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The Formation of Golgi Stacks from Vesiculated Golgi Membranes Requires Two Distinct Fusion Events

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Summary

We have reconstituted the fusion and assembly of vesiculated Golgi membranes (VGMs) into functionally active stacks of cisternae. A kinetic analysis of this assembly process revealed that highly dispersed VGMs of 60–90 nm diameter first fuse to form larger vesicles of 200–300 nm diameter that are clustered together. These vesicles then fuse to form tubular elements and short cisternae, which finally assemble into stacks of cisternae. We now provide evidence that the sequential stack formation from VGMs reflects two distinct fusion processes: the first event is N-ethylmaleimide (NEM)-sensitive factor (NSF) dependent, and the second fusion event requires an NSF-like NEM-sensitive ATPase called p97. Interestingly, while the earliest steps in stack formation share some similarities with events catalyzing fusion of transport vesicles to its target membrane, neither GTPγS nor Rab-GDI, inhibitors of vesicular protein traffic, inhibit stack formation.

Introduction

We have recently reconstituted the formation of Golgi stacks from vesiculated Golgi membranes (VGMs) in a permeabilized cell system to understand this process (Acharya et al., 1995). VGMs are produced by incubating intact NRK cells with ilimaquinone (IQ, Takizawa et al., 1993; Veit et al., 1993). These VGMs are about 60–90 nm in average diameter and are found dispersed throughout the cytoplasm. Following the removal of IQ, the cells are permeabilized by a rapid freeze–thaw and then washed to remove cytosolic proteins. The fusion and assembly of VGMs into functionally active stacks of cisternae can then be reconstituted by addition of cytosol (a high speed cell supernatant), an ATP-regenerating system, and incubation at 32°C. During the stack formation process, VGMs first fuse to form larger vesicles of about 200–300 nm diameter that are clustered together. These vesicular structures are then converted into tubular and short cisternal elements that finally assemble into stacks of cisternae. We have shown that N-ethylmaleimide (NEM)-sensitive factor (NSF) is required for initiating stack formation, since in the presence of inhibitory anti-NSF antibodies VGMs remain dispersed throughout the cytoplasm of permeabilized cell preparations (Acharya et al., 1995).

We now report that the larger vesicles of 200–300 nm average diameter are bona fide intermediates in the process of stack formation from VGMs. The formation of these structures requires soluble NSF attachment proteins (SNAPs) (Clary et al., 1990) in addition to the NEM-sensitive factor NSF (Block et al., 1988). The conversion of these larger aggregates of vesicles to stacks requires p97, an NEM-sensitive ATPase, highly homologous to NSF (Peters et al., 1990).

It is well documented that GTPγS inhibits vesicular protein traffic between membrane-bound compartments both of the exocytic (Ferro-Novick and Novick, 1993) and endocytic (Mayorga et al., 1989) pathways. While we could demonstrate that GTPγS inhibits transport from endoplasmic reticulum (ER) to Golgi in the permeabilized system, interestingly GTPγS does not inhibit stack formation from VGMs. Rab–GDI, a protein required for dissociation of GDP from a wide range of Rab proteins and, therefore, an inhibitor of the protein transport pathway (Sasaki et al., 1990; Elazar et al., 1994; Peter et al., 1994; Dirac-Svejstrup et al., 1994), also does not inhibit stack formation. The specific requirements for the fusion and assembly of VGMs into stacks are discussed.

Results

NEM Treatment Inhibits the Fusion of IQ-Derived VGMs

The morphological stages based on our previous kinetic characterization of stack formation from VGMs are outlined in Figure 1 (Acharya et al., 1995). The VGMs first fuse to form larger vesicles that then undergo further alterations before forming stacks of cisternae. This observation led us to test the hypothesis that stack formation requires distinct fusion events and that proteins other than NSF participate in these successive fusion events.

NRK cells are treated with IQ (Figure 2aA), washed to remove the drug, and then permeabilized by freeze–thaw as previously described (Acharya et al., 1995). The cells are fixed and visualized by immunofluorescence using an anti–mannosidase II (Man II) antibody (Velasco et al., 1993). If the cells are not depleted of the cytosolic pool of proteins, incubation of permeabilized cells at 32°C in the presence of an ATP-regenerating system causes fusion and assembly of VGMs into stacks of cisternae (Figure 2aB; Acharya et al., 1995). The change in the organization of Golgi membranes from completely dispersed to large aggregates of Man II as revealed by immunofluorescence microscopy is the basis of our assay. The completely dispersed phenotype corresponds to VGMs, and large aggregates are stacks of cisternae unless otherwise specified (Acharya et al., 1995). If the cells are treated

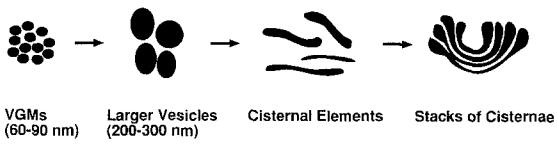

Figure 1. Intermediates in Stack Formation from VGMs

Based on kinetic analysis of the stack formation process (Acharya et al., 1995). VGMs of 60–90 nm average diameter are highly dispersed in the cytoplasm. VGMs first fuse to form larger vesicles of 200–300 nm average diameter, which are clustered together. This is evident in the first 20 min of the assembly process. The larger vesicles in the next 25 min fuse to form tubular elements and short stacks. In the subsequent 15 min, these structures assemble and mature into stacks of cisternae.

with 0.5 mM NEM on ice for 20 min followed by 4 mM dithiothreitol (DTT), to quench the excess NEM, and subsequently incubated in KHM buffer (25 mM HEPES [pH 7.2], 125 mM potassium acetate, 2.5 mM magnesium acetate, and 1 mg/ml glucose) at 32°C, VGMs remain dispersed in the cytoplasm (Figure 2aC). This indicated that a factor(s) sensitive to NEM was required for stack formation from VGMs, confirming our previous findings that stack formation from VGMs was inhibited by the anti-NSF inhibitory antibody (Acharya et al., 1995). The question we then addressed was whether the block in stack formation from VGMs by NEM was due to the inactivation of a single component such as NSF or reflected the involvement of other factors sensitive to NEM.

Reconstitution of the Fusion of VGMs into Larger Vesicles in NEM-Treated Cells

To test for the minimum set of components required for stack formation, NEM-treated cells (as in Figure 2aC) were incubated at 32°C for 60 min in the presence of an ATP-regenerating system with different purified proteins. The treatment included buffer alone (Figure 2bA), purified NSF alone (Figure 2bB), NSF plus α-SNAP (Figure 2bC), NSF plus β-SNAP (Figure 2bD), NSF plus γ-SNAP (Figure 2bE), and NSF plus α-SNAP plus γ-SNAP (Figure 2bF). The cells were fixed and visualized by immunofluorescence after staining for Man II. For each experiment, 200 cells on duplicate coverslips were counted, and greater than 90% of the cells showed the same phenotype. It is evident from the fluorescence data that addition of either purified NSF or SNAPs alone did not promote assembly of VGMs (Figures 2bB–2bE). However, addition of NSF along with both α-SNAP and γ-SNAPs to NEM-treated cells resulted in the assembly of Man II into discrete aggregates (Figure 2bF). Addition of α-SNAP plus β-SNAP plus γ-SNAP in the absence of NSF did not promote assembly from VGMs (data not shown).

To assess the morphological status of large aggregates of Man II–containing membranes formed in the presence of purified NSF and SNAPs, cells described in Figure 2bF were fixed and thin sections were visualized by electron microscopy after immunoperoxidase staining with anti–Man II antibody. Two hundred sections were scanned,

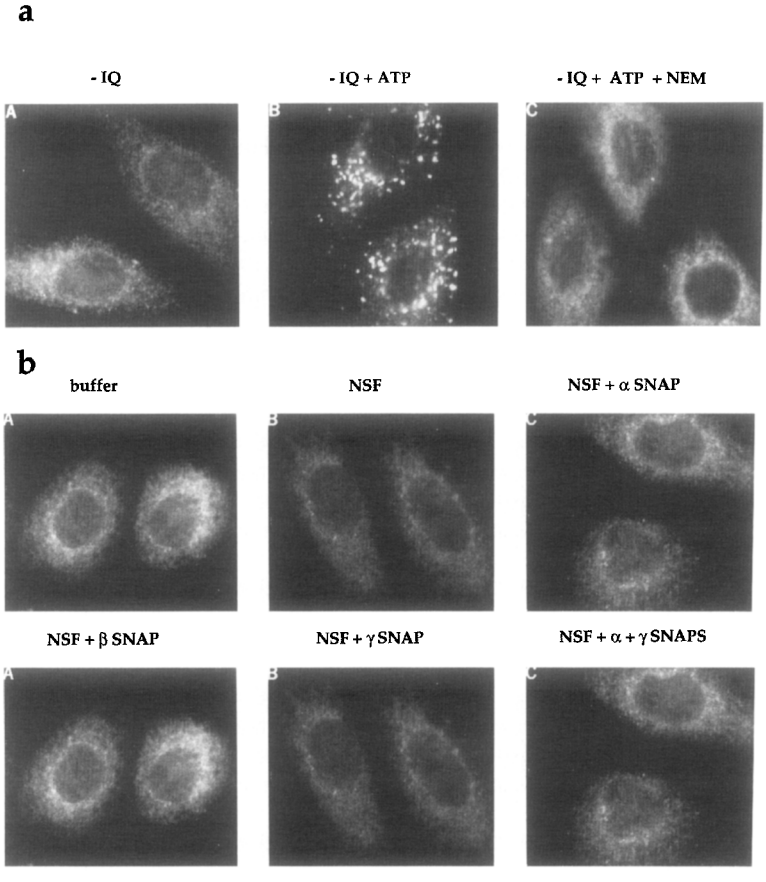

Figure 2. Reconstitution of Fusion and Assembly of VGMs in NEM-Treated Cells

(a) NRK cells grown on coverslips were incubated with IQ (A) to vesiculate the Golgi complex. The cells were washed to remove IQ, permeabilized by a rapid freeze–thaw, and then treated with either buffer (B) or 0.5 mM NEM (C) for 20 min on ice. The cells were then treated with 4 mM DTT to quench excess NEM and then incubated with an ATP-regenerating system at 32°C for 60 min. The cells were fixed for indirect immunofluorescence using the medial/*trans*-specific anti–Man II antibody, followed by rhodamine-conjugated goat anti-rabbit to visualize Golgi membranes. IQ treatment caused the Golgi complex to vesiculate (A), and if these cells are washed to remove IQ, permeabilized, and then incubated with ATP, the VGMs assemble into stacks of cisternae (B) (Acharya et al., 1995). NEM-treatment of permeabilized cells, however, inhibits the fusion process and the VGMs remain dispersed throughout the cytoplasm (C).

(b) NRK cells treated with IQ, and then washed to remove IQ, were permeabilized and treated with NEM as described above. The cells were then incubated with buffer (A), purified NSF (B), NSF plus α-SNAP (C), NSF plus β-SNAP (D), NSF plus γ-SNAP (E), or NSF plus α-SNAP plus γ-SNAP (F) and with an ATP-regenerating system for 60 min at 32°C. The cells were fixed and stained with anti–Man II antibody and visualized by fluorescence microscopy. Only addition of NSF plus α-SNAP plus γ-SNAP promotes assembly of VGMs into larger structures (F). Magnification, 530×.

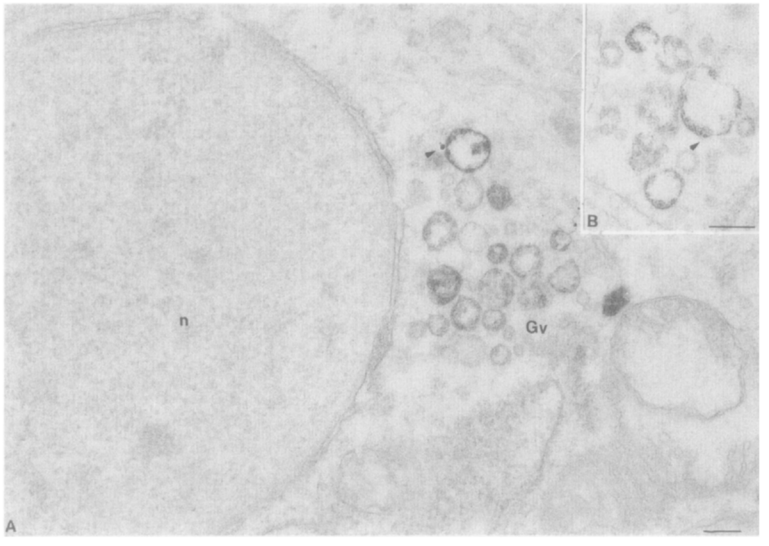

Figure 3. Morphological Analysis of NEM-Treated Cells Incubated with NSF and SNAPs

The sample from Figure 2bF was fixed and processed in parallel for immunoelectron microscopy using anti–Man II antibody as described previously (Acharya et al., 1995). Man II is contained in large vesicles (arrowheads) of about 200–300 nm diameter that are clustered together (labeled Gv). Thus, in NEM-treated cells, VGMs fuse to form clusters of larger vesicles in the presence of purified NSF plus SNAPs. There is no further progression along the stack formation process in these cells. Scale bar, 0.25 μm.

which revealed that addition of NSF and SNAPs alone to NEM-treated cells resulted in the fusion of VGMs into clusters of larger vesicles (Figures 3A and 3B). No stacks were found in the 200 sections visualized. These large vesicles (Gv, marked by arrowheads) are 200–300 nm in diameter, at least three times larger in diameter than the VGMs. Therefore, addition of purified NSF and SNAPs to NEM-treated cells was not sufficient for stack formation from VGMs.

To test the requirement of NEM-sensitive components other than NSF in stack formation, the following experiments were carried out. NEM-treated cells (as in Figure 2aC) were incubated with an ATP-regenerating system for 60 min at 32°C with the following components: KHM buffer (Figure 4A); bovine brain cytosol (4 mg/ml, Figure 4B); cytosol plus 1 M KCl supernatant from isolated rabbit liver Golgi membranes (1 mg/ml, termed "salt extract," Figure 4C); cytosol plus NEM-treated salt extract (Figure 4D); salt extract without exogenous cytosol (Figure 4E); NEM-treated salt extract alone (Figure 4F). The cells were fixed and visualized with anti–Man II antibody. Immunofluorescence analyses revealed that addition of cytosol alone to NEM-treated cells did not promote assembly of VGMs (Figure 4B). However, addition of the salt extract (obtained by stripping purified rabbit liver Golgi membranes with 1 M KCl in the presence of 1 mM ATP) with or without cytosol revealed aggregation of VGMs into larger discrete structures (Figures 4C and 4E). When NEM-treated salt extract in the presence (Figure 4D) or absence (Figure 4F) of exogenous cytosol was added to NEM-treated cells, VGMs remained completely dispersed as revealed by Man II staining. Cells as treated in Figure 4E were processed in parallel for immunoelectron microscopy (Figures 5A and 5B). Again, 200 sections were analyzed that revealed the presence of clusters of large vesicles (Gv, marked by arrowheads), indicating that inclusion of salt extract enabled (under these conditions) VGMs to fuse and assemble into clusters of larger vesicles. Stacks of Golgi cisternae were not found in these sections. Similar results were obtained when NEM-treated cells were incubated with salt extract and cytosol, corresponding to sample shown in Figure 4C (data not shown).

These results demonstrate that, in NEM-treated cells, VGMs do not initiate the stack formation steps unless the reaction is supplemented with purified NSF plus α- and γ-SNAPs. These components can also be provided by the salt extract from isolated Golgi membranes but not by the cytosol, since the preparation of cytosol in the absence of ATP inactivates NSF (Block et al., 1988). What is surprising in these results is that, under conditions in which the reaction is supplemented with the salt extract and cytosol, the maturation of VGMs does not proceed past clusters

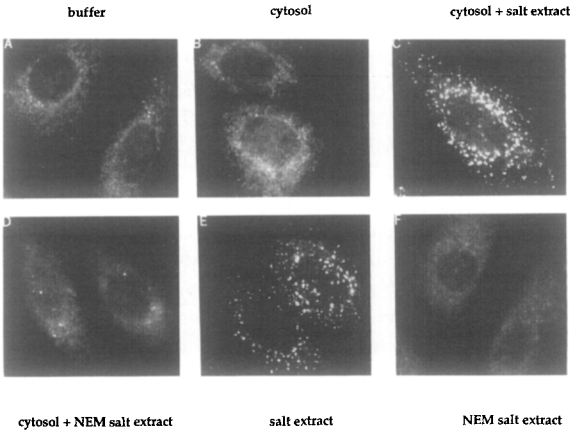

Figure 4. Peripheral Proteins Removed from Isolated Golgi Stacks with 1 M KCl Restore the Fusion of VGMs into Large Aggregates

Semi-intact NRK cells containing VGMs were treated with NEM as described in the legend to Figure 2a. After quenching excess NEM with DTT, the cells were incubated with buffer alone (A), cytosol (B), cytosol plus salt extract (C), cytosol plus NEM-treated salt extract (D), salt extract (E), and NEM-treated salt extract (F) for 60 min at 32°C. Addition of cytosol alone did not support the fusion and assembly of VGMs. Addition of the salt extract in the presence (C) or absence (E) of exogenous cytosol but not NEM-treated salt extract (D and F) caused the fusion and assembly of VGMs into larger structures. Magnification, 310×.

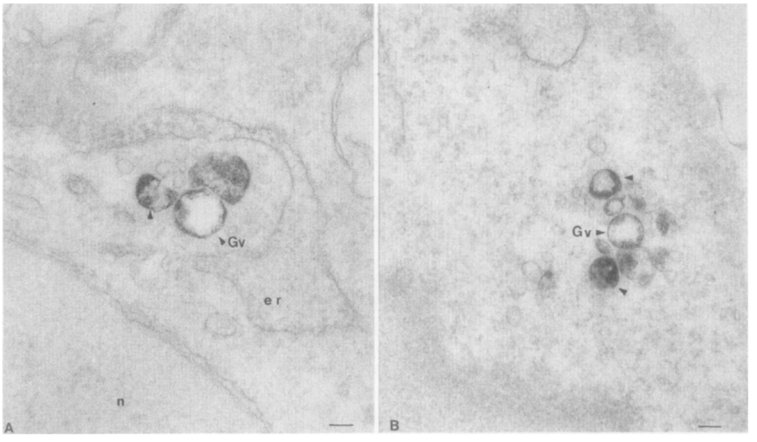

Figure 5. The Addition of Salt Extract from Isolated Golgi Stacks to NEM-Treated Cells Promotes the Fusion and Assembly of VGMs into Clusters of Larger Vesicles

The sample from Figure 4E was fixed and processed in parallel for immunoelectron microscopy using anti–Man II antibody. Man II under these conditions was localized to larger vesicles of about 200–300 nm diameter (labeled Gv). Therefore, the addition of the salt extract to NEM-treated cells restored the fusion and assembly of VGMs into larger vesicles, but there was no further progression of Golgi membranes along the stack formation pathway. Scale bar, 0.1 μm.

of larger vesicles. We have shown earlier that these clusters of larger vesicles are the predominant Man II–containing structures at 15–20 min of the assembly reaction under standard in vitro conditions (Acharya et al., 1995). These results indicate that addition of NSF and SNAPs promotes fusion of VGMs into larger clusters of vesicles but is not sufficient for further stages of stack formation. Subsequent completion of stack formation therefore requires NEM-sensitive component(s) tightly associated with the membrane (i.e., that cannot be stripped by 1 M KCl) and, thus, limiting in the salt extract and inactive in the cytosol preparation used in this assay.

Identification of the Second NEM-Sensitive Component Required for Stack Formation

NSF belongs to a family of ATPases that are sensitive to NEM, and we therefore reasoned that the second NEM-sensitive component required for stack formation might be another NSF-like ATPase. One such well-characterized NEM-sensitive ATPase is a 97 kDa protein purified from Xenopus oocytes (Peters et al., 1990). This protein, known as p97, forms homo-oligomers of 570,000 molecular weight. We provide evidence that p97 is the second NEM-sensitive component required for the formation of Golgi stacks.

As mentioned above, incubation of NEM-treated cells with purified NSF plus SNAPs or with 1 M KCl extract from isolated Golgi membranes resulted in the fusion of VGMs to form clusters of larger vesicles. Supplementing this reaction mixture with bovine brain cytosol (made in the absence of ATP) did not have any restorative activity for the fusion and maturation of larger vesicles into stacks. Thus, a key NEM-sensitive component that remained associated with the membranes was essential for the fusion of larger vesicles and was not present in the reaction mixture. To test whether this NEM-sensitive component was p97, the following experiments were carried out. NRK cells were treated with IQ, washed to remove IQ, permeabilized, and then treated with NEM. The reaction mixture was then incubated with the following components in the presence of an ATP-regenerating system at 32°C: p97 plus cytosol (Figure 6A); p97 plus cytosol plus salt extract (Figure 6B); NSF plus α-SNAP plus γ-SNAP plus cytosol (Figure 6C); NSF plus α-SNAP plus γ-SNAP plus cytosol plus p97 (Figure 6D); NSF plus α-SNAP plus γ-SNAP plus cytosol plus NEM-treated p97 (Figure 6E). Each of the samples mentioned above was fixed and visualized both by light microscopy and electron microscopy using anti–Man II antibody.

Immunofluorescence analysis revealed that addition of p97 alone to the reaction mixture did not promote any assembly from VGMs (VGMs remain completely dispersed in the cytoplasm; Figure 6A). All other combinations, however, resulted in the aggregation of VGMs into larger structures (Figures 6B–6E). Samples 6B–6E were processed for immunoelectron microscopy using anti–Man II antibody. We found that, in cells incubated with p97 plus salt extract and cytosol, VGMs fused to form stacks of cisternae (marked Gs in Figures 7A and 7B). In this experiment, again 200 sections were screened. Stacks of cisternae were observed in 40 sections, and some of the sections revealed the presence of more than one stack as shown in Figure 7A. This is in contrast with the electron micrographs shown in Figures 3A, 3B, 5A, and 5B, in which no stacks were observed in a similar number of sections. Clusters of cisternae (Gs) were also evident in about 40 out of 200 sections in samples incubated with cytosol and purified NSF plus SNAPs plus p97 (Figures 8A and 8B). Addition of NSF plus SNAPs plus cytosol or NSF plus SNAPs plus cytosol plus NEM-treated p97 only resulted in the formation of larger vesicles from VGMs (data not shown). These results clearly indicated that at the concentration of NEM used, NSF, SNAPs, and p97 were the NEM-sensitive components required for stack formation. When NSF and SNAPs were added to the reaction mixture, VGMs fused to form larger vesicles, but stack formation did not occur. Addition of p97, but not NEM-treated p97, to this reaction mixture removed this block and restored stack formation. These results, therefore, clearly demonstrate that p97 is required in addition to NSF for the stack assembly and that NSF and SNAPs act before the p97-requiring step in stack formation.

Western blot analysis of isolated rabbit liver Golgi membranes revealed the presence of p97 (data not shown). The data also indicated that when Golgi membranes were extracted with 1 M KCl in the presence of 1 mM ATP and centrifuged, virtually no p97 was detected in the superna-

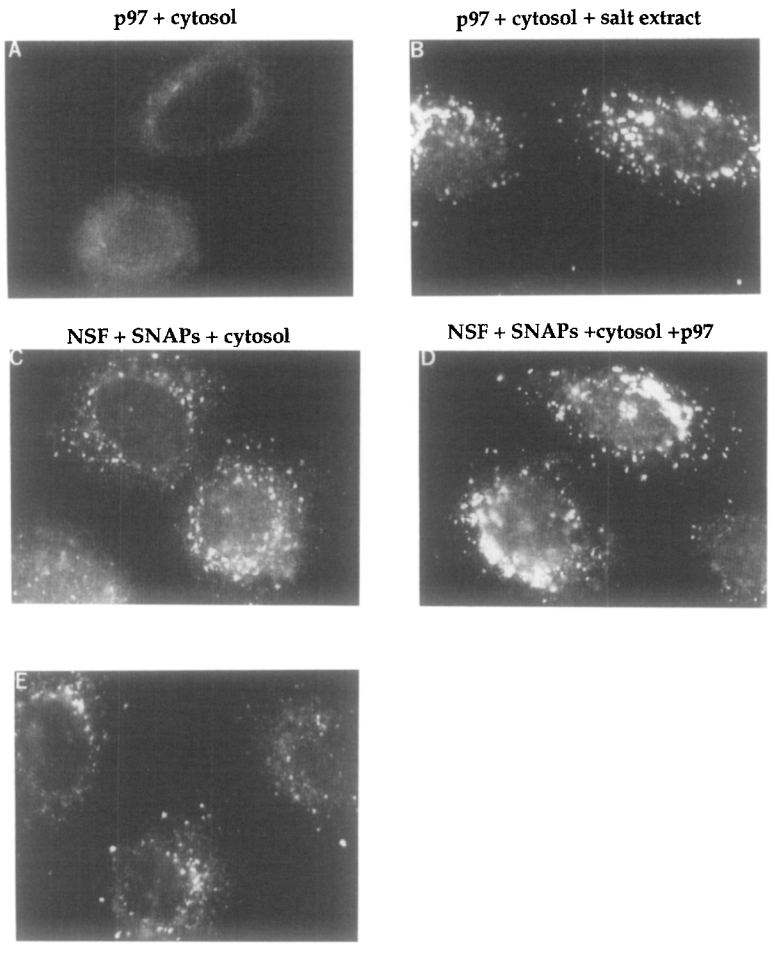

Figure 6. Addition of Purified p97 Either to Purified NSF and SNAPs or to Salt Extract in the Presence of Cytosol Promotes the Assembly of VGMs into Large Aggregates in NEM-Treated Cells

NRK cells treated with IQ, washed to remove IQ, permeabilized, and treated with NEM followed by DTT were incubated with the following reagents at 32°C for 60 min in the presence of an ATP-regenerating system: purified Xenopus p97 plus cytosol (A), p97 plus cytosol plus salt extract (B), NSF plus SNAPs plus cytosol (C), NSF plus SNAPs plus cytosol plus p97 (D), and NSF plus SNAPs plus cytosol plus NEM-treated p97 (E). The addition of p97 alone to NEM-treated cells did not promote the assembly of VGMs; however, in the presence of cytosol, addition of p97 to either salt extract or to NSF and SNAPs promoted the fusion and assembly of VGMs into larger aggregates. Magnification, 400×.

tant (data not shown). These results indicated that p97 was clearly limiting in the salt extract fraction; hence, the stack assembly did not proceed to completion when NEM-treated cells were supplemented with salt extract, but did so when purified p97 was added along with the extract.

Rab-GDI and GTPγS, Inhibitors of Vesicular Protein Transport, Do Not Inhibit Stack Formation from VGMs

The guanine nucleotide–dissociation inhibitor (GDI) dissociates GDP from a variety of Rab proteins (Sasaki et al.,

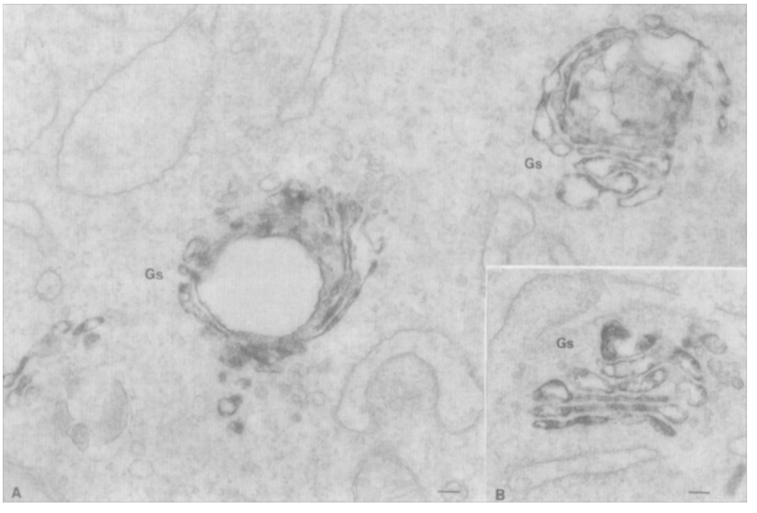

Figure 7. p97 along with Cytosol and the Salt Extract Restores Stack Formation

p97, cytosol, and salt extract were added to NEM-treated cells and (corresponding to the sample shown in Figure 6B) processed for immunoperoxidase staining using Man II antibody. These conditions promoted stack formation from VGMs (labeled Gs). Scale bar, 0.1 μm.

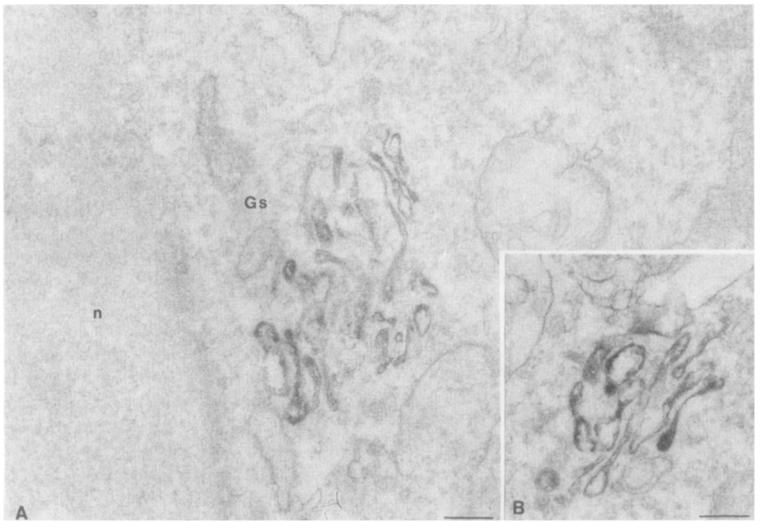

Figure 8. In NEM-Treated Cells, VGMs Form Clusters of Cisternae in the Presence of Purified NSF, SNAPs, p97, and Cytosol

NSF, SNAPs, p97, and cytosol were added to NEM-treated cells, and the cells (corresponding to sample shown in Figure 6D) were processed for immunoperoxidase staining using Man II antibody. These conditions promoted the formation of cisternal elements from VGMs. The cisternae, however, were found clustered together and not properly aligned into stacks (labeled Gs). Scale bar, 0.25 μm.

1990). The cytoplasmic Rab proteins in the GDP-bound form are found associated with GDI, and it also extracts the GDP-bound form of Rab proteins from the membranes (Novick and Brennwald, 1993). GDI has been shown to inhibit vesicular transport between Golgi cisternae (Elazar et al., 1994), ER to Golgi (Peter et al., 1994), and endosomes to Golgi (Dirac-Svejstrup et al., 1994). We tested the role of GDI in fusion and assembly of VGMs into stacks.

Permeabilized NRK cells containing VGMs along with cytosol and an ATP-regenerating system were incubated at 32°C (conditions that reconstitute stack formation) with detergent-free purified GDI (0.8 μg/50 μl reaction mix). Immunofluorescence analysis using anti–Man II antibody revealed assembly of VGMs into large aggregates in the absence (Figure 9aA) and presence (Figure 9aB) of GDI. These large aggregates also contain peripheral Golgi proteins such as coatomers as demonstrated by staining with anti-β-COP antibodies (data not shown). The cells were fixed and processed for immunoelectron microscopy. Stacks of Golgi cisternae were evident in these cells (data not shown). Thus, GDI did not inhibit the fusion and assembly of VGMs into stacks of Golgi cisternae.

To demonstrate that GDI was functional in the reaction mixture employed for the reconstitution of VGMs into stacks, its effect on the transport of newly synthesized proteins from ER to the newly formed stacks was tested both morphologically and biochemically. NRK cells were infected with the temperature-sensitive strain of vesicular stomatitis virus (VSV tsO45). This strain has a thermolabile defect in the transport of VSV G protein from ER to the Golgi (Lafay, 1974). At nonpermissive temperature (39.5°C), the VSV G protein is arrested in the ER. However, upon shifting the cells to permissive temperature (32°C), the G protein rapidly exits the ER and is transported along the secretory pathway to the plasma membrane (Balch et al., 1986). NRK cells infected with the tsO45 strain were treated with IQ to vesiculate Golgi membranes, the cells were washed to remove IQ, permeabilized, washed to remove cytosol, and then reincubated with GDI, exogenous cytosol, and an ATP-regenerating system at 32°C (permissive temperature) for 60 min. The cells were then double labeled with anti-VSV G protein antibody specific for the C-tail (P5D4) and anti-Man II antibody. Cells incubated without Rab–GDI show colocalization of Man II and VSV G protein (Figures 9bA and 9bB). In the presence of Rab–GDI, the Golgi membranes as revealed by the staining for Man II (Figure 9bC) were found in large aggregates. These large aggregates were stacks of cisternae (data not shown). VSV G protein, however, under these conditions was found dispersed throughout the cytoplasm, depicting an ER location (Figure 9bD).

The same experiment was carried out in parallel to monitor the maturation of VSV G protein as it moves from the ER to the Golgi cisternae. The VSV G protein acquires core glycosylation in the ER (Kornfeld and Kornfeld, 1985). The oligosaccharide chains of VSV G protein in the ER are sensitive to digestion with the enzyme endoglycosidase H (endo H). Upon exit and transfer of the VSV G protein to the *cis* and medial compartments of the Golgi, the oligosaccharide chains of the VSV G protein are processed by the Golgi-specific enzymes and the G protein acquires resistance to endo H. The acquisition of endo H resistance is used routinely as a criterion to monitor the trafficking of VSV G protein from ER to the Golgi cisternae. As shown in Figure 9bE, Rab–GDI inhibited the maturation of the oligosaccharide chains of VSV G protein in untreated (lanes 1 and 2) and drug-treated cells (lanes 5 and 6), whereas the G protein was endo H resistant in the absence of GDI in IQ-treated cells (lanes 3 and 4). These results provide additional evidence that, in the reaction mixture described here, Rab–GDI specifically inhibited ER-to-Golgi transport but not the fusion and assembly of VGMs into stacks of cisternae.

Similar experiments were carried out in the presence of GTPγS, and our results demonstrate that 1 mM GTPγS had no effect on the assembly of VGMs into stacks of Golgi cisternae, while the same concentrations of GTPγS completely inhibited protein transport from ER to the newly formed stacks of cisternae (data not shown). In this regard, it is interesting to note that the formation of Golgi stacks

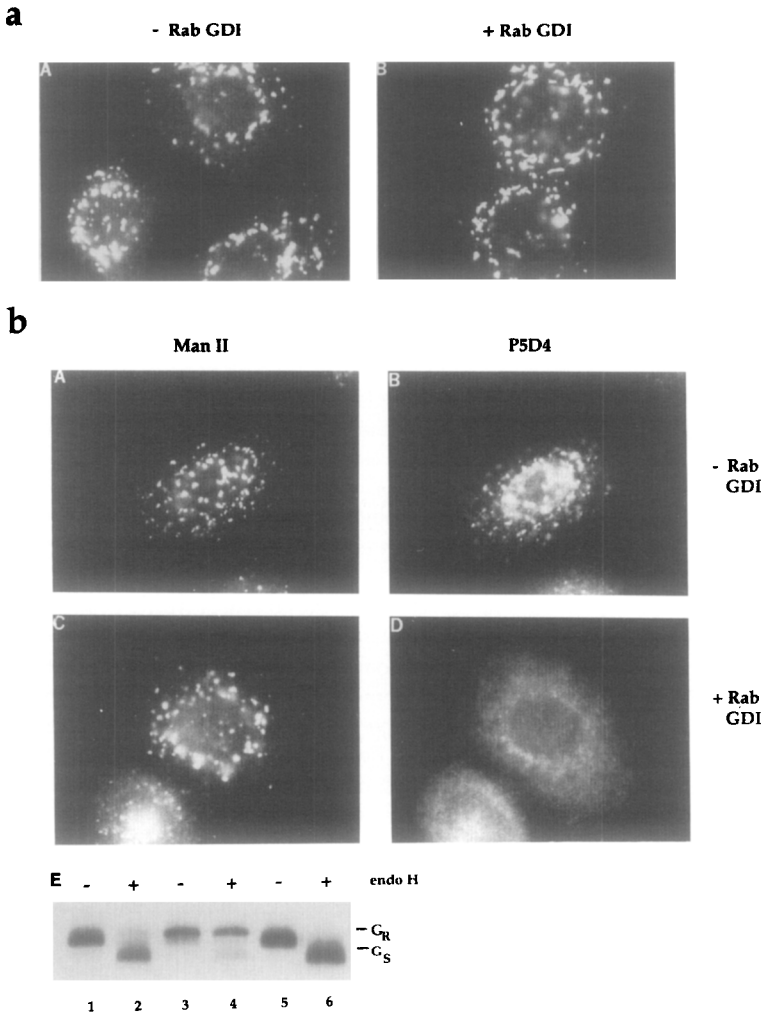

Figure 9. Rab–GDI Does Not Inhibit Stack Formation from VGMs but Inhibits ER-to-Golgi Transport

(a) NRK cells were treated with IQ, permeabilized, and then washed to remove the cytosolic proteins. Addition of cytosol and ATP at 32°C (A) results in the formation of large Man II–containing aggregates. We have shown earlier that these large aggregates are stacks of cisternae (Acharya et al., 1995). Addition of purified Rab–GDI (B) in the presence of cytosol and ATP at 32°C for 60 min also causes the formation of large aggregates of Man II. Immunoelectron microscopy with anti–Man II antibody revealed that these aggregates are stacks of cisternae (data not shown).

(b) NRK cells were infected with the tsO45 strain of VSV and kept at the nonpermissive temperature of 39.5°C. The cells were then incubated with IQ at 39.5°C, washed to remove IQ, permeabilized, washed to remove cytosolic proteins, and then incubated with cytosol and ATP-regenerating system at permissive temperature of 32°C. Addition of Rab–GDI to the reaction mixture blocks transport of VSV G protein from ER to the newly formed stacks (D) but does not block stack formation from VGMs (C). Control incubations lacking Rab–GDI are shown in (A) and (B). Magnification, 570×. A parallel set of coverslips after infection with wild-type VSV were incubated at 37°C with IQ, incubated with [^{35}S]methionine for 8 min, washed to remove IQ, permeabilized, washed to remove cytosolic proteins, and then incubated with cytosol and ATP at 32°C for 60 min in the absence or presence of Rab–GDI. VSV G protein was immunoprecipitated, and one half of the immunoprecipitate from each sample was incubated with endo H. The endo H–treated and untreated samples were analyzed by SDS–PAGE and fluorography. It is evident that, in the presence of Rab–GDI, VSV G protein remained sensitive to endo H treatment (E) (lanes 1 and 2, untreated cells; lanes 5 and 6, IQ-treated cells) demonstrating a block in transport to the medial compartment of the newly formed Golgi stacks. Lanes 3 and 4 show that the G protein is endo H resistant in the absence of Rab–GDI.

from mitotic Golgi fragments composed of cisternal elements and vesicular structures of various sizes was also not inhibited when GTPγS was added during the assembly reaction (Rabouille et al., 1995a).

These findings indicate two important points. First, protein transport from the ER and between Golgi membranes is not required for the fusion and assembly of IQ-derived VGMs into stacks. Second, while we cannot rule out the possibility that VGMs contain appropriate Rabs in the GTP-bound form, it is clear that new Rabs are not recruited during the fusion and assembly into stacks since this process is insensitive to Rab–GDI.

Discussion

VGMs undergo sequential changes in their size and morphology during stack formation (Acharya et al., 1995). Of interest was our observation that within 5 min of initiating the assembly reaction VGMs begin to fuse and are converted to clusters of larger vesicles of 200–300 nm average diameter. By 20 min, the clusters of larger vesicles are the predominant Man II–containing Golgi structures that subsequently become more tubular and finally assemble into stacks of cisternae. We proposed that VGMs may undergo two rounds of fusion: the first round forms larger vesicles that then fuse to form tubular and cisternal elements (Acharya et al., 1995).

We have shown before that, in the presence of anti-NSF inhibitory antibody, VGMs remain completely dispersed in the cytoplasm (Acharya et al., 1995). This indicated that NSF is required for the initial fusion of VGMs to produce larger vesicular intermediates. The question we wished to address was whether NSF is also required for the subsequent membrane fusion events to complete stack formation.

The Fusion of Larger Vesicles Is Catalyzed by an NSF-like ATPase

Treatment of permeabilized cells with NEM inactivates both the cytosolic and the membrane-associated pools of NSF. Incubation of such preparations with exogenously added cytosol in the presence of ATP does not rescue

stack formation, since the exogenous cytosol preparation employed in our assembly reaction does not contain functionally active NSF (Block et al., 1988; Malhotra et al., 1988). Adding a combination of purified NSF plus α- and γ-SNAPs to NEM-treated cells, however, promotes the fusion and assembly of VGMs into clusters of larger vesicles. Adding peripheral Golgi proteins (1 M KCl wash from isolated Golgi stacks) to NEM-treated cells also causes the fusion of VGMs, but again, the reaction does not proceed past the formation of clusters of larger vesicles. We have identified the limiting NEM-sensitive component for the second fusion event as the NSF homolog p97.

Our results clearly demonstrate that p97, in the absence of NSF and SNAPs, does not promote the fusion of VGMs into the larger vesicular intermediates. However, if larger vesicle clusters are allowed to form from VGMs, then adding p97 to NEM-treated cells restores stack formation to completion. Thus, clearly p97 acts only after VGMs have fused to form clusters of larger vesicles by NSF- and SNAP-dependent events. Two important conclusions can be made based on these observations. First, the large vesicular structures that form from VGMs are bona fide intermediates in the stack formation process. Second, while NSF and SNAPs are required for the fusion of VGMs to form larger vesicles, they are not sufficient for subsequent fusion of the larger vesicles to generate stacks of cisternae. Our results, therefore, demonstrate that stack formation is mediated by the sequential action of two homologous ATPases: NSF for the initial fusion of VGMs and p97 for the subsequent fusion of larger vesicles. Our results, however, do not exclude the possibility that NSF may also be required in combination with p97 for the fusion of larger vesicles.

Why Employ Two Distinct ATPases for Golgi Stack Formation

The Golgi is not the only organelle that requires two distinct fusion events for its formation. The biogenesis of peroxisomes in Pichia pastoris also requires two NSF-like ATPases, encoded by the *PAS1* and *PAS5* genes. Disruption of *PAS1* gene results in accumulation of small peroxisomal vesicles, whereas disruption of *PAS5* results in accumulation of larger vesicles that are clustered together (Spong and Subramani, 1993; Heyman et al., 1994). Disruption of both ATPases results in accumulation of smaller vesicles, indicating that Pas1p acts at a stage prior to Pas5p in the process of peroxisomal biogenesis (S. Subramani, personal communication). The morphological phenotype of the precursors that accumulate under these conditions and the sequential requirements of two distinct NSF-like ATPases is highly similar to that observed in Golgi stack biogenesis. The biogenesis of ER membranes in S. cerevisiae, on the other hand, is NSF independent but requires the p97 homolog Cdc48p (Latterich et al., 1995 [this issue of *Cell*]). Warren and colleagues have recently shown that cisternal growth from mitotic Golgi fragments (a heterogeneous population of cisternal elements and tubular–vesicular structures) can be achieved by addition of either p97 or NSF and SNAPs in vitro (Rabouille et al., 1995b [this issue of *Cell*]). How does one reconcile with these differential requirements, since our findings demonstrate that the assembly of Golgi stacks from VGMs requires the sequential action of NSF plus SNAPs followed by p97? Since the starting membranes in the two assays are different, it is possible that Warren and colleagues are not analyzing the initial stages of the reaction, the fusion of small vesicles (VGMs) to form 200–300 nm large vesicles that is NSF dependent.

The question still arises why employ two distinct NSF-like ATPases in the biogenesis of organelles such as the Golgi and peroxisomes and not the ER? Perhaps different fusion events require a particular member of the family of NSF-like proteins, and the number of NSF-like molecules involved simply reflect the collective fusion events required for forming the particular organelle. Indeed, it could be proposed that NSF may facilitate heterotypic membrane fusion, while another NSF-like molecule, such as p97, may promote homotypic membrane fusion. This hypothesis, although conceptually reasonable, may be an oversimplification in reality as illustrated below.

In contrast with the Golgi, peroxisomes are not compartmentalized, and thus, peroxisome formation is likely to involve fusion of homotypic membranes. According to the proposal, peroxisome biogenesis should require only one type of fusion, yet genetic analysis indicates that it requires two distinct fusion events. The ER, although a contiguous network of tubules, can be clearly separated into two distinct regions, the smooth and the rough ER. Thus, for ER formation, like the Golgi stack formation, more than one type of fusion event might be involved: one for smooth ER and another for rough ER. Evidence to date, however, indicates that Cdc48p is sufficient for the fusion of all ER membranes (Latterich et al., 1995).

The disparity in the specificity of NSF-like molecules involved in different types of fusion events during organelle biogenesis and membrane trafficking is not limited to the NSF ATPase family of proteins. It had been thought that membrane fusion events that require NSF or NSF-like proteins in general also display a requirement for GTP-binding proteins. The fusion of nuclear vesicles during nuclear envelop formation, fusion between ER membranes derived from mammalian tissue culture cells, and fusion between endosomes is inhibited by GTPγS (Newport and Dunphy, 1992; Boman et al., 1992; Mayorga et al., 1989; Gorvel et al., 1991). The fusion between endosomes is NSF dependent but also requires hitherto uncharacterized NEM-sensitive components (Diaz et al., 1989; Rodriguez et al., 1994). It is also not clear whether NSF is required for nuclear envelope formation. NSF is clearly not required for fusion of ER membranes in yeast, and this process is also insensitive to GTPγS (Latterich and Schekman, 1994). Vesicular transport from the *trans*-Golgi network to the basolateral plasma membrane in MDCK cells is inhibited by anti-NSF antibodies and Rab–GDI; in contrast, apical transport is insensitive to both anti-NSF antibodies and Rab–GDI (Ikonen et al., 1995). Our results show that, although the first fusion event requires NSF, the reaction is insensitive to GTPγS and Rab–GDI. These findings clearly indicate a lack of obvious consensus for the requirement of these proteins in the homotypic (presumed during or-

ganelle biogenesis) or heterotypic (vesicular transport) mode of membrane fusion. It might therefore be more appropriate to assign these proteins to fusion events defined on the basis of the size of fusing partners, involvement of specific docking mechanisms, i.e., interaction with cytoskeletal or other cellular proteins, and whether other accessory proteins are also required (for example, SNAPs in the case of NSF). In the case of NSF, it is also clear that it binds to a preformed complex of proteins assembled at the vesicle–target membrane junction (Söllner et al., 1993). The binding occurs in the absence of ATP, and subsequent ATP hydrolysis releases NSF from the remaining protein complex. Based on these observations, it is obvious that NSF (and by analogy p97) appears to regulate the function and stability of large protein complexes. Identification of proteins that interact with p97 in a similar manner should, therefore, provide further insight into the mechanisms by which these ATPases regulate membrane fusion events.

Experimental Procedures

Unless otherwise noted, all chemicals and reagents were obtained from sources described earlier (Acharya et al., 1995). The procedures for cell permeabilization, immunofluorescence microscopy, immunoperoxidase staining, infection with tsO45, and labeling of cells were as described earlier (Acharya et al., 1995).

NEM Treatment of Cells

NRK cells were treated with IQ, washed to remove the drug, and permeabilized by freeze–thaw. The cells were then incubated with 0.5 mM NEM for 20 min on ice. The cells were then incubated for 15 min on ice with 4 mM DTT to quench excess NEM. Purified NSF, α-SNAP, β-SNAP, γ-SNAP were added at a final concentration of 500 ng/50 μl reaction mix and p97 at 1 μg/50 μl reaction mix. p97 was inactivated by incubation with 2 mM NEM for 20 min on ice.

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