Tyrosine phosphorylation of p97 regulates transitional endoplasmic reticulum assembly in vitro


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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 infected with anti-β-coatomer 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 relocation from the ER to the nucleus (yeast) or to the centosome (mammalian WISH cells). Tyrosine phosphorylation of p97 in T cells has been observed as a consequence of p60^fyn 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 PTPH1, 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
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 μg 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 μg 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 (×550). Five micrographs were counted for each sample in each of the three independent experiments.

Protein Purification. Low-density microsomes from rat liver (600 μg 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 mM bpV(phen) (20, 21)/1 μg/ml leupeptin/5 kallikrein-inhibiting units/ml aprotinin (buffer A) for 30 min. Lysate was centrifuged for 30 min at 100,000 × g, 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 μCi [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 8.0/100 mM MgCl₂/5 mM MnCl₂/1 μCi [γ-³²P]ATP. After reaction, substrates were precipitated with 5% trichloroacetic acid, washed four times with the same buffer. Proteins pulled-down were 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 of or in the absence of 100 μM bpV(phen) and 100 μCi [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 50,000 × g for 20 min, and then incubated in the presence of glutathione-Sepharose beads carrying 15 μg 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 on 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 μCi [γ-³²P]ATP. Phosphorylated material was either directly resolved by SDS-PAGE or released with 1% 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 constitutes the assembly of 1.5-μm-diameter transitional ER networks from a starting preparation of low-density microsomes (0.08–0.12-μm 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-
to the highest tyrosine kinase specific activity (Fig. 2). Fractions extracted with 0.45 and 0.6 M NaCl, which contained activity with poly Glu-Tyr as substrate (Fig. 2), were eluted by an NaCl step gradient and tested for tyrosine 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 μg 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).

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), were pooled, concentrated, dialyzed, and then analyzed by SDS/PAGE after in vitro autophosphorylation with [γ-32P]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-32P]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 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.

Fig. 2. Purification of tyrosine kinase activities from LDM. (A) Low-density microsomes from rat liver (600 μg 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 μg 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).

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 μM bpV(phen) and 100 μCi [γ-32P]ATP. Lysates were then incubated in the presence of glutathione-Sepharose beads carrying 15 μg 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.
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), and smooth endoplasmic reticulum membranes in tER networks reconstituted directly in Laemmli buffer. The proteins in the supernatant pellets were 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 ± SD. Closed symbols stand for membrane-associated p97 and open symbols for p97 released from the membranes. The bars represent the mean ± 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. S4). The experimental design was to phosphorylate LDM in vitro by exogenous 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. S3 A 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 were 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. S3B), 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. 4 A–C); similar results were obtained by incubating LDM with anti-PTPase 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. 4 D 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. 5 A and B). Although p97 is serine phosphorylated by p34cdc2 (23), no effect on the Ser/Thr phosphatases inhibitor, okadaic acid (10–30 μM), 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 μM genistein, or 30 μg of GST-PTPH1, p97 association with syntaxin 5 was evaluated by coimmunoprecipitation. Minimal stable 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 α2p24 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 (NSF) 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). Dephosphorylation in vitro 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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