Modulation of N-Ethylmaleimide-sensitive Factor Activity upon Amino Acid Deprivation*

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Adaptation of eukaryotic cells to changing environmental conditions entails rapid regulation of protein targeting and transport to specific organelles. Such adaptation is well exemplified in mammalian cells exposed to nitrogen starvation that are triggered to form and transport autophagosomes to lysosomes, thus constituting an inducible intracellular trafficking pathway. Here we investigated the relationship between the general secretory machinery and the autophagic pathway in Chinese hamster ovary cells grown in the absence of amino acid. Utilizing VSVG-YFP (vesicular stomatitis virus G protein fused to yellow fluorescent protein) and norepinephrine as markers for constitutive and regulated exocytosis, respectively, we found that secretion is attenuated in cells grown in media lacking amino acid. Such decrease in exocytosis stems from partial inhibition of N-ethylmaleimide-sensitive factor ATPase activity, which in turn causes an accumulation of SNARE complexes at both the Golgi apparatus and the plasma membrane of the starved cells. These findings expose a novel cellular strategy to attenuate secretion of proteins under conditions of limited amino acid supply.

Specific recognition between an intracellular vesicle carrying cargo molecules and its appropriate target membrane involves the interaction between v-SNAREs, integral membrane proteins located on the vesicle, and t-SNAREs, located at the target membrane (1). These interactions form a SNARE "core complex," which consists of four entwined α-helix bundles of typically three Q-SNARE helices and one R-SNARE helix, a classification based on conserved glutamine or arginine residues at the center of their SNARE-binding domain. The SNARE core complex is stabilized mainly by hydrophobic interactions between the four helices and by a central ionic layer consisting of one arginine and three glutamine residues contributed by each of the four α-helices (2). Formation of trans-SNAREs from opposing membranes yields a close, stable proximity between the two membranes, which facilitates overcoming the energy barrier required for membrane fusion (3–5). Furthermore, using three sets of functionally identified yeast t-SNAREs to mediate the fusion of ER-derived transport vesicles with the Golgi, the homotypic fusion of vacuoles, and the fusion with the plasma membrane (PM), it was demonstrated that isolated SNARE proteins encode compartmental specificity and mediate the actual fusion event (6–8). Additional proteins are required for targeting and tethering of these transport vesicles with their specific targets (9).

The hexameric ATPase N-ethylmaleimide-sensitive factor (NSF) utilizes ATP hydrolysis to dissociate cis-SNARE complexes after membrane fusion, allowing the individual SNARE proteins to be recycled for subsequent rounds of fusion (10, 11). Whereas specific v- and t-SNAREs are associated with each intercompartmental transport step, NSF is a general cytosolic factor that can disassemble SNARE complexes from most intracellular transport steps. The ATPase activity of NSF is enhanced by α-SNAP (soluble NSF attachment protein), which mediates NSF binding to different SNARE complexes (12), and possibly by other factors such as GATE-16 (13, 14) and rab-6 (15). Notably, evidence for direct negative regulation of NSF activity in vivo has been suggested by Matsushita et al. (16), whereby S-nitrosylation of specific cysteine residues on NSF leads to attenuation of triggered exocytosis of endothelial granules. However, it is not known yet whether NSF S-nitrosylation controls other cellular membrane fusion processes such as constitutive exocytosis or vesicular transport along the secretory pathway.

Direct regulation of vesicular trafficking is crucial not only for the spatially and temporally controlled secretion of bioactive molecules but also for controlling the levels of various molecules (e.g. receptors, transporters) on the PM (17). A variety of regulatory events that operate at different steps of vesicular transport control the trafficking along specific cellular pathways in response to different signals. Developmental, environmental, and cell cycle-related signals have differential effects on different vesicular transport pathways. For example, transport along the secretory pathway in Xenopus oocytes is blocked between the trans-Golgi and the PM during meiotic maturation (18). On the other hand, transport of special vesicles to lysosomes is enhanced upon amino acid deprivation in a process known as autophagy (19).

Autophagy is a bulk protein degradation process in which newly formed double membrane vesicles, termed autophagosomes, deliver cytoplasmic contents and organelles for lysosomal degradation (19). There are at least three types of autophagy: macroautophagy (referred to as autophagy hereafter), microautophagy (20), and chaperon-mediated autophagy (21). Autophagy, a relatively understudied membrane-trafficking mechanism, coordinates autophagy via the hexameric ATPase NSF, which can also disassemble SNARE complexes (22).

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process, may be regarded as a unique vesicular transport pathway that is triggered in response to stress conditions such as nitrogen starvation (19). Recent studies implicated transport factors such as Sec18p (NSF yeast homologue), SNAREs, Rab5, and members of GATE-16 family in fusion steps associated with autophagy (22–26). In the present study we have investigated whether deprivation of amino acid also affects other vesicular transport pathways such as constitutive and regulated secretion. By comparing exocytosis in starved versus non-starved control cells, we conclude that the Golgi to PM transport is significantly attenuated under amino acid starvation conditions. Structural and functional assays suggest that this attenuation is directly caused by inhibition of NSF ATPase activity during starvation. Our data provide, for the first time, a molecular explanation for the attenuation of exocytosis under these physiological stress conditions.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Induction of Amino Acid Starvation**—CHO cells were grown in α-MEM containing 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin, either on plates or in suspension. PC12 cells were grown in DMEM containing 6% FCS, 6% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. To induce autophagy by amino acid deprivation, cells were washed three times with Earle’s balanced salt solution (EBSS) and then incubated in EBSS (supplemented with vitamins and pyruvate to the same concentrations present in the rich media) for the indicated time periods at 37 °C. As a control, washed cells were incubated with α-MEM or DMEM in the absence of FCS for the indicated starvation periods.

**Degradation and Secretion Assays**—Measurement of long-lived protein degradation in CHO cells was based on an assay previously described by Ogier et al. (27). Briefly, CHO cells plated on 6-well plates were prelabeled with [14C]valine (Amersham Biosciences) for 24 h. Cells were then washed three times with phosphate-buffered saline and then preincubated for 1 h in either α-MEM or DMEM, both containing 0.1% bovine serum albumin and 10 mM cold valine. After 1 h of incubation, the culture media were replaced with identical fresh media, and the cells were incubated for an additional 2 or 4 h. The media were collected and centrifuged for 3 min at 2200 rpm, and the supernatant radioactivity was determined by liquid scintillation counting.

**Preparation of Cytochalasin and Membrane Extracts**—Cells were washed twice with phosphate-buffered saline and then twice in a homogenization buffer containing 0.25 M sucrose, 25 mM Tris-HCl, pH 7.4, and 50 mM KCl. The cells were then homogenized in the same buffer containing 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml apro- tinin, and 2 μM pepstatin A using a Balch homogenizer (for cells grown in suspension) or Dounce homogenizer (for cells grown on plates). The homogenates were centrifuged for 5 min at 2500 rpm, and the supernatant was further centrifuged for 30 min at 200,000 × g. The supernatant containing the cytosol was collected. The pellet, containing total membranes, was resuspended in extraction buffer containing 20 mM Hepes, pH 7.0, 20 mM KCl, and 0.5% Triton X-100. To obtain cytosol and membrane extracts in the presence of ATP, CHO cells were washed twice with phosphate-buffered saline, then twice in a homogenization buffer containing 20 mM PIPES, pH 7.2, 10 mM MgCl₂, 5 mM ATP, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM KCl, and homogenized and fractionated as described above.

**SNARE Complex Disassembly Assays**—To compare SNARE complex localization in control versus starved cells, membrane extracts from CHO or PC12 cells were incubated in SDS-sample buffer at either 30 °C or 100 °C for 5 min and then subjected to immunoblotting. To examine the capacity of cytosolic fractions to dissociate SNARE complexes, rat brain membranes containing SNARE complexes were pretreated on ice with 1 mM N-ethylmaleimide (NEM) for 10 min followed by treatment with 2 mM dithiothreitol for 15 min. NEM-treated membranes were incubated with 50 μg of cytosol obtained from control or starved CHO cells in a reaction buffer containing 0.5 μg of α-SNAP, 0.5 mM ATP, 2 mM MgCl₂, and ATP regeneration buffer at 30 °C for 30 min. In the indicated reactions, 1.2 μg of recombinant NSF was added to the disassembly assay. The membranes were then isolated by centrifugation (14,000 rpm, 10 min at 4 °C), resuspended in SDS-sample buffer, and processed for immunoblotting without prior boiling unless mentioned.

**Proteolytic Digestion**—Recombinant NSF proteins and cytosolic fractions obtained from starved or non-starved CHO cells were incubated at 30 °C with trypsin (ratio of 1:20 and 1:200 trypsin/protein (w/w), respectively). Progress of the proteolysis was assessed by removing aliquots at the indicated time points and quenching the digestion with a mixture of soybean trypsin inhibitor and 2× SDS-PAGE sample buffer. The aliquots were subjected to 12% SDS-PAGE and analyzed by Western blot using a mixture of 2E5, 2C8, and 6E6 anti-NSF monoclonal antibodies.

**Immunoprecipitation**—Monoclonal antibodies (anti-NSF) and polyclonal antibodies (anti-Goe-28 or anti-syntxin-5) were covalently coupled by dimethyl pimelimidate (Sigma) to protein G- and protein A-agarose beads (Santa Cruz Biotechnology). The coupled beads were then incubated either with recombinant His-NSF-Myc protein (0.5 μg) or cytosolic (200 μg) and membrane extract fractions (200 μg) obtained from control or starved CHO cells at 30 °C for 30 min prior to the immunoprecipitation.

**ATPase Activity Assay**—Prior to the ATPase activity assay, NSF (6 μg) was preincubated with cytosolic fractions (40 μg) obtained from control or starved CHO cells at 30 °C for 20 min in a total volume of 100 μl of 25 mM Tris-HCl, pH 7.4, 50 mM KCl, 2 mM MgCl₂, and 0.5 mM ATP. The assay was initiated by adding residual amounts of [γ-32P]ATP (0.25 Ci/sample), carried out for 30 min at 30 °C, and stopped by adding 100 μl of ice-cold perchloric acid to a final concentration of 10%. Nucleotides were bound to 500 μl of ice-cold charcoal, and samples were centrifuged at 14,000 rpm for 15 min at 4 °C. The resulting supernatants containing released 32P were quantified by liquid scintillation counting. Background activities of control and starved cytosol alone were subtracted (0.08 and 0.079 μCi/sample), and the radioactivity remaining in the cells at the end of the experiment was recorded.

**RESULTS**

**Secretion Is Inhibited in Response to Amino Acid Deprivation**—Autophagy triggered by starvation may be regarded as a
Valine release to the medium) and total cells radioactivity. The radioactivity found in the trichloroacetic acid-soluble fraction (free degradation of prelabeled proteins was measured as the ratio between the radioactivity found in the trichloroacetic acid-insoluble fraction and total cells radioactivity. Data are the means ± S.D. of triplicates from a representative experiment repeated three times.

To identify the step along the secretory pathway that is most affected by amino acid starvation, we utilized [14C]valine-prelabeled tissue culture cells to examine the effects of amino acid starvation on intracellular protein degradation and simultaneously on protein secretion. In this system the autophagic activity was determined by monitoring the bulk protein degradation of long-lived proteins (27), whereas exocytosis was determined by monitoring the release of secreted proteins from the cells. For this purpose, prelabeled CHO cells were incubated for different time periods in either α-MEM (without FCS) or EBSS (starvation medium), and the levels of secreted labeled proteins and amino acid into the media were determined. As depicted in Fig. 1A, bulk degradation of long-lived proteins, measured by the release of trichloroacetic acid-soluble [14C]valine from the cells, was about 50% higher in cells incubated in medium lacking amino acid compared with control medium, indicating significant enhancement of autophagy in starved cells. Concomitantly, the radioactivity measured in the trichloroacetic acid-insoluble pellet was significantly reduced (~30%) in cells deprived of amino acid (Fig. 1B). Similar results were obtained when CHO cells were prelabeled with [35S]methionine, as well as in different cell lines such as COS-7 and PC12 cells that were treated similarly (data not shown). Taken together, these results indicate that during amino acid starvation protein degradation is stimulated, whereas the constitutive secretion of newly synthesized proteins is largely inhibited.

Vesicular Transport to the Plasma Membrane Is Inhibited during Starvation—To identify the step along the secretory pathway that is most affected by amino acid starvation, we utilized the well studied vesicular stomatitis virus ts045 G protein fused to yellow fluorescent protein (VSVG-YFP) to monitor trafficking through the various compartments of the secretory pathway in living cells. We took advantage of the fact that at 40 °C the ts045 VSVG mutant protein is retained within the ER, whereas upon a shift to 32 °C it moves as a synchronous population to the Golgi complex before being transported to the PM (28). Hence, CHO cells were transfected with VSVG-YFP, incubated in normal or starvation medium for 3 h at 40 °C, and then shifted to 32 °C to trigger transport. The transport of the VSVG-YFP ts045 protein was monitored by confocal microscopy. At each of several selected time points, the percentage of cells in which the VSVG-YFP protein was localized to the ER, Golgi, or the PM was calculated. As shown in Fig. 2, the VSVG protein accumulated in the Golgi apparatus in about 60% of the control cells within a 5-min incubation period at 32 °C and in 100% of these cells within 20 min. During this period, no significant difference in the rate of transport of VSVG protein from the ER to the Golgi was detected in the PM of more than 60% of the control cells, whereas only 25% of the starved cells exhibited PM labeling. From this analysis we conclude that although ER-to-Golgi transport is only marginally affected by amino acid starvation, the transport from the Golgi apparatus to the PM is significantly attenuated in starved cells.

The effect of amino acid deprivation on exocytosis was studied further in PC12 cells, a rat adrenal phaeochromocytoma cell line, which expresses several well defined neuronal properties in culture, including the regulated secretion of neurotransmitters. To examine the effects of amino acid starvation on exocytosis in this cell line, release of [3H]norepinephrine from prelabeled cells was measured in control versus starved cells. Hence, prelabeled cells were incubated for 3.5 h in either DMEM or EBSS supplemented with glucose (starvation media) and then subjected to time course secretion analysis. As depicted in Fig. 3, the basal rates of NE release were low and similar for both control and starved cells. However, when NE secretion was stimulated by directly depolarizing the PC12...
the \([3H]\)NE content was secreted within the first 2 min and to inducing secretion at time 0 with 55 mM K
PC12 cells prelabeled with \([3H]\)norepinephrine (NE) were incubated in low K⁺ medium for 10 min prior to inducing secretion at time 0 with 55 mM K⁺. Media were collected and replaced every 2 min. Data are the means ± S.D. of triplicates from a representative experiment repeated three times.

FIG. 3. Regulated norepinephrine secretion is inhibited in PC12-starved cells. A schematic representation of the time course of the experiment is depicted. PC12 cells prelabeled with \([3H]\)norepinephrine (NE) were preincubated for 3.5 h in DMEM (control medium, gray columns) or EBSS supplemented with glucose (starvation medium, black columns), and then incubated in low K⁺ medium for 10 min prior to inducing secretion at time 0 with 55 mM K⁺. Media were collected and replaced every 2 min. Media counts (cpm) were normalized to total cell counts. Data are the means ± S.D. of triplicates from a representative experiment repeated three times.

cells with elevated extracellular K⁺ ions (55 mM KCl), 14% of the \([3H]\)NE content was secreted within the first 2 min and cumulatively up to 31% within 12 min. Under these conditions, however, secretion of NE from the starved cells was inhibited by about 32%, indicating that triggered exocytosis too is sensitive to amino acid starvation.

Disassembly of SNARE Complexes Is Inhibited during Amino Acid Starvation—A normal vesicular transport cycle requires rapid and regulated disassembly of SNARE complexes, mediated by the ATPase activity of NSF and its co-factor α-SNAP (reviewed in Ref. 29). Exocytosis in PC12 cells, for example, requires the formation and subsequent dissociation of a specific SNARE complex, including the vesicle-associated membrane protein (VAMP) and the PM proteins syntaxin and SNAP-25. To examine whether lack of amino acid affected the dissociation of these complexes, we relied on the fact that SNARE complexes are SDS-resistant at 37 °C and thus can be detected as high molecular weight complexes by immunoblotting (30). As shown in Fig. 4A, the level of SNARE complexes found in the starved cells was much higher than in the control cells, indicating that disassembly of SNARE complexes involved in exocytosis is inhibited under amino acid starvation conditions.

To further examine the effect of amino acid deprivation on the oligomeric state of SNARE molecules, we isolated membrane extracts from control and starved CHO cells and analyzed the oligomeric states of GOS-28, a v-SNARE participating in intra-Golgi transport, and its cognate t-SNARE, syntaxin-5. Consistent with the results obtained with the PC12 cells (Fig. 4A), we observed significantly more GOS-28/syntaxin-5 SDS-resistant complexes in the starved cells (Fig. 4B), indicating that the disassembly of SNARE complexes containing GOS-28 and syntaxin-5 was impaired under these conditions. The effect of starvation on the interaction between GOS-28 and syntaxin-5 was further analyzed by co-immunoprecipitation experiments. For this purpose, agarose-protein A beads coupled to anti-syntaxin-5 or anti-GOS-28 antibodies, as indicated, were mixed with membrane extracts obtained from control or starved CHO cells, and the eluted material was subjected to Western blot analysis. The results presented in Fig. 4C demonstrate that the interaction between GOS-28 and syntaxin-5 in a complex containing NSF is increased during starvation.

NSF Activity Is Inhibited in Starved Cells—The accumulation of different SNARE complexes in the starved cells may best be explained by a reduction in NSF activity. To test this hypothesis, we determined the ability of cytosolic fractions obtained from control or starved cells to dissociate endogenous SNARE complexes. Rat brain membranes were first treated with NEM to abolish intrinsic NSF activity. Next, the treated membranes were incubated with cytosol obtained from starved or control CHO cells, and the level of SNARE complexes was analyzed by Western blotting. As shown in Fig. 5, incubation of NEM-treated membranes with cytosol obtained from control cells resulted in a significant dissociation of high molecular weight SNARE complexes detected in this system. In contrast, no substantial dissociation of SNARE complexes was observed upon incubation with cytosol obtained from starved cells, suggesting that NSF activity was inhibited in this fraction. Moreover, the addition of recombinant NSF to the starved cytosol recovered most of the SNARE complex disassembly activity of this fraction, indicating that the inhibition of NSF in starved cells led to the accumulation of SNARE complexes in these cells. Notably, the recovery of the disassembly activity was not complete as compared with the disassembly activity found in the control cytosol, suggesting partial inactivation of the recombinant NSF proteins by the starved cytosolic fraction (see below).

NSF Undergoes a Conformational Change in Response to Starvation—NSF is a homohexamer in which each of the subunits consists of three domains: an N-terminal domain, NSF-N, which is responsible for the interaction with the α-SNAP-SNARE complex, and two homologous ATP-binding
domains, NSF-D1 and NSF-D2 (31, 32). ATP binding to the D1 domain is crucial for NSF ATPase activity, leading to dissociation of the SNARE complex. In response to ATP binding to its D1 domain, NSF undergoes a major conformational change (33). Thus, in the presence of ATP, recombinant NSF is relatively resistant to limited proteolysis by trypsin, whereas in a buffer lacking ATP it is rapidly degraded (Fig. 6A and Ref. 33).

To determine whether the inability of NSF to disassemble SNARE complexes was correlated with structural changes induced by starvation conditions, cytosols obtained from control and from starved CHO cells were treated with a mild trypsin concentration for different time periods, and the proteolytic profile of NSF was analyzed by Western blot using anti-NSF antibodies. It turned out that relative to NSF from starved cells, NSF obtained from cytosol of non-starved control cells was more sensitive to the mild trypsin treatment (Fig. 6B). In addition, the proteolytic products of ~35 kDa appeared much more resistant in cytosolic NSF from starved cells. Accordingly, the proteolytic profile of NSF obtained from starved cells was similar to that obtained from NSF in ATP buffer, whereas the profile of NSF obtained from the control cells was similar to that of NSF in a solution of low ATP concentration. We therefore propose that amino acid starvation induces changes in cytosolic NSF, stabilizing the ATP-bound form of the protein. Notably, in different cytosols NSF is probably found in equilibrium between its two states, thus obliterating the differences in the trypsinolysis patterns of the crude cytosols (Fig. 6B) relative to the pure recombinant protein (Fig. 6A).

We also tested whether anti-NSF monoclonal antibodies could distinguish between the different conformations of NSF. Hence, we examined the ability of monoclonal anti-NSF antibodies to immunoprecipitate recombinant NSF in a nucleotide dependent manner. As shown in Fig. 7A, recombinant NSF immunoprecipitated more efficiently when incubated with ATP. When cytosolic fractions obtained from starved and control CHO cells were tested in this system, significantly more NSF was precipitated in the cytosol obtained from the starved cells, although no difference was detected in the total level of NSF found in different cytosolic fractions (Fig. 7B, left panel). Similar results were obtained with cytosolic fractions from control versus starved PC12 cells (data not shown). To further determine whether NSF conformational changes triggered by amino acid starvation were related to the nucleotide-bound state of NSF, cytosolic fractions obtained from starved and control cells were prepared in the presence of 5 mM ATP and then subjected to immunoprecipitation by anti-NSF antibodies. In the presence of high ATP concentration, no difference in the amount of immunoprecipitated NSF from the different cytosolic fractions was observed (Fig. 7B, right panel). Notably, the amounts of NSF precipitated in ATP-rich cytosol from control and starved cells were similar to the amount precipitated from starved cells in the absence of ATP, suggesting that under starvation conditions NSF is mostly stabilized in a nucleotide-bound state (Fig. 7B).

Next we utilized the immunoprecipitation assay to study the conformational changes of NSF in a cell-free system. To that end, recombinant NSF fused at its C terminus to the Myc epitope tag was incubated with cytosolic fractions obtained from control or starved CHO cells for 30 min at 30 °C followed by immunoprecipitation with anti-NSF antibodies. As shown in Fig. 7C, significantly more recombinant NSF immunoprecipitated after treatment with the cytosol ob-
modulation of NSF activity upon amino acid deprivation

The basic mechanism of intracellular membrane trafficking is not only evolutionary conserved between yeast and man but is also remarkably similar in different cellular membrane transport pathways. Autophagy is believed to represent a special membrane-trafficking route for the delivery of cytosolic proteins and organelles for degradation in lysosomes (34). Gathering data indicate that many of the factors involved in vesicular transport also play an important role in formation and subsequent transport of autophagosomes to their target membranes (22–26). However, the relationships between vesicular transport and autophagy are yet unknown. In the present study we show that amino acid starvation induces autophagy while attenuating exocytosis. This attenuation is accompanied by the accumulation of SNARE complexes at both the Golgi apparatus and the PM, caused by inhibition of NSF ATPase activity. Together, these findings reveal a novel strategy utilized by cells to attenuate secretion of proteins under conditions in which amino acid supply is limited. Considering the changes in the equilibrium between biosynthesis and catabolism of proteins during nitrogen supply is limited, the controlled inhibition of NSF ATPase is to allow the dissociation of such cis-SNARE complexes while attenuating exocytosis. This attenuation is accompanied by the accumulation of SNARE complexes at both the Golgi apparatus and the PM, caused by inhibition of NSF ATPase activity. Together, these findings reveal a novel strategy utilized by cells to attenuate secretion of proteins under conditions in which amino acid supply is limited. Considering the changes in the equilibrium between biosynthesis and catabolism of proteins during nitrogen starvation and therefore that the protein is found in an ATP-bound state.

DISCUSSION

The basic mechanism of intracellular membrane trafficking is not only evolutionary conserved between yeast and man but is also remarkably similar in different cellular membrane transport pathways. Autophagy is believed to represent a special membrane-trafficking route for the delivery of cytosolic proteins and organelles for degradation in lysosomes (34). Gathering data indicate that many of the factors involved in vesicular transport also play an important role in formation and subsequent transport of autophagosomes to their target membranes (22–26). However, the relationships between vesicular transport and autophagy are yet unknown. In the present study we show that amino acid starvation induces autophagy while attenuating exocytosis. This attenuation is accompanied by the accumulation of SNARE complexes at both the Golgi apparatus and the PM, caused by inhibition of NSF ATPase activity. Together, these findings reveal a novel strategy utilized by cells to attenuate secretion of proteins under conditions in which amino acid supply is limited. Considering the changes in the equilibrium between biosynthesis and catabolism of proteins during nitrogen starvation and therefore that the protein is found in an ATP-bound state.
indicated that Sec-18 activity is not required for autophagosome formation, although it is essential for their subsequent fusion with the vacuole (24). Moreover, the v-SNARE Vti1p, localized on autophagosomes outer membrane, and the t-SNAREs Vam3p and Vam7p, localized on the vacuole membrane, were suggested to be involved in this fusion process in yeast (23, 37, 38). The only mammalian factor known to reside on autophagosomes membrane is LC-3, which is likely to take part in one or more of these fusion steps (26). LC-3 shares high similarity with GATE-16, a low molecular weight factor involved in intra-Golgi transport (14). GATE-16 function is coupled with NSF ATPase-independent activity, shown to be involved in homotypic fusion between fragmented Golgi membranes (13). Taking these findings together, it is tempting to speculate that similarly to its function in reassembly of mitotic Golgi membranes, the ATPase activity of NSF is not required for autophagosomes/lysosomes fusion processes. Alternatively, under conditions of reduced NSF activity, these processes may involve other AAA ATPase fusion factors. For example, P97, a close homologue of NSF that mediates several homotypic membrane fusion events (39, 40), may mediate homotypic fusion of autophagosomes. Another AAA ATPase protein that may be involved in this process is SKD1, an essential factor for endosomal trafficking (41). Thus, overexpression of a dominant negative SKD1 mutant defective in its ATP hydrolysis results in accumulation of autophagosomes that cannot fuse with the lysosomes (42).

NSF forms homohexamers in which each subunit is composed of three domains (12): an N-terminal domain that interacts with the SNAP family, found associated with the SNARE complexes; a D1 domain that hydrolyzes ATP and provides the mechanical force for SNARE complex disassembly; and a D2 domain that tightly binds ATP and is responsible for the hexamization of NSF subunits. Nucleotide binding to the D1 domain induces dramatic conformational changes in the N-terminal domain. These changes are assumed to be the driving force for the capability of NSF to stimulate the dissociation of cis-SNARE complexes. In the present study, we show that amino acid starvation stabilizes the ATP-bound conformation of NSF by inhibiting its ATPase activity. We propose that this effect may block the ability of NSF to change further its conformation upon ATP hydrolysis and therefore may hinder its ability to dissociate SNARE complexes. The exact mechanism by which starvation induces such an effect on NSF is yet unclear. The conformational change in NSF probably does not simply stem from changes in the levels of available ATP, as no significant differences between ATP levels were found in cytosols obtained from control versus starved CHO cells. Interestingly, Matsushita et al. (16) have recently reported for aortic endothelial cells that S-nitrosylation of NSF induced by nitric oxide inhibits the exocytosis of Weibel-Palade bodies. The effects reported here for amino acid starvation, including inhibition of exocytosis, accumulation of SNARE complexes, and inhibition of NSF, resemble those reported for nitric oxide. It is therefore conceivable that similar molecular mechanisms may regulate both processes. Indeed, it has been reported that nitrogen starvation is accompanied by changes in the redox state (43), which in turn may affect essential cysteine residues on NSF. Further study is required to determine whether nitric oxide or other reactive oxygen species molecules are involved in the regulation of NSF activity during starvation.

The fact that NSF, being a general fusion factor, is a target for regulating exocytosis under starvation conditions is rather surprising. It should be noted, however, that amino acid starvation induces only partial inhibition of the secretory pathway, predominantly affecting transport to the PM. Moreover, it is possible that the affected steps are those that have a particularly high demand for NSF, and partial inhibition of NSF activity may therefore result in a selective effect. In accord with this suggestion, reduced levels of NSF expression have been detected in schizophrenic patients (44). Alternatively, NSF inactivation under amino acid starvation may occur locally, at the vicinity of the affected SNARE complexes. Such a scenario may require the involvement of lipids and/or other components with a confined partition to specific microdomains within the membrane. In summary, our results describe for the first time a mechanism by which environmental conditions directly control the activity of NSF, a key factor required for most intracellular trafficking processes.

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REFERENCES

1. Bonifacino, J. S., and Glick, B. S. (2004) Cell 116, 153–166
2. Sutton, R. B., Fasshauer, D., Jahn, R., and Brunger, A. T. (1998) Nature 395, 347–353
3. Weber, T., Zemelman, B. V., McNee, J. A., Westermann, B., Gnaschi, M., and Rothman, J. E. (1998) Cell 92, 759–772
4. Parlati, F., Weber, T., McNee, J. A., Westermann, B., Sollner, T. H., and Rothman, J. E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12565–12570
5. Nickel, W., Weber, T., McNee, J. A., Parlati, F., Sollner, T. H., and Rothman, J. E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12571–12576
6. McNee, J. A., Parlati, F., Fukuda, R., Johnston, R. J., Paz, K., Paumet, F., Sollner, T. H., and Rothman, J. E. (2000) Nature 407, 153–159
7. Parlati, F., McNee, J. A., Fukuda, R., Miller, E., Sollner, T. H., and Rothman, J. E. (2000) Nature 407, 194–198
8. Parlati, F., Varlamov, O., Paz, K., McNee, J. A., Hurtado, D., Sollner, T. H., and Rothman, J. E. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5424–5429
9. Waters, M. G., and Hughson, F. M. (2000) Traffic 1, 588–597
10. Mayer, A., Wickner, W., and Haas, A. (1996) Cell 85, 83–94
11. Sollner, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H., and Rothman, J. E. (1993) Cell 75, 409–418
12. Whiteheart, S. W., Schraw, T., and Matveeva, E. A. (2001) Int. Rev. Cytol. 207, 71–112
13. Muller, J. M., Shortier, J., Newman, R., Deinhardt, K., Sagiv, Y., Elazar, Z., Warren, G., and Shimizu, D. (2002) J. Cell Biol. 157, 1161–1173
14. Sagiv, Y., Legesse-Miller, A., Porst, A., and Elazar, Z. (2000) EMBO J. 19, 1494–1504
15. Han, S. Y., Park, D. Y., Park, S. D., and Hong, S. H. (2000) Biochem. J. 352, 165–173
16. Matsushita, K., Morrell, C. N., Cambien, B., Yang, S. X., Yamakuchi, M., Bao, C., Haro, M. R., Quick, R. A., Cao, W., O’Rourke, B., Lowenstein, J. M., Pevisner, J., Wagner, D. D., and Shima, D. T. (2002) J. Cell Biol. 157, 139–150
17. Gruisven, J., and Macaulay, S. L. (2003) Front. Biosci. 8, 620–641
18. Leaf, D. S., Roberts, S. J., Gerhart, J. C., and Moore, H. P. (1990) Dev. Biol. 141, 1–12
19. Mizushima, N., Ohsumi, Y., and Yoshimori, T. (2002) Cell Struct. Funct. 27, 421–429
20. Kunz, J. B., Schwarz, H., and Mayer, A. (2004) J. Biol. Chem. 279, 9987–9996
21. Cuervo, A. M., Gomes, A. V., Barnes, J. A., and Dice, J. F. (2000) J. Biol. Chem. 275, 33329–33335
22. Abeliovich, H., Dunn, W. A., Jr., Kim, J., and Kiontsev, D. J. (2000) J. Cell Biol. 151, 1025–1034
23. Darsow, T., Rieder, S. E., and Emr, S. D. (1997) J. Cell Biol. 138, 517–529
24. Ishihara, N., Hamasaki, M., Yokota, S., Suzuki, K., Kamada, Y., Kihara, A., Pevisner, J., Wagner, D. D., and Lowenstein, J. C. (2003) Cell 115, 139–150
25. Kominami, E., Ohsumi, Y., and Yoshimori, T. (2000) J. Cell Biol. 149, 529–535
26. Ogier-Denis, E., Houri, J. J., Bauray, C., and Codogno, P. (1996) J. Biol. Chem. 271, 28593–28600
27. Presley, J. F., Smith, C., Hirschberg, K., Miller, C., Cole, N. B., Zaal, K. J. M., and Lippincott-Schwartz, J. (1999) Mol. Biol. Cell 10, 1617–1626
28. May, A. P., Whiteheart, S. W., and Weis, W. I. (2001) J. Biol. Chem. 276, 21991–21994
29. Harash, T., Yamakawa, S., Nauenburg, S., Bins, T., and Niemann, H. (1995) EMBO J. 14, 2317–2325
30. Nagiec, G., Fernández, A., and Whiteheart, S. W. (1995) J. Biol. Chem. 270, 29182–29189
31. Whiteheart, S. W., Rossnagel, K., Buhrow, S. A., Brunner, M., Jaenicke, R., and Rothman, J. E. (1994) J. Cell Biol. 126, 945–954
32. Hanson, P. I., Roth, R., Morisaki, H., Jahn, R., and Heuser, J. E. (1997) Cell 90, 523–535

2 H. Shorer and Z. Elazar, unpublished observation.
34. Reggiori, F., and Klionsky, D. J. (2002) *Eukaryot. Cell* 1, 11–21
35. Sollner, T. H. (2003) *Mol. Membr. Biol.* 20, 209–220
36. Liou, W., Geuze, H. J., Geelen, M. J., and Slot, J. W. (1997) *J. Cell Biol.* 136, 61–70
37. Sato, T. K., Darsow, T., and Emr, S. D. (1998) *Mol. Cell. Biol.* 18, 5308–5319
38. Isahara, K., Ohsawa, Y., Kanamori, S., Shibata, M., Waguri, S., Sato, N., Gotow, T., Watanabe, T., Momei, T., Urase, K., Kominami, E., and Uchiyama, Y. (1999) *Neuroscience* 91, 233–249
39. Latterich, M., Frohlich, K. U., and Schekman, R. (1995) *Cell* 82, 885–893
40. Rabouille, C., Levine, T. P., Peters, J. M., and Warren, G. (1995) *Cell* 82, 905–914
41. Yoshimori, T., Yamagata, F., Yamamoto, A., Mizushima, N., Kabeya, Y., Nara, A., Miwako, I., Ohashi, M., Ohsumi, M., and Ohsumi, Y. (2000) *Mol. Biol. Cell* 11, 747–763
42. Nara, A., Mizushima, N., Yamamoto, A., Kabeya, Y., Ohsumi, Y., and Yoshimori, T. (2002) *Cell Struct. Funct.* 27, 29–37
43. Ollinger, K., and Roberg, K. (1997) *J. Biol. Chem.* 272, 23707–23711
44. Mirnics, K., Middleton, F. A., Marquez, A., Lewis, D. A., and Levitt, P. (2000) *Neuron* 28, 53–67
45. Nichols, B. J., Kenworthy, A. K., Polishchuk, R. S., Lodge, R., Roberts, T. H., Hirschberg, K., Phair, R. D., and Lippincott-Schwartz, J. (2001) *J. Cell Biol.* 153, 529–541

Modulation of NSF Activity upon Amino Acid Deprivation