trapping strategy (16, 22). In this approach, the invariant aspartate residue of the PTP signature motif, which facilitates protonation of the tyrosyl

leaving group and hydrolysis of the cysteinyl-phosphate intermediate, respectively, is mutated to alanine. This mutation dramatically slows catalysis without affecting ligand affinity, allowing the identification of the major substrates of PTPH1. An association of the tyrosine-phosphorylated p97 with this substrate-trapping mutant of PTPH1 (PTPH1 D811A) (16) was observed in the membranes used here (Fig. 3A). The experimental design was to phosphorylate LDM *in vitro* by endogenous kinases with endogenous PTPases inhibited by bpV(phen). A GST-PTPH1 D811A was then used to trap any PTPH1 substrate after inactivation of bpV(phen). Three bands were observed on the radioautogram, with one band migrating at a mobility corresponding to 97 kDa. This was confirmed as p97 by immunoblotting (Fig. 3A Middle). These studies predict PTPH1 as the membrane-associated phosphatase responsible for p97 dephosphorylation. An assessment of the distribution of PTPH1 in LDM was attempted by analytical fractionation. LDM was separated on a linear sucrose gradient (density 1.05 to 1.28). Fractions were analyzed by immunoblotting with antibodies against PTPH1, JAK-2, p97, and Bip. PTPH1, JAK-2, and p97 revealed similar median densities, respectively, of 1.160, 1.164, and 1.162 (Fig. 3B), which corresponded to that of Syntaxin 5 as previously reported (12). The ER marker Bip revealed a similar distribution with a median density of 1.169, whereas the protein content showed a median density of 1.161. We conclude that the tyrosine kinase JAK-2, the tyrosine phosphatase PTPH-1, and their substrate p97 are present in the same subcompartment of the tER.

p97 has been previously implicated in membrane fusion leading to tER assembly *in vitro* (12). Hence, the role of p97 phosphorylation was assessed using *in vitro* tER reconstitution as a read out. BpV(phen) inhibited the assembly of the smooth tubule domain within tER networks, thereby implicating a PTPase (Fig. 4A–C); similar results were obtained by incubating LDM with anti-phosphotyrosine antibodies (data not shown). Smooth tubular membranes associated with the reconstituted tER networks were reduced 6-fold following incubation in the presence of bpV(phen) (Fig. 4E). Furthermore, the total number of tER networks was significantly decreased compared with control (Fig. 4F). In contrast, the tyrosine kinase inhibitor, genistein, did not affect significantly the amount of smooth membranes associated with any single tER network (Fig. 4D and E) but increased 2.5 times the total number of tER networks (Fig. 4F).

Hence, genistein induced membrane fusion and coincidentally increased the amount of nonphosphorylated p97. Dissociation of p97 from membranes was accelerated in the presence of the PTPase inhibitor bpV(phen), whereas in the presence of genistein, p97 dissociation from membranes was inhibited (Fig. 5A and B). Although p97 is serine phosphorylated by p34cdc2 (23), no effect on the Ser/Thr phosphatases inhibitor, okadaic acid (10–30 μ M), was observed (data not shown).

p97 has been found in a complex with p47 and syntaxin 5 (24). Following solubilization of reconstituted tER after incubation in the presence or not of 350 μ M genistein, or 30 μ g of GST-PTPH1, p97 association with syntaxin 5 was evaluated by coimmune precipitation. Minimal stable association of p97 and syntaxin 5 was found under conditions used to generate tER networks (Fig. 5C, control). However, association was enhanced (4- to 5-fold) after incubation in the presence of genistein or GST-PTPH1, whereas the amount of p97 immunoprecipitated remained the same (Fig. 5C). We observed also that incubation with 100 μ M bpV(phen) abolished completely the association of p97 with syntaxin 5 (data not shown). Hence, preventing tyrosine phosphorylation of p97 or increasing its dephosphorylation enhanced the steady-state stability

of p97 (and p47):syntaxin 5 complexes, which coincided with an enhanced number of tER networks.

Discussion

The present study is the first to demonstrate JAK-2 and PTPH1 as ER-associated constituents implicated in tER formation. This was deduced from a cell-free system that faithfully reconstitutes cargo sorting as well as enhanced tER concentration of the recycling membrane proteins α 2p24 and p58 (ERGIC 53) (25). The starting membrane preparation (LDM) has been documented as ER derived with little contamination from the plasma membrane or the Golgi apparatus (11, 12). LDM are isolated from liver parenchyma of young adult rats. These cells are largely in G₀ (26), and the events described here are therefore unlikely to be related to the postmitotic assembly of tER. Moreover, p97 accumulates in nonproliferating cells and is not detectable in proliferating cells in *Drosophila* (27, 28). Genetic studies have shown that the p97 ortholog and the putative p47 ortholog *Bag-of-marbles* are involved in membrane fusion events (27). Therefore, the reconstitution of the differentiated early secretory pathway by fusion of fragmented LDM likely reflects dynamic membrane transformation events of the early secretory apparatus. PTPH1 and JAK-2 share a band 4.1-related domain at their N-termini (29–31). This domain has been implicated in targeting proteins to the membrane/cytoskeleton interface (32), but it may also be a motif for directing association with the tER membrane because of its low affinity for phosphatidylinositol (4,5)P₂ (33). Indeed, phosphatidylinositol (4,5)P₂ is known to be synthesized in the ER membrane (34) and has also been shown to regulate homotypic vacuole membrane fusion events in yeast (35). The effect of genistein on membrane fusion observed in this study could also be linked to its role in phosphatidylinositol turnover.

These data indicate a relationship between signal transduction events (tyrosine phosphorylation) and SNARE-regulated fusion and extend the signal transduction pathways to membrane elements of the early secretory pathway. Tyrosine phosphorylation does not affect the ATPase activity of p97 (36). Thus, the regulation of its localization in proximity to the fusogenic apparatus is likely relevant to SNARE priming (Fig. 5D). Dephosphorylation of p97 stabilized p97 (and p47):syntaxin 5 association. A similar scenario has been observed for α -SNAP, whose phosphorylation by protein kinase A decreased the SNARE binding capacity of *N*-ethylmaleimide sensitive factor: α -SNAP complexes (37). In our assay, dephosphorylation of p97 was linked to membrane association of the ATPase through stabilization of the p97 (and p47):syntaxin 5 complexes. This coincided with an enhanced number of fusogenic events that would be a predicted outcome of enhanced docking of smooth membrane vesicles in LDM to reconstituting rough cisternae, leading to an increased number of tER networks (Fig. 4). Phosphorylation/dephosphorylation of p97 may be a means of controlling the number of rounds of fusion ultimately limiting the size of the tER compartment, which is known to vary widely within different cell types (3, 7–9).

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