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Inhibition of the membrane fusion machinery prevents exit from the TGN and proteolytic processing by furin

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Abstract The Semliki Forest virus (SFV) glycoprotein precursor p62 is processed to the E2 and E3 during the transport from the *trans*-Golgi network (TGN) to the cell surface. We have studied the regulation of the membrane fusion machinery (Rab/*N*-ethylmaleimide (NEM)-sensitive fusion protein (NSF)/soluble NSF attachment protein (SNAP)–SNAP receptor) in this processing. Activation of the disassembly of this complex with recombinant NSF stimulated the cleavage of p62 in permeabilized cells. Inactivation of NSF with a mutant α-SNAP(L294A) or NEM treatment inhibited processing of p62. Rab GDP dissociation inhibitor inhibited the cleavage. Inactivation of NSF blocks the transport of SFV glycoproteins and vesicular stomatitis virus G-glycoprotein from the TGN membranes to the cell surface. The results support the conclusion that inhibition of membrane fusion arrests p62 in the TGN and prevents its processing by furin. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Semliki Forest virus; Furin; Transport; Membrane fusion; Membrane trafficking

1. Introduction

Multiple membrane fusion events depend on the *N*-ethylmaleimide (NEM)-sensitive factor (NSF), soluble NSF attachment proteins (SNAPs), SNAP receptors (SNAREs), and Rab GTPases. NSF is a homohexameric ATPase [1,2] which is stimulated by α-, β- and γ-SNAP [3]. Before NSF can interact with SNAP isoforms, SNAPs have to be bound to the membrane by SNAREs. NSF, SNAPs, and SNAREs form a stable 20S complex which can be disassembled by the ATPase activity of NSF [4,5]. The formation and disassembly of the 20S complex facilitate the fusion of the target and vesicle membranes. Rabs, small GTPases, regulate the tethering/docking reactions [6]. In this study, we have used bacterial toxin streptolysin O (SLO)-permeabilized cells and in vitro reconstitution assays to study whether the Rab–NSF–SNAP–SNARE complex is involved in regulation of p62 processing when it is released from the *trans*-Golgi network (TGN). Semliki Forest virus (SFV) is an α-virus widely used in membrane traffic studies. The SFV spike protein complex consists of two integral membrane glycoproteins, E1 and E2, and a peripheral protein E3. The E2/E3 proteins are first synthesized as a 62 kDa precursor in the endoplasmic reticulum (ER) where it forms a complex with E1 [7]. During transport to the plasma membrane, where the budding of the virus occurs [8], p62 is cleaved at the C-terminal side of the sequence -Arg-His-Arg-Arg- [9] most likely by furin which recognizes the consensus sequence Arg-Xaa-(Lys/Arg)-Arg. This cleavage was first believed to occur on the outer surface of the plasma membrane because addition of spike protein specific antisera to the culture medium of virus-infected cells inhibited the proteolytic processing [10,11]. When p62 is accumulated in the TGN by the aid of a 20°C transport block, it remains uncleaved [12,13]. Studies in permeabilized cells [12] and in live cells [14] have shown that the processing of p62 takes place during the transport from the TGN to the plasma membrane. Endocytosis of antibodies against the SFV envelope glycoproteins as well as antibodies against the enzymatically active motif of furin inhibited the cleavage, suggesting that furin and p62 meet each other at an intracellular site distal to the TGN [14]. As suggested by kinetic experiments this interaction between the exocytic and endocytic routes occurs at the level of early endosomes. To obtain further information on the intracellular processing site, we investigated whether the membrane fusion machinery is involved in p62 processing.

The results indicate that inhibition of the membrane fusion machinery by different means prevents the exit from the TGN and the cleavage of the SFV p62. Inactivation of NSF in live cells by NEM treatment or by microinjection of the α-(SNAP) mutant (L294A) prevents the transport of p62 from the TGN region to the cell surface.

2. Materials and methods

2.1. Materials

Plasmids encoding the His_6-α-SNAP(L294A) mutant and His_6-NSF were gifts from Dr. R. Burgoyne (University of Liverpool, Liverpool, UK). The His_6-GDP dissociation inhibitor (GDI) plasmid was a gift from Dr. M. Zerial (European Molecular Biology Laboratory, Heidelberg, Germany). Plasmids were expressed in *Escherichia coli* and recombinant proteins purified on a Ni-NTA-agarose batch. His_6-α-SNAP and His_6-GDI were purified with modifications as previously described [15] and His_6-NSF according to Whiteheart et al. [16]. Plasmid encoding early endosomal autoantigen 1 (EEA1; 1257–1411)-GST was a gift from Dr. H. Stenmark (Radium Hospital, Montebello, Norway) and the recombinant protein was purified according to the purification protocol from the manufacturer (Amersham Pharmacia

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Abbreviations: EEA1, early endosomal autoantigen 1; GDI, GDP dissociation inhibitor; NEM, *N*-ethylmaleimide; NSF, *N*-ethylmaleimide-sensitive fusion protein; SFV, Semliki Forest virus; SLO, streptolysin O; SNAP, soluble NSF attachment protein; TM, transport medium; VSV, vesicular stomatitis virus

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Biotech, Uppsala, Sweden). SLO was purchased from Dr. S. Bhakdi (University of Mainz, Mainz, Germany). Polyclonal antibodies against SFV glycoprotein E2 [14] were a kind gift from Dr. S. Keränen (VTT Biotechnology and Food Research, Espoo, Finland). Vesicular stomatitis virus (VSV) tsO45 mutant and polyclonal antiserum were gifts from Dr. K. Metsikkö (University of Oulu, Oulu, Finland). Mouse monoclonal anti-rat TGN38 was a gift from Dr. G. Banting (University of Bristol, Bristol, UK). Mouse monoclonal anti-human IgG was from Medix Biochemica (Kauniainen, Finland). Lissamine rhodamine (LRSC)-conjugated and fluorescein (FITC)-conjugated antibodies were purchased from Jackson Immuno Research (West Grove, PA, USA). All other reagents were of analytical grade and were obtained from commercial sources.

2.2. Viral infection and pulse-chase experiments

BHK21 cells were infected with the wild type SFV as described previously [17]. Pulse labeling with [^{35}S]methionine and immunoprecipitation with SFV-E2 specific antiserum were performed according to Sariola et al. [14]. The gels were dried and exposed to Fujifilm Imaging Plate and analyzed with Bio-Imaging Analyzed Bas-1500 (Fuji Photo Film Co., Ltd., Japan). Images were quantitated using Tina version 2.09c software (Isotopenmessgeräte GmbH, Germany). The percentage of the cleavage of p62 at 37°C was calculated as the percentage of E2 formed from p62. Some of the p62 leaks out from the TGN and is cleaved to E2 during the 20°C block. Therefore E2 produced at 20°C has to be deducted from E2 formed at 37°C to be able to estimate the real cleavage during 37°C incubation.

2.3. SLO permeabilization

The cells were permeabilized with modifications as previously described [18,19]. Transport medium (TM) contained 115 mM K-acetate, 2.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 5 mM EGTA, 2.5 mM $CaCl_2$, 25 mM HEPES-KOH pH 7.4. The cytosol extract was prepared from rat brains in cytosol buffer (155 mM K-acetate, 3.5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1 mM phenylmethylsulphonyl fluoride, 25 mM HEPES-KOH pH 7.4) according to Miller and Moore [20]. The ATP regenerating system was 1 mM ATP, 8 mM phosphocreatine, 50 μg/ml creatine phosphokinase.

2.4. Microinjection and immunocytochemistry

BHK21 and NRK cells were grown as confluent monolayers on coverslips and infected with the SFV ts-1 mutant as previously reported [17]. The rabbit polyclonal antiserum against the 29S complex of SFV spike glycoproteins was prepared as described [21]. VSV tsO45 mutant infection was performed as previously described [22]. The polyclonal antiserum against VSV G-protein has been described by Metsikkö et al. [23]. 300–400 cells were microinjected on each coverslip (three coverslips/experiment) with test substances. α-SNAP mutant concentration was 2–3 mg/ml. Microinjections were performed using an Eppendorf semi-automated micromanipulator injector (Transjector 5246) with femtotip needles (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). Conventional fluorescence images were viewed using an Olympus AX70 fluorescence microscope (×60; 1.4 NA) with a SenSys CCD camera (Photometrics, Ltd., Munich, Germany). Images were converted using the Image-Pro Plus version 3.0 software (Media Cybernetics, Silver Spring, MD, USA). Confocal laser scanning microscopy was performed on a Bio-Rad MRC-1024 system (Bio-Rad Laboratories, Hercules, CA, USA) attached to an Axiovert 135 M compound microscope (Carl Zeiss, Inc., Thornwood, NY, USA) (×100; 1.4 NA). Confocal images were converted using LaserSharp MRC-1024 software (Bio-Rad Laboratories, Hercules, CA, USA).

3. Results

3.1. NEM inhibits the processing of p62

We have studied involvement of the membrane fusion machinery in the cleavage of the SFV spike glycoproteins precursor p62 to E2 and E3 in BHK cells. When the cells are infected with SFV, and labeled viral glycoproteins are accumulated at 20°C in the TGN, there is no cleavage of p62 (Fig. 1A). The cleavage of p62 occurs when the 20°C transport block is released by increasing the temperature to 37°C (Fig.

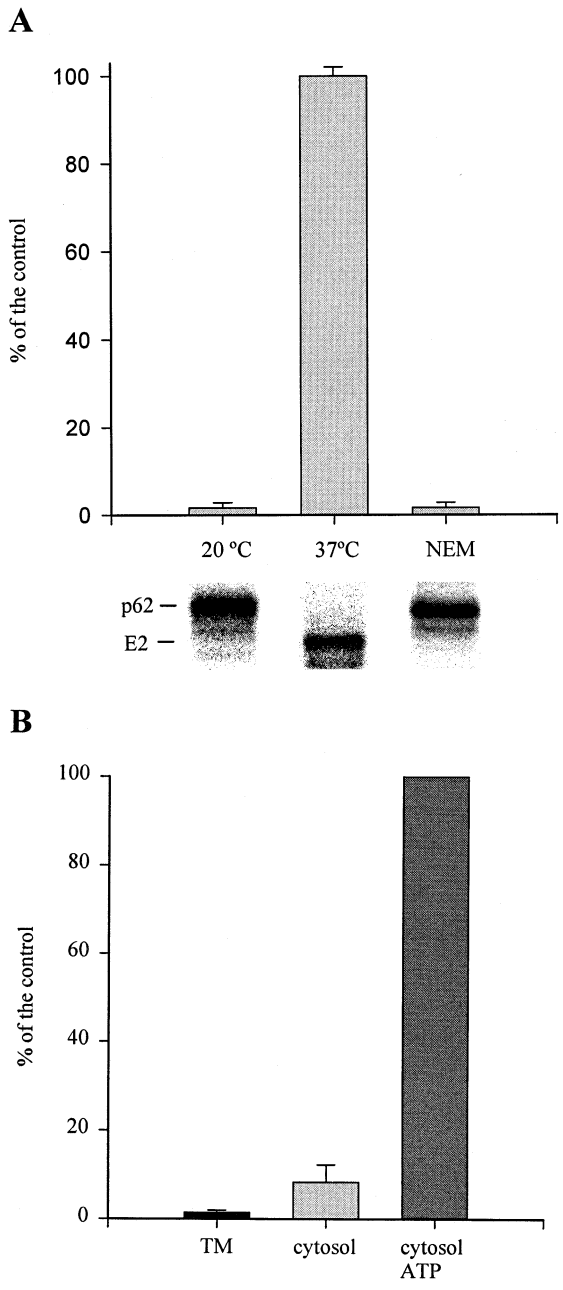

Fig. 1. The cleavage of SFV p62 in BHK21 cells. A: The cells were infected with SFV and pulse-labeled for 10 min with [^{35}S]methionine. The labeled virus glycoproteins were accumulated in the TGN by incubating the cells at 20°C for 2 h. No cleavage of p62 occurs. When the transport block is released by increasing the temperature to 37°C for 2 h, p62 is cleaved to E2 and E3. The analysis of the cleavage was carried out by immunoprecipitation of p62 and E2 and separation by 10% SDS–PAGE. The upper panel shows quantitation by phosphoimager of the p62 and E2 bands seen in the lower panel. The cleavage is inhibited in the presence of 1 mM NEM. NEM treatment was performed by incubating the cells first for 90 min at 20°C then NEM was added and the cells were further incubated for 30 min at 20°C and then for 2 h at 37°C. The cleavage of p62 in the control was 88%. B: The labeled virus glycoproteins were accumulated in the TGN and the cells were permeabilized with SLO followed by incubation at 37°C for 2 h in the presence of TM, cytosol, or cytosol with ATP regeneration system. The amount of cleavage of p62 in the complete medium was expressed as 100%. The cleavage of p62 in control was 28%. Values are means ± S.E.M., $n = 4$ observations. Data are representative of two similar experiments.

1A) [12,14]. The possible role of the membrane fusion, occurring before the processing of the TGN-accumulated p62, was studied by using a sulphydryl alkylating reagent NEM. NEM has been reported to inactivate the membrane fusion component NSF [24] as well as membrane transport in several systems such as between the ER and the Golgi stacks [25] and intra-Golgi transport [26]. In intact BHK cells 1 mM NEM totally blocked the cleavage of p62 indicating the possible involvement of membrane fusion before processing of the TGN-located precursor (Fig. 1A).

3.2. Reconstitution of p62 cleavage in SLO-permeabilized cells

We studied the cleavage of SFV p62 to E2 and E3 further by using SLO-permeabilized BHK cells. SFV-infected BHK cells were first pulse-labeled and chased for 2 h at 20°C to accumulate labeled glycoproteins in the TGN. The cells were then SLO-permeabilized and further incubated for 2 h at 37°C to allow the transport of the spike glycoproteins to the plasma membrane. When the SLO-permeabilized cells were incubated in the TM, the cleavage of p62 did not take place (Fig. 1B). In the presence of cytosol and ATP regeneration system, about 30% of p62 was cleaved. In intact cells the cleavage was about 70% on average. Cleavage of p62 was ATP dependent since only 8% of p62 was cleaved in the absence of ATP regener-

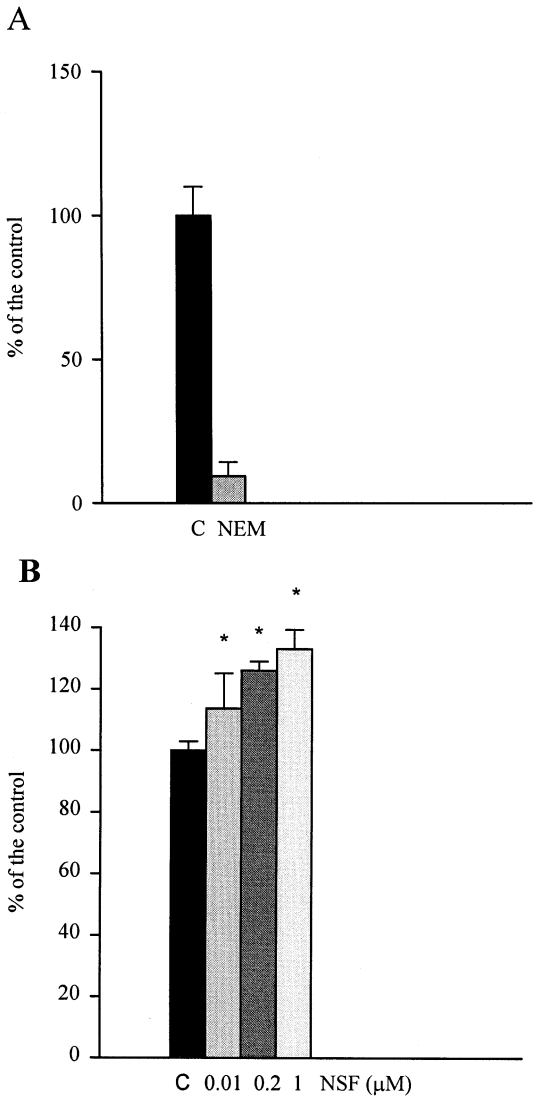

Fig. 2. NSF stimulates the cleavage of SFV p62. BHK cells were infected with SFV, labeled virus glycoproteins were accumulated in the TGN, and the cells were permeabilized with SLO followed by incubation at 37°C for 2 h in the presence of cytosol, ATP regeneration system, and NEM (A) or with an increasing concentration of NSF (0.01, 0.2 or 1 μM) (B). NEM treatment was performed by incubating the cells and cytosol with 1 mM NEM for 15 min at 4°C and then quenching with 2 mM glutathione. The cleavage of p62 in controls (C) was 27% and 28%, respectively. Values are means ± S.E.M., $n = 3$–7 observations. *$P < 0.01$ versus control, Student's t-test. Data are representative of two similar experiments.

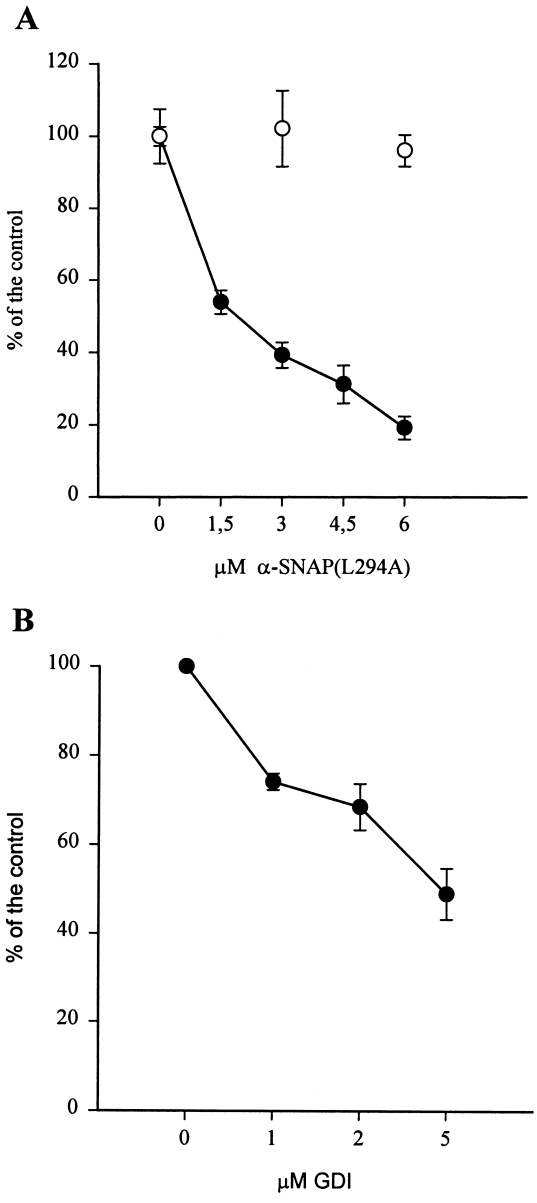

Fig. 3. The effect of the α-SNAP(L294A) mutant (A) or GDI (B) on the cleavage of p62. BHK cells were infected with SFV, labeled virus glycoproteins were accumulated in the TGN, and the cells were permeabilized with SLO and followed by incubation at 37°C for 2 h in the presence of cytosol, ATP regeneration system, and increasing concentration of the α-SNAP(L294A) mutant (●) or α-SNAPwt (○) (A) or GDI (B). The cleavage of p62 in controls (A and B) was 26% and 39%, respectively. Values are means ± S.E.M., $n = 3$ observations or three separate experiments, respectively. Data are representative of two similar experiments in A.

ation system when compared with the cleavage of p62 in the presence of ATP (Fig. 1B).

3.3. Involvement of NSF, α-SNAP and Rabs in the cleavage of p62

The possible role of NSF in the cleavage of p62 was investigated by treating the cells and cytosol with NEM. Incubating cytosol with 1 mM NEM at 4°C for 15 min did not repeatedly inhibit cleavage of p62 when added to SLO-permeabilized BHK21 cells (data not shown). When both the cytosol and the cells were treated with NEM, the cleavage was totally inhibited (Fig. 2A). The recombinant NSF stimulated the cleavage (Fig. 2B), suggesting that NSF is involved and that it is a rate limiting factor in this system. However, NSF was unable to reverse the inhibitory effect of NEM. This could be due to the observation that endogenous NSF remains associated with membranes and that the recombinant NSF may not be able to replace NEM-inactivated endogenous NSF as suggested previously [27]. This suggests that membrane bound NSF alone is sufficient to support the membrane fusion step required prior to the p62 cleavage. The tight membrane association of NSF is supported by an experiment with endocytic vesicles. The stimulation of their fusion by recombinant NSF could only be obtained after removal of endogenous NSF by high salt treatment [28].

α-SNAP stimulates exocytosis in digitonin-permeabilized adrenal chromaffin cells [27]. We used a recombinant α-SNAP mutant protein (L294A, leucine 294 changed to alanine) that has been reported to be able to bind NSF but not to stimulate NSF [29]. This mutant caused a dose-dependent inhibition of the cleavage of p62 in SLO-permeabilized BHK cells (Fig. 3A). 6 μM α-SNAP mutant inhibited 80% of the cleavage of p62, indicating that the α-SNAP(L294A) mutant can replace endogenous α-SNAP and stop membrane fusion. Wild type α-SNAP had no affect on the processing of p62 at 6 μM (Fig. 3A).

There are over 40 different Rab GTPases which have been implicated to play a role in membrane docking [30]. The possible role of Rabs in the cleavage of p62 was investigated by using recombinant Rab-GDI. Since Rab-GDI binds to and inactivates several different Rabs, it acts as a general regulator [31]. Rab-GDI has been reported to inhibit intracellular vesicle trafficking in several systems, for instance between the ER and Golgi [32], inter-cisternal Golgi transport [33], and basolateral transport of MDCK cells [34]. In our reconstituted system, the addition of 5 μM recombinant Rab-GDI with the cytosol to SLO-permeabilized cells caused 50% reduction in the cleavage of p62 (Fig. 3B), suggesting that a Rab protein is involved in the membrane fusion/communication step prior to the p62 processing. This result is in agreement with a previous report that Rab-GDI inhibited the transport of SFV glycoproteins E1 and E2 from the TGN to the plasma membrane in SLO-permeabilized BHK cells [19].

3.4. Effect of the α-SNAP mutant on the transport from the TGN region

Since the results from the reconstitution assays clearly indicated that the membrane fusion machinery is involved in the

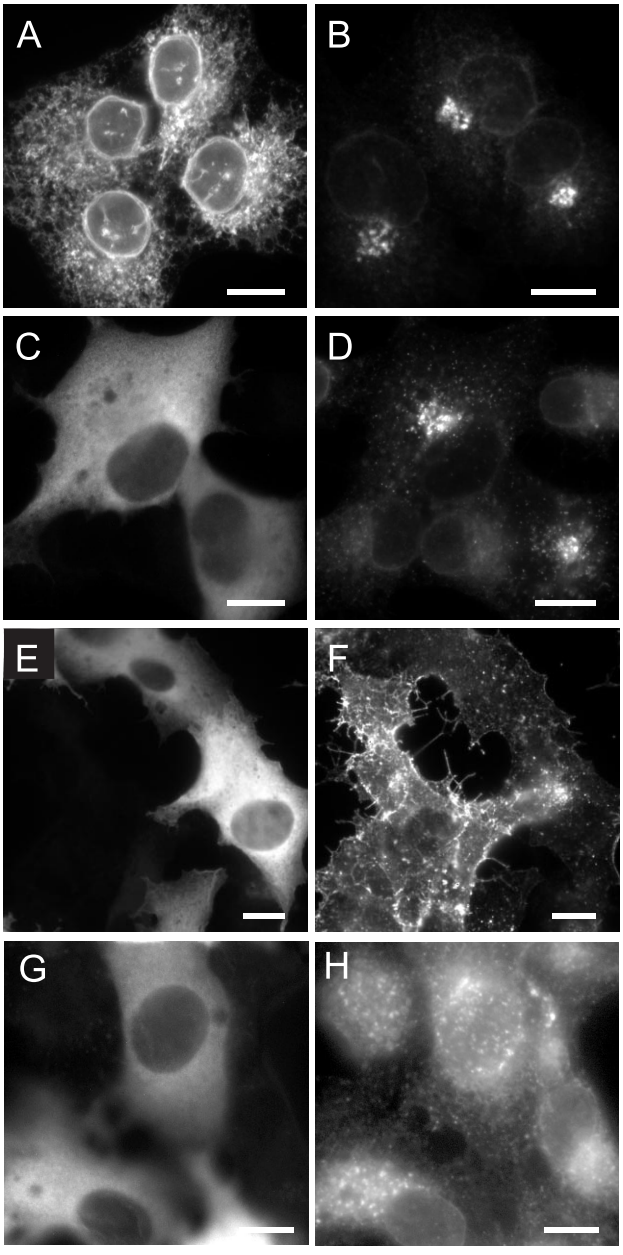

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Fig. 4. The effect of the α-SNAP(L294A) mutant on the localization of SFV ts-1 glycoproteins. BHK cells were infected with the temperature-sensitive SFV mutant ts-1 (A), viral glycoproteins were accumulated in the TGN at 20°C (B). The cells were microinjected with the α-SNAP(L294A) mutant (C, E) or α-SNAPwt (G) together with monoclonal mouse anti-human IgG as a microinjection marker. The temperature was raised to 28°C for 2 h (C–H). The cells were fixed with 3% PFA and permeabilized with 0.05% Triton X-100. The cells were stained with polyclonal anti-SFV glycoprotein antibody followed by LRSC-conjugated goat anti-rabbit IgG. The injector marker was visualized using FITC-conjugated goat anti-mouse IgG (C, E, G). Microinjection marker did not affect the transport of the viral glycoproteins and about 75% of the α-SNAP mutant-microinjected cells contained the accumulation of the viral glycoproteins in the TGN. A shows virus glycoproteins in the ER; B shows virus glycoproteins in the TGN; C and D represent the same microscopic field where C shows the α-SNAP(L294A)-microinjected cells and D shows the localization of virus glycoproteins in those cells; E and F show another field (α-SNAP(L294A)-microinjected cells) where F shows virus glycoproteins on the cell surface before permeabilization with Triton X-100 to reveal the injection marker (E). G and H represent the same microscopic field where G shows the α-SNAPwt-microinjected cells and H shows the localization of virus glycoproteins in those cells. Bars, 10 μm.

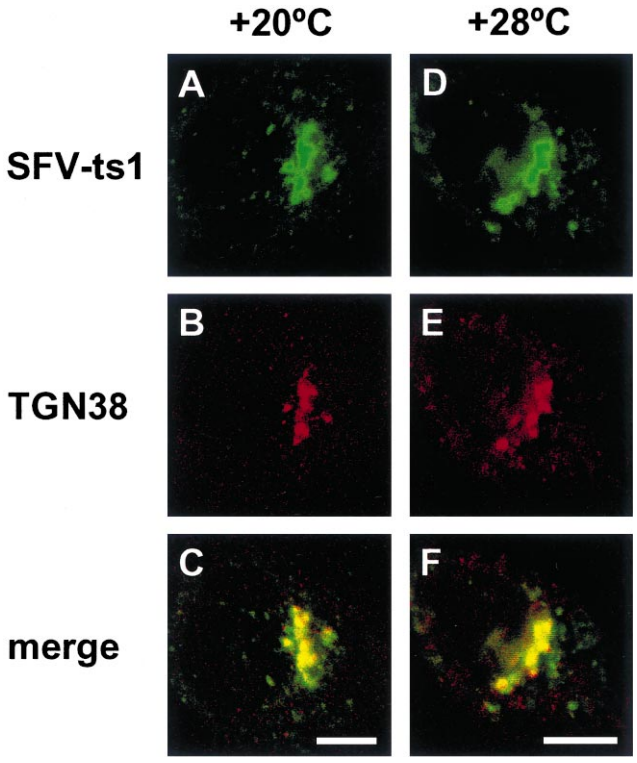

Fig. 5. Colocalization of virus glycoproteins and TGN38 by confocal microscopy. SFV ts-1 mutant viral glycoproteins were accumulated in the TGN in NRK cells cultured on cellogate coverslips. Cells were microinjected with the α-SNAP(L294A) mutant and incubated further at 28°C. The cells were fixed with 3% PFA and permeabilized with 0.05% Triton X-100. The cells were stained with polyclonal anti-SFV glycoprotein antibody followed by FITC-conjugated goat anti-rabbit IgG (A, D) as well as monoclonal anti-TGN38 and TRITC-conjugated goat anti-mouse IgG (B, E). The yellow color in C and F reveals the colocalization. Bars, 10 μm.

regulation of the cleavage of p62, and since the cleavage of p62 as such is not required for the transport to the plasma membrane [35], we wanted to study whether inhibition of membrane fusion affects the transport of p62 from the TGN. We studied the transport of p62 from the TGN under conditions where membrane fusion is inhibited by the α-SNAP mutant. As shown by in vitro reconstitution experiments, p62 remains uncleaved in the presence of the α-SNAP mutant. BHK cells were first infected with a temperature-sensitive SFV ts-1 mutant and incubated at 38.5°C to arrest SFV glycoproteins to the ER [21] (Fig. 4A). The SFV proteins were then allowed to be transported to the TGN at 20°C [17] (Fig. 4B). After microinjection with the α-SNAP mutant, the low temperature transport block was released by increasing the temperature to 28°C. In the uninjected control cells, the viral glycoproteins were transported to the plasma membrane (Fig. 4F). The cells injected with the α-SNAP mutant showed greatly reduced plasma membrane staining of virus glycoprotein, indicating that the transport of viral proteins to the cell surface was markedly inhibited (Fig. 4F). Instead, bright intracellular staining of perinuclear elements was observed (Fig. 4D).

We used the indirect double staining technique and confocal laser scanning microscopy to study the colocalization of viral glycoproteins in the perinuclear elements (Fig. 4D) with the TGN marker TGN38. Because the anti-rat TGN38 antibody did not stain BHK cells, NRK cells were used. After 2 h incubation at 20°C, colocalization of the viral glycoproteins and the TGN38 marker was observed in the TGN membranes (Fig. 5C). After microinjection with the recombinant α-SNAP mutant protein and incubation for 2 h at 28°C, the viral glycoproteins and the TGN38 protein were still mostly colo-

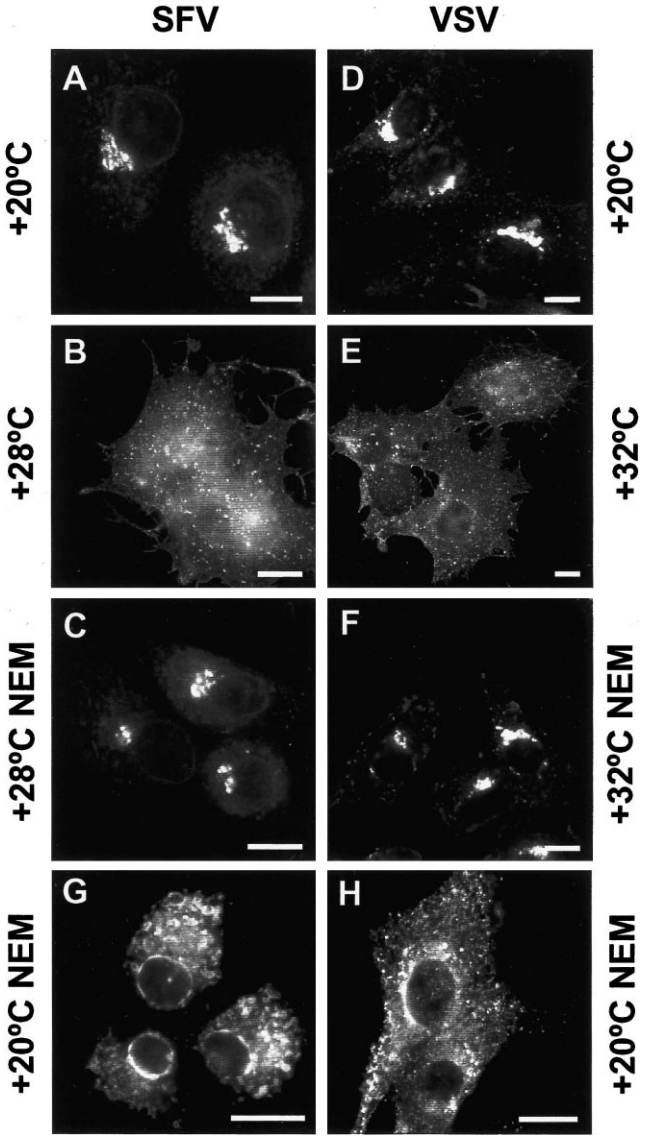

Fig. 6. NEM blocks the transport of viral glycoproteins from the TGN to the plasma membrane as well as from the ER to the TGN. SFV ts-1-infected BHK cells were incubated at 20°C for 2 h to accumulate viral glycoproteins to the TGN (A) and then the temperature was shifted to 28°C for 2 h (B). In NEM-treated cells viral glycoproteins were first incubated at 20°C for 1.5 h and then 1 mM NEM was added and further incubated at 20°C for 0.5 h before the shift to 28°C (C). Similarly VSV-infected BHK cells were incubated at 20°C (D) and shifted to 32°C in the absence (E) and the presence of 1 mM NEM (F). G and H show the inhibition of the viral glycoprotein transport from the ER to the TGN in the presence of NEM. The infected BHK cells were incubated 30 min with 1 mM NEM before shifting to 20°C for 2 h. The cells were fixed with 3% PFA and permeabilized with 0.05% Triton X-100. The cells were stained with polyclonal anti-SFV glycoproteins antibody or polyclonal anti-VSV G antibody followed by LRSC-conjugated goat anti-rabbit IgG. Bars, 10 μm.

calized (Fig. 5F), indicating that the viral protein transport from the TGN to the plasma membrane was prevented.

A similar inhibitory effect on the protein transport was observed in NEM-treated cells. As shown by immunofluorescence microscopy NEM (1 mM) blocked the transport of the SFV glycoproteins from the TGN to the plasma membrane in intact BHK cells (Fig. 6C). The same TGN export inhibition by NEM was observed when the VSV tsO45 mutant was used in the transport assay (Fig. 6F). NEM also inhibited the transport from the ER to the TGN and caused the vesiculation of the ER membranes (Fig. 6G,H). These results are in agreement with the previous in vitro transport assays. These studies have shown that membrane transport between the ER and Golgi [32] and intra-Golgi transport [33] are inhibited with NEM.

4. Discussion

Previously it has been shown using reconstitution experiments in SLO-permeabilized MDCK cells that Rab-GDI, NSF, SNAREs and α-SNAP are involved in the transport from the TGN to the basolateral plasma membrane but not to the apical plasma membrane [34]. In addition to dissecting different pathways SLO-permeabilized cells can be used to study separate steps in late biosynthetic transport. In this report, we show that function of the membrane fusion machinery is required for the processing of the SFV p62 precursor accumulated in the TGN at 20°C. Inactivation of NSF both in live cells with NEM, and in SLO-permeabilized cells with NEM or the α-SNAP mutant (L294A), inhibited the cleavage of p62 when the low temperature-induced transport block in the TGN was released. Addition of recombinant NSF to the reconstitution assay stimulated the processing of p62. Moreover, depletion of Rab-GTPases with recombinant GDI protein inhibited the processing of p62 when the transport block was released. We studied also whether p62 was exported from the TGN in the absence of membrane fusion. For this purpose we used the SFV ts-1 transport mutant and immunofluorescence assays. Unexpectedly, inhibition of NSF function in live cells either with NEM treatment or with microinjection of the α-(SNAP) mutant (L294A) protein inhibited the transport of SFV glycoproteins from the TGN to the cell surface. Since previous studies have demonstrated that cleavage of p62 is not a prerequisite for its transport to the plasma membrane [35], it appears likely that inhibition of the fusion machinery results in inhibition of membrane export from the TGN. This conclusion was supported by the observation that inactivation of NSF inhibited also the export of the VSV G-protein from the TGN. This would suggest either direct or indirect participation of the fusion machinery in vesicle budding. Although the involvement of NSF and α-SNAP in SNARE complex disassembly and exocytosis has been well established the exact site of disassembly of the 20S complex is still unknown. According to the original SNARE hypothesis the disassembly of 20S complex by NSF ATPase activity provides the energy for the fusion and is the last step in membrane fusion [36].

Later other hypotheses have been presented suggesting that NSF activity is needed after membrane fusion to disassemble the SNARE complex and to allow the recycling of v-SNAREs to its membrane of origin [37], or that SNARE complex disassembly occurs before the docking of the vesicle to prime SNAREs [38]. Recently, more evidence has appeared suggesting a much earlier role for NSF/α-SNAP. For instance, synaptic vesicles and chromaffin granules contain most of the components of the 20S complex including α-SNAP, NSF v-SNAREs, and t-SNAREs [39–42]. Additionally, the recent kinetic studies in catecholamine release in chromaffin cells suggest that the disassembly of the 20S complex occurs at an early stage of vesicle recruitment rather than at the late step for fusion itself [43]. For these reasons the possible involvement of the NSF and α-SNAP in vesicle budding cannot be ruled out. The other possibility is that exit from the TGN is critically dependent on retrograde traffic which supplies components needed for budding. For example, inhibition of membrane fusion will prevent recycling of budding factors from the endosomal recycling compartment to the TGN. This possibility clearly needs further investigations. In conclusion, the present biochemical and morphological results together indicate that inhibition of membrane fusion prevents the exit of p62 from the TGN and its subsequent proteolytic processing.

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