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Distinct Roles for the AAA ATPases NSF and p97 in the Secretory Pathway

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NSF and p97 are related AAA proteins implicated in membrane trafficking and organelle biogenesis. p97 is also involved in pathways that lead to ubiquitin-dependent proteolysis, including ER-associated degradation (ERAD). In this study, we have used dominant interfering ATP-hydrolysis deficient mutants (NSF(E329Q) and p97(E578Q)) to compare the function of these AAA proteins in the secretory pathway of mammalian cells. Expressing NSF(E329Q) promotes disassembly of Golgi stacks into dispersed vesicular structures. It also rapidly inhibits glycosaminoglycan sulfation, reflecting disruption of intra-Golgi transport. In contrast, expressing p97(E578Q) does not affect Golgi structure or function; glycosaminoglycans are normally sulfated and secreted, as is the VSV-G ts045 protein. Instead, expression of p97(E578Q) causes ubiquitinated proteins to accumulate on ER membranes and slows degradation of the ERAD substrate cystic-fibrosis transmembrane-conductance regulator. In addition, expression of p97(E578Q) eventually causes the ER to swell. More specific assessment of effects of p97(E578Q) on organelle assembly shows that the Golgi apparatus disperses and reassembles normally after treatment with brefeldin A and during mitosis. These findings demonstrate that ATP-hydrolysis–dependent activities of NSF and p97 in the cell are not equivalent and suggest that only NSF is directly involved in regulating membrane fusion.

INTRODUCTION

Membrane fusion is an essential step in all forms of vesicle trafficking and organelle assembly. Fusion is driven by a series of regulated protein–protein interactions. Many participating proteins have been identified, and their specific roles are gradually coming to light (reviewed in Jahn et al., 1999). Among these proteins are a number of ATPases.

N-ethyl maleimide sensitive factor (NSF) was one of the first proteins specifically linked to membrane fusion (Wilson et al., 1989). It belongs to a family of chaperone-like ATPases known as AAA (ATPases associated with a variety of cellular activities; proteins (Neuwald et al., 1999). NSF together with α-SNAP (soluble NSF attachment protein) dissociates the SNARE (SNAP receptor) complexes that promote association and fusion of cellular membranes. More recently, NSF has also been implicated in other cellular processes on the basis of its ability to bind the AMPA receptor GluR2, β-arrestin 1, and GATE-16 (reviewed in Whiteheart et al., 2001). However, its role in SNARE disassembly and membrane fusion remains its best understood function.

Not all ATP-requiring steps that lead to membrane fusion can be attributed to NSF (Goda and Pfeffer, 1991; Latterich and Schekman, 1994; Rodriguez et al., 1994; Wilson, 1995). Other ATPases must therefore be involved. One AAA protein thought to be an alternate to NSF is known as p97 (also referred to as valosin-containing protein, VCP). p97 is an abundant and highly conserved protein (Peters et al., 1990) whose cellular function has been the subject of much debate. A break in the mystery of p97’s function came when it turned up as a factor involved in NSF-independent in vitro fusion between membranes of the ER (Latterich et al., 1995), mitotic Golgi fragments (Rabouille et al., 1995), illamaquione-generated Golgi fragments (Acharya et al., 1995), low-density microsomes (Roy et al., 2000), and fragments of the nuclear envelope (Hetzer et al., 2001). The additional finding that p97 could bind to syntaxin 5 in vitro (Rabouille et al., 1998) led to the proposal that p97 might carry out a reaction similar to the SNARE complex disassembly mediated by NSF, but on different SNAREs (Patel et al., 1998; Rabouille et al., 1998). However, although p97 has been shown to release the t-SNARE syntaxin 5 from a complex with p47 and VCP (Uchiyama et al., 2002), it has so far not been found to disassemble complexes consisting of multiple SNARE proteins. Therefore the mechanism by which p97 participates in in vitro fusion reactions remains unknown.

Meanwhile, a variety of seemingly unrelated activities of p97 have emerged. These include roles in ubiquitin- and proteasome-dependent degradation of cytosolic proteins (Ghislain et al., 1996; Dai et al., 1998; Dai and Li, 2001), ER-associated degradation (ERAD; reviewed in Bays and Hampton, 2002; Tsai et al., 2002), and regulated ubiquitin-dependent processing (Rape et al., 2001). It is believed that different adaptor proteins direct p97 to perform these varied cellular activities. These adaptors include p47 (Kondo et al., 1998).
1997), Ufd1/Npl4 (Meyer et al., 2000, 2002), VCIP135 (Uchiyama et al., 2002), and SVIP (Nagahama et al., 2003). Although the mechanism by which AAA ATPases such as NSF and p97 use ATP to modulate the structure of their substrates is not known, the overall homology between these two enzymes makes the idea that they might operate by a similar mechanism attractive. Each consists of an N domain and two adjacent AAA domains referred to as D1 and D2. The homologous N domains are attached by flexible linkers to hexameric rings formed by the AAA domains and are important in allowing the enzymes to bind their intended substrates (Nagiec et al., 1995; Dai and Li, 2001; Yuan et al., 2001). Conformational changes take place in both enzymes coincident with the ATP-hydrolysis cycle (Hanson et al., 1997; Rouiller et al., 2000, 2002; Beuron et al., 2003) and are presumably transmitted to bound substrate proteins, resulting in their modification.

To compare the functions of p97 and NSF in the secretory pathway, we looked at the effects of expressing dominant interfering mutants of these enzymes in mammalian cells. We find that each mutant inhibits distinct and nonoverlapping cellular processes. Our results support a direct role for NSF in membrane fusion. More strikingly, they suggest a different mode of action for p97 in which its primary role is in the handling of ubiquitinated proteins.

**Materials and Methods**

**Cloning and DNA Manipulation**

For mammalian expression of NSF and NSF(E329Q) GFP fusions, Chinese hamster ovary NSF and NSF(E329Q) were PCR-amplified and cloned between XhoI and BamHI sites in EGFPN1 (Clontech, Palo Alto, CA). This resulted in a C-terminal GFP fusion with a linker of VDPPVAT between the last residue of NSF (K) and the first residue of GFP. Tryptophan-containing plasmids were made as follows: NSF-GFP fusion constructs were excised from EGFPN1 using XhoI and XbaI and subcloned into pcDNA4/TTO (Invitrogen, San Diego, CA). NSF and NSF(E329Q) myc constructs were created by subcloning from the NSF pcDNA4/TTO constructs using BamHI to excise the NSF. This was introduced into the BamHI site of pcDNA4/TTO myc/HisC resulting in a C-terminal His fusion and a linker sequence of SD-PLVQQCGGILQISSTVAAARA between NSF and myc.

A mammalian expression construct for p97 was made by PCR amplification from mouse p97 DNA and cloning between BamHI and Nofl in pcDNA3.1 myc/HisB. This results in a C-terminal myc fusion with a linker sequence of GGRSLSGPRF between the last amino acid of p97 (G) and the first amino acid of the myc tag. p97 mutants (p97(E305Q), p97(E578Q), and p97(E305/578Q)) were generated by Stratagene Quickchange (La Jolla, CA) in pcDNA4/TO myc/HisB (Invitrogen). This results in a C-terminal myc/His fusion with a linker sequence of SD-PLVQQCGGILQISSTVAAARA between NSF and myc.

**Expression and Purification of Recombinant p97**

Proteins were expressed in BL21(DE3) at room temperature by induction with 0.4 mM IPTG at an OD600 of ~1.2. Cells were harvested 4 h after induction. Proteins were purified essentially as described (Meyer et al., 2000). After elution with imidazole, fractions containing p97 were pooled and concentrated (Vivascence, Hanovery, Germany). They were further purified on a Superdex 200 HR 10/30 gel filtration column (Amersham Biosciences, Piscataway, NJ) in gel filtration buffer (50 mM Hepes, 150 mM KCl, 1 mM β-mercaptoethanol, 2 mM MgCl2, 5% glycerol, 0.5 mM NaN3, pH 7.4). Peak protein fractions were then snap-frozen in liquid nitrogen.

Protein concentrations were determined by Bradford assay using bovine serum albumin (BSA) as a standard (Bio-Rad, Cambridge, MA). Quick-freeze deep-etch EM of the p97 molecules was performed as described (Hanson et al., 1997).

**ATPase Activity Assay**

ATPase activities of p97, p97(E305Q), p97(E578Q), and p97(E305/578Q) were assayed at 37 °C in assay buffer containing 25 mM HEPES, 0.5 mM dithiothreitol (DTT), 100 mM NaCl, 1 mM glycerol, 10 mM MgCl2, pH 7.4, and 2 μM ATP. Aliquots removed at 0, 15, 30, 45, and 60 min were quenched in 50 mM EDTA on ice. Released phosphate was detected using a colorimetric assay as described (Lill et al., 1990).

**Generation of Cell Lines and Cell Culture Techniques**

U2OS T-REx cells stably transfected with a tetracycline repressor plasmid (Invitrogen) were transfected to generate cell lines expressing NSF and p97. Medium containing 50 μg/ml hygromycin B (Invitrogen) and 125 μg/ml zeocin (Invitrogen) was added to select the stable cell lines. Approximately 12, 14, and 7 colonies for each desired cell line were transferred into separate wells and screened. In the case of NSF(E329Q), 50 colonies were screened. Immunofluorescence and Western blot analysis were used to identify lines that expressed protein only in the presence of tetracycline. Cells were maintained with 50 μg/ml hygromycin B and 65 μg/ml zeocin. Protein expression was induced by the addition of 1 μg/ml tetracycline for the indicated times. At the standard times used for experiments (12 h for p97 cell lines and 5.5 h for NSF cell lines), 80–90% of the cells expressed the exogenous, tagged protein.

**Immunofluorescence, Immunoprecipitation, and Western Blots**

Immunofluorescence was performed on cells fixed in 4% paraformaldehyde in ice-cold sucrose and permeabilized with 0.1% Triton X-100 with the exception of those cells stained with anti-β-COP which were permeabilized with a mixture of 0.1% Triton X-100/0.5% SDS. For saponin (Sigma, St. Louis, MO) treatment of cells, coverslips were briefly (~5 s) exposed to 0.5% saponin/phosphate-buffered saline (PBS) sucrose before fixation.

Immunoprecipitations were carried out with cells solubilized in 0.5% Triton X-100 under ATP-hydrolyzing conditions (30 μM HEPES, 100 mM NaCl, 5 mM MgCl2, 2 mM ATP, 1 mM DTT, EDTA-free protease inhibitors, pH 7.4). 9E10 anti-myc or affinity-purified polyclonal anti-β-COP antibodies (BS) were used and collected with protein G-Sepharose beads (Amersham). Immunoblots were developed as described (Hanson et al., 1997).

The following antibodies were used in this study: Mouse monoclonal anti-β-COP (Research Diagnostics, Flanders, NJ), rabbit anti-α-tubulin (Cell Signaling Technology, Beverly, MA), rabbit anti-giantin (Covance, Richmond, CA), mouse monoclonal anti-PDI and rabbit anti-calnexin (SPA-860; Stressgen, Victoria, BC, Canada), mouse monoclonal ubiquitin FK2 (A.R. Research Products, Exeter, UK), mouse monoclonal anti-α-tubulin (clone DM 1A) and anti-β-COP (clone maD; Sigma), rabbit anti-COPII (Affinity Bioreagents Inc., Golden, CO), rabbit polyclonal (B1) and mouse monoclonal anti-NSF (CBS311, gift from Reinhard Jahn), mouse monoclonal anti-αSNAP (CIP72, available from S. Dalal), goat anti-α-tubulin (Santa Cruz), mouse monoclonal anti-NSF (CBS311, gift from Reinhard Jahn), mouse monoclonal anti-αSNAP (CIP72, available from S. Dalal), mouse monoclonal anti-myc (9E10), rabbit anti-GFP (BS), rabbit anti-p47 (gift from Graham Warren), mouse monoclonal anti-p115 (BD Transduction Labs, Lexington, KY), sheep anti-TGN46 (Serotec, Oxford, UK). Secondary goat anti-mouse, goat anti-rabbit, and donkey anti-sheep antibodies conjugated to Alexa 488 or Alexa 568 were purchased from Molecular Probes (Eugene, OR).

**CFTR Degradation**

p97(E578Q) cells were grown in six-well trays and infected with 1.2 × 10⁹ adenoviral particles containing a ΔS508 CFTR expression vector (provided by the Vector Core of the Institute for Human Gene Therapy at the University of Pennsylvania School of Medicine, Philadelphia, PA). Thirty-six hours after infection, tetracycline was either added (ON) or not (OFF). Twelve hours after infection, cells were starved in methionine-free media for 45 min, and then labeled with 100 μCi trans-35S-label (ICN, Costa Mesa, CA) per well for 30 min. After labeling, cells were washed and incubated in media containing 25 μg/ml cycloheximide for the indicated chase times. Cells were solubilized in 700 μl of RIPA buffer (150 mM NaCl, 50 mM HEPES, pH 7.4, 1 % NP-40, 0.5 % deoxycholate, 0.2 % SDS, 0.2 % IBSA, 0.5 mM phenylmethylsulfonyl fluoride) with 1 μg/ml protease inhibitor (Roche, Nutley, NJ) for 30 min at 4°C. To preclar the cell lysates, they were incubated with 30 μl of a 50 % slurry of Pansorbin cells (Calbiochem, La Jolla, CA) at 4°C. Pansorbin cells and insoluble material were removed by centrifugation at 10,000 rpm for 10 min. Immunoprecipitation of the ΔS508 CFTR was carried out with 5 μl of polyclonal antiserum raised against the N-terminus of CFTR (Meacham et al., 1999). Immunoprecipitated material was eluted from the beads by heating at 37°C for 15 min in 2x SDS sample buffer. Eluted material was analyzed by SDS-PAGE and fluorography. Densitometry was carried out on a Bio-Rad GS-700 densitometer.

**GAG Sulfation Assay**

To monitor sulfated GAG synthesis, we measured 35S-labeled sulfated GAGs as follows (based on Millar and Moore, 1992). Cells were incubated in 5 mM xylene (4-methylumbelliferyl-β-D-xyloside, Sigma), for 30 min to initiate
GAG synthesis, and then labeled with 200 μCi/ml 35S-sulfate in DME/HEPES at 37°C for 2 min. Reactions were terminated by adding wash solution containing 4 mM unlabeled Na2SO4. Cells were lysed in KHMgE (70 mM KCl, 30 mM HEPES, 5 mM MgCl2, 3 mM EGTA, pH 7.4) containing 1% Triton. Samples were digested with pronase E (Sigma) for 1 h and then precipitated with 15% cetylpyridinium chloride (Sigma) and chondroitin sulfate (Sigma) for 1 h. Samples were collected on nitrocellulose filters and counted in scintillation fluid.

Secretion of 35S-labeled sulfated GAGs was measured by labeling sulfated GAGs for 2 min with 35S-sulfate as above, and then chasing in DME/HEPES at 37°C for 0, 5, 10, 15, and 20 min. 35S-labeled sulfated GAGs were then collected separately from the cell media and the lysed cells as above. The results of all experiments shown were confirmed in three independent trials.

**VSV-G ts045 Trafficking**

p97(E578Q) and U2OS cells were transfected with VSV-G ts045-GFP (Presley et al., 1997) using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. After transfection, cells were shifted to 38.5°C. Four hours later, tetracycline was added and coverslips were incubated at 38.5°C for an additional 12 h. Cycloheximide (100 μg/ml) was then added for 30 min before moving the coverslips to 32°C. Coverslips were removed, fixed, and processed for immunofluorescence at 0, 30, 60, and 120 min later, as described above, using rabbit anti-GFP antibody.

**Brefeldin A Treatment of Cells**

Cells were incubated in 5 mM brefeldin A (BFA; Molecular Probes) in media at 37°C. After 40 min, coverslips were either fixed directly or allowed to recover for 2 h and then fixed.

**Electron Microscopy**

For traditional thin sections, cells grown to ~70% confluence in a 35-mm dish were treated with tetracycline, washed in phosphate-buffered saline, fixed in 2.5% glutaraldehyde in Na-cacodylate in situ, embedded, sectioned, and stained with uranyl acetate according to standard procedures. For freeze-substitution, cells were grown and induced on 4-mm glass coverslips. Coverslips were rapidly frozen and transferred to liquid nitrogen (Heuser et al., 1979). Freeze-substitution was performed by exposing the coverslip to frozen 4% osmium tetroxide in anhydrous acetone (Heuser and Reese, 1981). Coverslips were removed, fixed, and processed for immunofluorescence at 0, 30, 60, and 120 min later, as described above, using rabbit anti-GFP antibody.

**Light Microscopy**

Confocal microscopy was performed on a Radiance 2000 Bio-Rad microscope using 488- and 543-nm laser lines. All images were acquired with sequential scans using LaserSharp 2000. Images were prepared using Adobe Photoshop (Adobe Systems, San Jose, CA).

**RESULTS**

**Inverse Arrangement of AAA Domains in NSF and p97**

NSF and p97 share a common domain structure, consisting of N domains followed by two AAA domains referred to as D1 and D2. Previously, it has been shown that the D1 domain is responsible for NSF and p97 share a common domain structure, consisting of N domains followed by two AAA domains referred to as D1 and D2. Previously, it has been shown that the D1 domain is responsible for NSF’s ATPase activity (Whiteheart et al., 1994). In contrast, NSF’s D2 domain stabilizes the hexameric state of the enzyme but does not hydrolyze ATP (Nagiec et al., 1995).

In the case of p97, it was not clear at the start of this study which (or both) of the two AAA domains is directly responsible for the enzyme’s ATPase activity. Both of p97’s AAA domains are more homologous to each other and the D1 domain of NSF than to NSF’s D2 domain. There are precedents in other two domain AAA proteins for D1, D2, or both being critical for function (Vale, 2000).

To define the relative contributions of the AAA domains to p97’s ATPase activity, we mutated residues in each that are known to be important for function in related ATPases. The second acidic residue in each Walker B DExx motif was replaced with a glutamine to generate potential hydrolysis defective mutants (Hung et al., 1998). We created three p97 variants (p97(E305Q), p97(E578Q), and p97(E305/578Q)) and purified them after expression in *Escherichia coli*. All assembled into oligomers that eluted from a size exclusion column at a position consistent with a 540-kDa hexamer (our unpublished observations). Deep-etch electron microscopy confirmed that all variants assembled into cylinders indistinguishable from those formed by wild-type p97 (Figure 1A).

Measurements of ATP hydrolysis by wild-type and the mutants revealed differences in their ability to hydrolyze ATP (Figure 1B). p97(E305Q) was similar to wild-type p97 and hydrolyzed ATP at a rate comparable to that previously reported for p97/VCAP (~7 nmol/h/μg p97; Peters et al., 1992; Egerton and Samelson, 1994; Meyer et al., 1998), whereas p97(E578Q) had severely impaired ATPase activity. The double mutant p97(E305/578Q) had no ATPase activity. These data show that the D2 domain of p97 is responsible for most of the enzyme’s ATPase activity and are consistent with data recently reported by others (Lamb et al., 2001; Kobayashi et al., 2002; Song et al., 2003; Ye et al., 2003). The dominance of the D2 domain in p97 contrasts with that of the D1 domain in NSF, indicating that the N-domains of the respective enzymes must be coupled to their hydrolytically

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**Figure 1.** The D2 domain of p97 is its dominant ATPase domain. (A) Deep-etch electron microscopy of bacterially expressed and purified p97 demonstrates that Walker B DExx box mutations in D1(p97(E305Q)), D2 (p97(E578Q)), or both (p97(E305/578Q)) do not alter its normal hexameric cylindrical structure. Molecules are 13–15 nm in diameter. (B) Normal ATPase activity of wt p97 (•) and p97(E305Q) (○) versus diminished ATPase activity in p97(E578Q) (●) and p97(E305/578Q) (□) indicates that its D2 domain is responsible for the majority of the enzyme’s ATPase activity. Data shown are an average of two independent measurements and are representative of four independent trials.
active ATPase domains in different ways. Possible mechanisms for this are suggested by the recent structure of p97 (DeLaBarre and Brünger, 2003).

**NSF and p97 Walker B Mutants Accumulate on Membranes**

To compare the roles of NSF and p97 in vivo, we generated cell lines in which expression of wild-type or hydrolysis-defective mutants could be induced by addition of tetracycline. Induced proteins were marked with C-terminal epitope tags, either myc (both enzymes) or GFP (NSF). Shown in Figure 2, A and E, is the time course of protein expression in each of the cell lines. p97 is more abundant than NSF in the cell (comprising ~1% of cell protein vs. ~0.1% for NSF), and thus it took longer for induced p97 to reach endogenous levels. Experiments with NSF cell lines were typically done 5.5 h after induction, whereas those with p97 cell lines were done 12 h after induction. For the NSF(E329Q) and p97(E578Q) cell lines, these times are twice as long as it takes for the mutant protein to reach a level equivalent to endogenous protein. To confirm that most or all holoenzymes contain some mutant subunits, we immunoprecipitated induced proteins and found that endogenous NSF or p97 coprecipitated with their tagged counterparts at a ratio similar to that of the proteins in the extract (Figure 2, B and F). This has previously been shown that the presence of any inactive subunits within a holoenzyme destroys NSF (Whiteheart et al., 1994) and p97 (Wang et al., 2003) function. Based on this and the fact that ATP-bound NSF has highest affinity for its substrates (Hanson et al., 1997), we anticipated that Walker B mutations in each enzyme’s active AAA domain would function as dominant inhibitors and accumulate on their respective substrates in the cell leading to effective inhibition even in the presence of endogenous enzyme.

What effect do these mutations have on protein localization? As expected, wtNSF was cytosolic and excluded from the nucleus (Figure 2D). Brief permeabilization with saponin before fixation depleted the cell of wtNSF, leaving only a small amount on the membranes of the Golgi apparatus. In contrast, NSF(E329Q) was enriched on various structures throughout the cell and was only minimally released by saponin permeabilization (Figure 2D). All known substrates
for NSF are membrane proteins (primarily the SNAREs); hence the shift of NSF(E329Q) from cytosol to membranes is consistent with binding and retention on these membrane associated substrates. Furthermore, when NSF was immuno-precipitated from detergent-solubilized cell extracts in the presence of Mg$^{2+}$-ATP, α-SNAP coprecipitated with NSF(E329Q) but not with wtNSF (Figure 2C). This shows that normal ATP-sensitive interactions of NSF persist under hydrolyzing conditions with NSF(E329Q) and confirms that NSF(E329Q) functions as a “substrate trap.”

Similar experiments with cell lines expressing p97 and its mutants showed that all variants of p97 were present in both the cytosol and nucleus, but the predicted substrate trap p97(E578Q) was selectively retained on reticular membranes after saponin permeabilization (Figure 2G). These membranes could be costained with markers of the ER (unpublished data). These experiments show that mutation of the D2 AAA domain of p97 traps a significant portion of p97 on membranes in vivo. Note that in contrast to the behavior of NSF(E329Q), there is also a sizeable pool of soluble p97(E578Q), possibly reflecting the presence of soluble substrate traps. Together with experiments described below, these results demonstrate that D2 is the AAA domain most critical for p97’s activity in vivo as well as in vitro. The presence of p47 in immunoprecipitates of all three p97 variants confirmed that physiologically relevant interactions are maintained (Figure 2F).

**NSF(E329Q) Disrupts Golgi Structure**

To monitor effects of expressing NSF(E329Q) on organelle morphology, we carried out immunofluorescence using markers for a variety of cellular compartments. Induction of myc- or GFP-tagged wtNSF had no effect on the normal morphology of either the Golgi or the ER (Figure 3, A and C). In contrast, expression of myc- or GFP-tagged NSF(E329Q) disrupted the organization of the Golgi apparatus (Figure 3, B and D). Membranes containing giantin (a Golgi membrane protein) quickly dispersed throughout the cell into small and large punctate structures and then over several hours coalesced into discrete clusters concentrated near the nucleus and the microtubule organizing center. Other markers for the Golgi apparatus and the TGN, including p115, β-COP, and TGN46, were similarly dispersed in NSF(E329Q) cells (not shown). Not all NSF(E329Q) containing structures colocalized with giantin (Figure 3B), suggesting that NSF(E329Q) was also trapped on other membranes. The structure of the ER was not apparently perturbed by expression of NSF(E329Q) (Figure 3D). ER exit sites marked by the COPII coat complex were present at the usual density throughout the cell, and the nuclear envelope appeared intact (our unpublished observations).

To further define the changes caused by NSF(E329Q), we examined the ultrastructure of cells expressing wtNSF or NSF(E329Q) by thin-section electron microscopy (EM; Figure 4). U2OS cells contain well-organized, and abundant Golgi stacks, with variable degrees of dilation at their rims, and these were unaffected by expression of wtNSF (Figure 4A). The ribosome studded ER and nuclear envelope in these cells were similarly typical. After induction of NSF(E329Q), likely Golgi remnants (Figure 4B) and pools of vesicles (Figure 4C) were seen in the perinuclear region normally occupied by Golgi stacks, which were now absent. These results confirm that NSF activity is needed to maintain Golgi structure and imply that it is not required for vesicle formation from Golgi stacks.

**p97(E578Q) Does Not Disrupt Golgi Structure But Causes the ER to Swell**

To compare cellular activities of p97 with those of NSF, we examined the morphology of the ER and Golgi in cells expressing wt-p97, p97(E305Q), or the hydrolysis-deficient p97(E578Q) mutant. This showed that expression of myc-tagged wt-p97 or p97(E305Q) had no effect on either the ER (Figure 5, A and B) or the Golgi (Figure 5, E and G). Likewise, expression of p97(E578Q) had no effect on the Golgi (Figure 5, F and H) and the ER remained normal looking for several hours after induction. However, over time many cells expressing p97(E578Q) developed large vacuoles (Figure 5, C and D). These vacuoles stained in their interior with an antibody recognizing the luminal ER protein PDI and on their limiting membrane with the ER membrane protein calnexin (Figure 5D), demonstrating that they derive from the ER. Expression of p97(E578Q) did not, however, affect distribution of the COPI coat protein β-COP (Figure 5G vs. H) or the COPII coat component Sec23 (Figure 5I vs. J).

Examination of p97(E578Q)-expressing cells by thin-section EM confirmed the presence of large ribosome studded vacuoles, clearly representing dilated endoplasmic reticu-
ubiquitin conjugates (Fujimuro et al., 1994; Figure 7A). This showed that expressing p97(E578Q) caused a significant build-up of ubiquitinated proteins on intracellular membranes. Importantly, p97(E578Q) itself was not ubiquitinated (unpublished data).

The accumulation of ubiquitinated proteins on the ER in p97(E578Q)-expressing cells may reflect a defect in dislocation and subsequent degradation of membrane proteins targeted for the proteasome such as has been shown for H-2Kb in vitro (Ye et al., 2001). To test this, we infected cells with adenovirus encoding the ERAD substrate ΔF508 CFTR and examined the effect of inducing either wild-type p97 or p97(E578Q) on its turnover (Figure 7B). p97(E578Q) significantly slowed degradation of ΔF508 CFTR (Figure 7B), whereas wild-type p97 did not have an effect on the turnover of ΔF508 CFTR (our unpublished observations). This confirms that ATPase activity in the D2 domain of p97 is important for efficient degradation of a known multi-transmembrane domain-containing ERAD substrate.

Effects of Inhibiting NSF and p97 on Secretory Pathway Function

The differing effects of NSF and p97 mutants on ER and Golgi structure suggest that these mutants will affect trafficking in the secretory pathway differently as well. We monitored intra-Golgi transport and secretion by measuring synthesis and release of sulfated glycosaminoglycans (GAGs; Miller and Moore, 1992; Fernandez and Warren, 1998). We found that GAG sulfation was decreased in cells expressing NSF(E329Q). Shown in Figure 8A is the inhibition in GAG synthesis seen after induction of NSF(E329Q) for different times. This time course parallels the morphological disruption of the Golgi apparatus described above and demonstrates that intra-Golgi transport is impaired by NSF(E329Q).

In contrast, GAG synthesis was not affected by expressing p97(E578Q), demonstrating that intra-Golgi transport remained normal (unpublished data). We therefore measured secretion of the sulfated GAGs to examine transport between the trans-Golgi network and the plasma membrane. Secretion was unaffected by p97(E578Q) (Figure 8B), suggesting that even when p97 activity is suppressed, the cell can transport material within its Golgi apparatus and to the cell surface.

To test the possibility that p97 might be more specifically involved in trafficking between ER and Golgi (Zhang et al., 1994; Roy et al., 2000), we monitored the delivery of GFP-tagged VSV-G ts45 protein from the ER to the cell surface in individual cells (Presley et al., 1997). Regardless of whether p97(E578Q) was expressed or not, transferring the cells from nonpermissive to permissive temperature allowed movement of VSV-G bo45 from ER to Golgi and then to the cell surface (Figure 8C), except in cells in which the ER was fully vacuolated and thereby occupying most of the cell’s volume. These experiments demonstrate that the ER remains able to concentrate and secrete cargo even when p97 activity is impaired.

Effects of p97(E578Q) on Golgi Reassembly In Vivo

Nothing in our analysis so far would suggest that p97 activity is necessary for normal function of membranes in the secretory pathway. However, because p97 has most clearly been implicated in organelle assembly reactions reconstituted in vitro (Acharya et al., 1995; Lutterich et al., 1995; Rabouille et al., 1995; Roy et al., 2000; Hetzer et al., 2001), we specifically asked whether p97(E578Q) expression affects de novo organelle assembly in vivo. Both p97 and NSF are
needed to form Golgi stacks from mitotic Golgi fragments in vitro (Rabouille et al., 1995). To our surprise, p97(E578Q) expressing cells assembled normal-looking Golgi ribbons rimmed by giantin during telophase, as assessed by light microscopy (Figure 9). Although we have not looked at these Golgi membranes by EM, we conclude from their regular organization that impairing p97 activity has little or no effect on the organization of membranes through mitosis.

A variety of pharmacologic agents also reversibly disrupt Golgi structure, including BFA, ilimaquinone, okadaic acid, and nocodozole (Dinter and Berger, 1998). We asked whether p97 is involved in Golgi reassembly after treatment with BFA. Both control U2OS and p97(E578Q)-expressing cells responded to BFA with rapid dispersal of their Golgi membranes (Figure 10). Removing BFA from these cells led to the prompt reformation of normal-looking Golgi apparatus even in cells whose ER had visibly swollen. Hence, assembly of Golgi ribbons after mitotic or chemical dispersion does not appear, in our in vivo studies, to require p97’s ATPase activity.

DISCUSSION

In this study, we have used dominant interfering mutants to define the roles that the AAA ATPases NSF and p97 play in the structure and function of the secretory pathway in animal cells. We confirm that NSF’s ATPase activity is essential for transport through the Golgi apparatus and show that interfering with this activity promotes disassembly of Golgi stacks. Surprising in comparison is the finding that inhibit-

Figure 5. p97(E578Q) perturbs the ER but does not affect Golgi morphology. (A) p97myc (anti-myc, red) and (B) p97(E305Q)myc (anti-myc, red) do not affect normal ER morphology as observed using the ER marker anti-PDI (green). (C) p97(E578Q)myc (antimyc, green) leads to swelling of the ER (anti-PDI, red) in ~37% of cells after a 12-h induction. (D) Staining of p97(E578Q)myc with an ER membrane marker (anti-calnexin, green) and lumenal marker (anti-PDI, red) confirms that vacuoles are ER derived. (E, G, and I) p97myc (anti-myc, red) and (F, H, and J) p97(E578Q)myc (anti-myc, red) do not perturb (E and F) giantin (anti-giantin, green), (G and H) COPI (anti-β-COP, green), and (I and J) COPII (anti-Sec23, green). These images were collected on a confocal microscope. Each box is 83 x 83 μm.

Figure 6. Dilated ER and nuclear envelope in p97(E578Q) expressing cells. Traditional thin section EM of (A) a p97(E578Q)myc expressing cell in which the ER looks relatively normal, (B) a p97(E578Q)myc expressing cell containing swollen ribosome-studded ER. (C) Thin-section EM of freeze substituted p97(E578Q)myc expressing cell showing swollen ER and nuclear envelope together with apparently normal Golgi. Scale bars, 0.2 μm.
would impair movement between intracellular compart-
ments and disrupt the organization of the secretory path-
way. This was indeed the case. The ultrastructural appearance
of membranes in NSF(E329Q)-expressing cells (Figure 4)
recapitulates the accumulation of vesicles seen after NEM
treatment of Golgi membranes in vitro as well as after treat-
ment of whole cells with NEM (Orci et al., 1989). Interest-
ingly, Golgi membranes dispersed by NSF(E329Q) (Figure 3)
did not appear to fuse with the ER, but instead moved
around the cell and ended up clustered near the nucleus
(our unpublished observations). A likely explanation for the
lack of fusion between these Golgi-derived vesicles and the
ER is that they contain inappropriate cis-SNARE complexes
that cannot participate in fusion. Such an accumulation of
SNARE complexes has been shown to happen after inactiv-
vating NSF in Drosophila (Littleton et al., 1998; Tolar and
Palanick, 1998; Mohtashami et al., 2001) and yeast (Grote et
al., 2000).

The affinity of AAA proteins for substrates is typically highest
in the ATP-bound state (Hanson et al., 1997; Vale,
2000); hence the localization of hydrolysis deficient (presum-
ably ATP-bound) NSF(E329Q) should reflect the distribution
of NSF’s substrates in the cell. In fact, this mutant was almost
completely retained on membranes (Figure 2). Al-
though we could not identify all of the membranes that
recruited NSF(E329Q), many contained markers for the
Golgi apparatus (Figure 3) or the late endosome (LAMP2,
our unpublished observations). Consistent with this recruit-
ment to endosomal membranes, NSF(E329Q) has previously
been shown to interfere with phagocytosis and endosomal
mature (Coppolino et al., 2001). Golgi and endosome
membranes are among the most dynamic in the cell and thus
are likely to have the highest concentration of SNAREs to
recruit NSF. ER membranes in contrast recruited little
NSF(E329Q), despite the known ability of their SNAREs to
bind α-SNAP and NSF (Hay et al., 1997; Hatsuzawa et al.,
2000), indicating that they may have a low concentration of
SNAREs.

p97 Function in U2OS Cells
p97 (Cdc48p in yeast) has been implicated in membrane
fusion based on its role in assays that reconstitute aspects of
organelle assembly in vitro (see Introduction and below). We
therefore expected that inhibiting p97 in vivo would at
least partially disrupt ER and Golgi structure and function.
When this did not happen (Figures 5 and 8), we looked
specifically at Golgi reassembly under circumstances similar
to those in which p97 had been studied in vitro (Acharya et
al., 1995; Rabouille et al., 1995). Hydrolysis-deficient p97 did
not block the cells’ ability to progress into and through
mitosis, allowing successful reassembly of Golgi apparati
during telophase (Figure 9). Similarly, mutant p97 did not
interfere with BFA-induced disassembly or postwashout re-
cov ery of the Golgi ribbon (Figure 10).

However, before concluding from these results that p97 is
not directly involved in the membrane fusion needed to
assemble and restructure the Golgi, we had to be sure that
expressing p97(E578Q) inhibited some known function of
p97. Indeed, cells induced for p97(E578Q) accumulated
ubiquitin-conjugated proteins on their ER (Figure 7). p97
(E578Q) causes accumulation of ubiquitinated pro-
teins on ER membranes and a delay in ΔF508 CFTR degradation. (A) After saponin permeabilization, retained ubiquitin-conjugates (FK2,
green), and p97myc, p97(E305Q)myc, or p97(E578Q)myc (anti-myc,
red) were visualized by immuno-
fluorescence. These images were
collected on a confocal microscope. Each box is 73 × 73 μm. (B)
Pulse chase analysis of ΔF508 CFTR turnover in cells expressing
(ON) or not (OFF) p97(E578Q). Immunoprecipitated ΔF508 CFTR is
shown in the autoradiographs on the left (OFF, top; ON, bottom),
and quantitated by densitometry on the right. The data presented
are representative of four independent trials.

Effect of ATPases in vivo suggest that NSF and p97 have primary
roles in different cellular reactions.

NSF and Membrane Fusion

Many experiments have shown that NSF is essential for
proper assembly and function of organelles in the secretory
and endocytic pathways (reviewed in Whiteheart et al.,
2001). NSF operates in these reactions at least in part by
dissociating SNARE complexes that form during membrane
fusion (Sollner et al., 1993). Thus, we expected that inhibiting
NSF activity by expressing a dominant interfering mutant
would impair movement between intracellular compart-
ments and disrupt the organization of the secretory path-
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ubiquitin-conjugated proteins on their ER (Figure 7). Accumulation of ΔF508 CFTR in
cells overexpressing a p97 Walker A motif mutant has also recently been reported (Kobayashi et al., 2002). Additional evidence that p97(E578Q) interferes with p97’s normal activity came from finding that it causes the ER to vacuolate. Although the secretory pathway remained functional, expressing p97(E578Q) did lead to dramatic dilation of the ER (Figures 5 and 6; see also Hirabayashi et al., 2001; Kobayashi et al., 2002). Why the ER swells after inhibiting p97 is not clear, but may be related to the activity associated with one of its cofactors, SVIP, because manipulating SVIP causes a similar dilation of the ER (Nagahama et al., 2003). These morphological changes seem unlikely to be caused by a defect in membrane fusion between tubules of the ER. The predicted outcome of such a block in fusion would be an ER network with a lower than normal number of 3-way junctions and not the dilated ER that we observed. One possibility is that the ER dilates because of the impairment in ERAD or related degradative processes (Figure 7). This is suggested by the fact that similar swelling can be induced by treating cells with the proteasome inhibitors PSI and MG115 and antagonized by overexpressing wild-type p97 (Hirabayashi et al., 2001). We do not, however, think that ER dilation is the result of global accumulation of misfolded proteins in the ER lumen because adding cycloheximide after a short induction of p97(E578Q) did not prevent it (our unpublished observations). Another possible explanation for the swelling would be a change in the organization of ER subdomains. Such disorganization could result in changes in the ratio,
brane trafficking reactions and our finding that dominant interfering mutants do not readily disrupt membrane trafficking or organelle structure in vivo? Formal possibilities include 1) the small proportion of unpoisoned endogenous p97 hexamer remaining after induction of p97(E578Q) can support membrane fusion but not ubiquitin related processes, 2) membrane fusion but not ubiquitin related processes can persist in the presence of mixed hexamers containing endogenous and p97(E578Q) subunits, and 3) membrane fusion can selectively proceed in the absence of ATPase activity. However, we think these explanations are rather unlikely. Most endogenous p97 subunits coassemble with p97(E578Q) subunits. Moreover, even if endogenous holoenzymes remain, they would be unlikely to function normally because of the persistent interaction between mutant p97 and substrates.

To reconcile our results with previous studies, we first review the evidence that implicates p97 in membrane fusion. p97 participates in reassembly of l DMAquinosine dispersed Golgi membranes into Golgi stacks (Acharaya et al., 1995), reformation of unfenestrated Golgi cisternae from mitotic Golgi fragments (Rabouille et al., 1995, 1998), fusion of yeast ER membranes (Latterich et al., 1995; Lin et al., 2001), formation but not maintenance of tubulated ER from low-density microsomes (Lavoie et al., 2000; Roy et al., 2000), and formation and growth of the nuclear envelope and associated ER network (Hetzer et al., 2001). These in vitro assays all reconstitute complex processes related to organelle assembly. The membranes involved must undergo not only fusion but also profound shape changes to generate the structures that are the end points of the assays. These systems have led to discovery of proteins that directly participate in membrane fusion, but it may be difficult to distinguish between such proteins and others that are less directly involved.

More recently, Kondo and coworkers have shown that the p97 adapter proteins p47 and VCIP135 are required in vivo for reassembly of the Golgi apparatus and formation of the ER network upon exit from mitosis (Uchiyama et al., 2002, Uchiyama et al., 2003). Although these studies may point to a specific role for p97 in membrane fusion, both adapter proteins are also directly linked to ubiquitin pathways, p47 and VCIP135 each contain a ubiquitin-like domain, and p47 also contains a ubiquitin binding domain (Meyer et al., 2002; Uchiyama et al., 2002). Perturbations in ubiquitin homeostasis caused by interfering with these proteins during cell division may complicate analysis of p97's function in post-mitotic organelle assembly.

All of the above studies taken together have led to the current model that p97 participates in both membrane fusion and protein degradation via interaction with distinct adaptor proteins (Meyer et al., 2002). The direct inhibition of p97 reported in this study (without changes in the adapter proteins) suggests that p97's central function in the cell is in

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**Figure 9.** p97(E578Q) does not affect Golgi distribution mitosis. p97(E578Q)myc cells at different stages of mitosis stained with anti-giantin (green) and anti-a-tubulin (red). (A) metaphase, (B) anaphase, (C) telophase I, (D–F) telophase II. Images were obtained on a confocal microscope. (A–C) Each box is 40 × 40 μm. (D–F) Each box is 83 × 83 μm.

**Figure 10.** Disruption and reassembly of Golgi ribbon in response to BFA are unaffected by p97(E578Q). p97myc cells (top panel) and p97(E578Q)myc cells (bottom panel) are shown from left to right before BFA addition, after BFA treatment for 40 min, and after recovery from BFA for 120 min, respectively. Giantin is in green (anti-giantin) and p97(E578Q)myc is in red (anti-myc). Images were obtained on a confocal microscope. Each box is 83 × 83 μm.
controlling ubiquitin-dependent processes. Although this places p97 in a position to regulate a large number of cellular events, we do not see evidence for an obvious role in membrane fusion. This is in contrast to NSF, for which our experiments point convincingly to a primary and essential role in membrane fusion. We propose that p97’s normal role in controlling membrane fusion is therefore likely to be different from that of NSF. An interesting possibility is that p97-controlled protein degradation could regulate ER, nuclear envelope, and Golgi assembly.

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