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Vimentin Filaments in Fibroblasts Are a Reservoir for SNAP23, a Component of the Membrane Fusion Machinery

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Soluble *N*-ethyl maleimide-sensitive fusion protein attachment protein receptors (SNAREs) are core machinery for membrane fusion during intracellular vesicular transport. Synaptosome-associated protein of 23 kDa (SNAP23) is a target SNARE previously identified at the plasma membrane, where it is involved in exocytotic membrane fusion. Here we show that SNAP23 associates with vimentin filaments in a Triton X-100 insoluble fraction in fibroblasts in primary culture and HeLa cells. Upon treatment of human fibroblasts with *N*-ethyl-maleimide, SNAP23 dissociates from vimentin filaments and forms a protein complex with syntaxin 4, a plasma membrane SNARE. The vimentin-associated pool of SNAP23 can therefore be a reservoir, which would supply the plasma membrane fusion machinery, in fibroblasts. Our observation points to a yet unexplored role of intermediate filaments.

INTRODUCTION

SNAP23 is a ubiquitously expressed isoform of synaptosomal-associated protein of 25 kDa (SNAP25) (Ravichandran *et al.*, 1996), a component of the whole neuronal plasma membrane (Oyler *et al.*, 1989; Galli *et al.*, 1995). SNAP25 plays a role as a target soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein (SNAP) receptor (t-SNARE), through the formation of a ternary complex with syntaxin 1, another neuronal plasma membrane protein, and synaptobrevin 2, a synaptic vesicle SNARE (Sollner *et al.*, 1993). Formation of trans-SNARE complexes between adjacent membranes mediates lipid bilayer fusion (Weber *et al.*, 1998; Bock and Scheller, 1999; Nickel *et al.*, 1999). SNAP23 localizes to the apical and lateral plasma membranes in epithelial cells and it is involved in exocytosis at both domains (Galli *et al.*, 1998; Leung *et al.*, 1998; Low *et al.*, 1998a; Lafont *et al.*, 1999). SNAP23 is also involved in granule exocytosis in nonpolarized cell types, including mast cells (Guo *et al.*, 1998), adipocytes (Foster *et al.*, 1999), and platelets (Chen *et al.*, 2000).

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The mechanism and regulation of targeting of SNAP25 and SNAP23 to the plasma membrane is not fully understood. Palmytoylation of cysteine residues located in the center of these proteins is required (Gonzalo and Linder, 1998; Gonzalo *et al.*, 1999) but the interaction with a plasma membrane syntaxin (Veit, 2000) and phosphorylation by SNAP kinase (Cabaniols *et al.*, 1999) are also important in this process. It was recently reported that SNAP23 partially sediments with cytoskeletal elements in a Triton X-100 insoluble fraction (Guo *et al.*, 1998; Foster *et al.*, 1999). In this study we show that the cytoskeleton-associated pool of SNAP23 is bound to vimentin filaments in fibroblasts in primary culture and may be recruited to form SNARE complexes at the plasma membrane.

MATERIALS AND METHODS

Cells

HeLa cells, human fibroblasts, and embryonic mouse fibroblasts were used. Human fibroblasts were obtained from Dr. G. de Saint Basile (Hôpital Necker Enfants Malades, Paris, France) and cultured in RPMI 1640, 10% fetal calf serum, 5 mM glutamine. Embryonic mouse fibroblasts from wild-type and vimentin knockout mice were

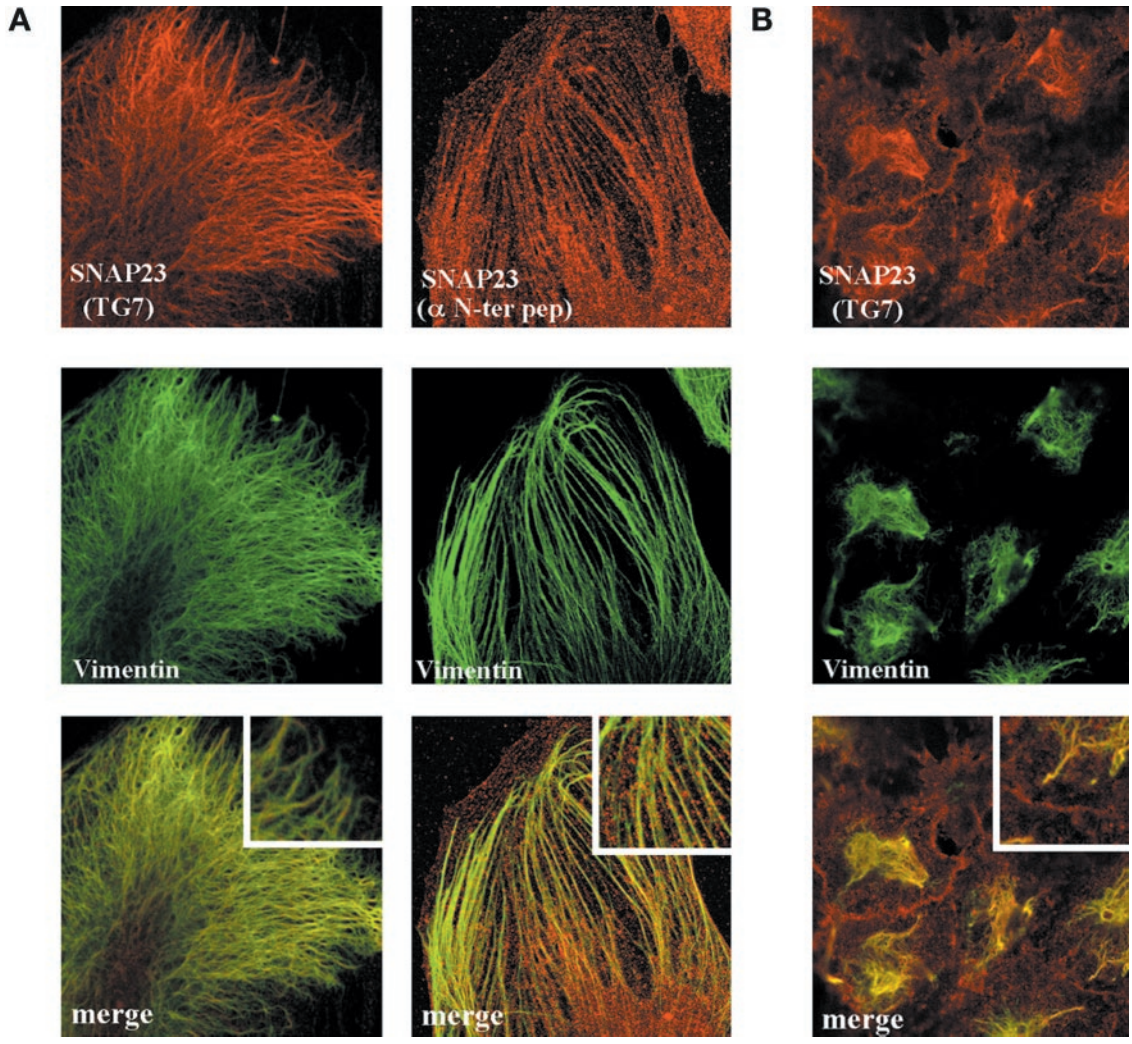


Figure 1. SNAP23 is targeted to vimentin filaments in fibroblasts. (A) Confocal sections of human and murine fibroblasts in primary culture double-stained for vimentin and SNAP23 by using two different anti-SNAP23 antibodies: TG7, directed against the full recombinant human protein, and α-Nter, directed against the first 17 N-terminal amino acids of human SNAP23. In both cases, SNAP23 immunoreactivity appears as discontinuous spots along vimentin filaments. (B) Confocal section of HeLa Cells double-stained for SNAP23 (with TG7) and vimentin. SNAP23 immunoreactivity is present both at the plasma membrane and along vimentin filaments.

prepared from E13.5 embryo as described (Gillard *et al.*, 1998) and cultured in Dulbecco's modified Eagle's medium, 10% fetal calf serum, 5 mM glutamine, 1 mM sodium pyruvate.

Antibodies

Primary antibodies included rabbit affinity-purified anti-SNAP23 recombinant protein (TG7); anti-Nter peptide (Nter, 1–17 residues; generous gift of Dr. P. Roche, National Institutes of Health, Bethesda, MD; Low *et al.*, 1998b); anti-peptide (residues 196–211; Synaptic Systems, Göttingen, FRG); and mouse antibodies directed against β-tubulin (clone tub2.1; Sigma, St. Louis, MO), syntaxin 4 (clone 49; Transduction Laboratories, San Diego, CA), vimentin (clone7A3), and keratin (anti pan-keratin, clone PCK-26; Sigma) in phosphate-buffered saline (PBS). Secondary antibodies included Texas Red-coupled anti-rabbit $F(ab')_2$ (Jackson Laboratories, West Grove, PA) or rhodamine-coupled phalloidin (Sigma), and Alexa 488-coupled donkey anti-mouse secondary antibodies (Molecular Probes, Leiden, The Netherlands). Horseradish peroxidase-coupled antibodies against mouse and rabbit was purchased from Jackson Laboratories.

Immunofluorescence

Cells were grown on glass coverslips and fixed in methanol at −20°C for 3–5 min, incubated in PBS/bovine serum albumin/saponin (0.2%/0.05%) for 10 min, and subsequently with the different antibodies for 20–30 min at each step. The coverslips were mounted in Mowiol. Analysis of the samples was performed on a TCS confocal microscope (Leica, Heidelberg, FRG). In certain experiments, cells were incubated during 16 h in RPMI containing 4 mM acrylamide before fixation (Eckert, 1985; Olink-Coux *et al.*, 1992).

N-*ethyl Maleimide (NEM) Treatment*

Human fibroblasts were treated with 1 mM NEM in PBS for 15 min on ice and then with 2 mM dithiothreitol (DTT) in PBS for another

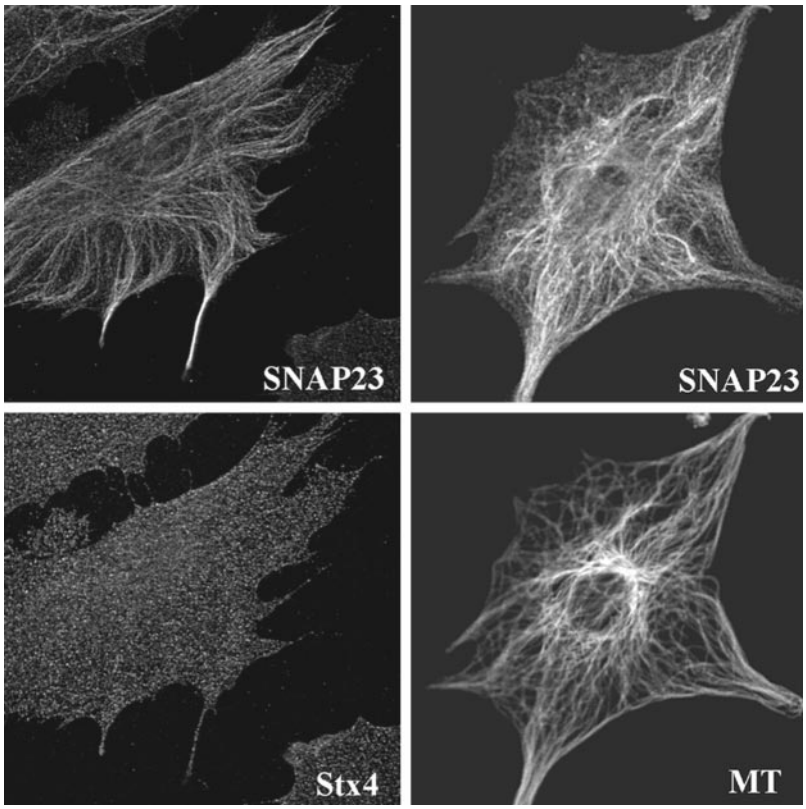


Figure 2. Confocal sections of human and murine fibroblasts in primary culture double-stained for SNAP23 and α-tubulin (MT), or syntaxin 4 (Stx4). Note that SNAP23 does not significantly colocalize with α-tubulin or syntaxin 4.

15 min on ice, or with 1 mM NEM + 2 mM DTT in PBS for 30 min on ice, washed extensively with ice-cold PBS, and incubated in culture medium at 37°C for 30 min. They were lysed with 1% Triton X-100 in 50 mM Tris, 150 mM NaCl, 5 mM EDTA, and a cocktail of protease inhibitors (Sigma). The extract was centrifuged at 100,000 $\times$ *g* for 30 min, resulting in Triton X-100 insoluble and soluble fractions. Immunoprecipitation was carried out from the Triton X-100 soluble fraction with antibody-coated magnetic beads (Dynal, Oslo, Norway)

Electron Microscopy

The protocol was previously published by Maison *et al.* (1993). Briefly, cells were incubated for 45 min at 37°C in 2 ml of culture medium containing 20 ng/ml nocodazole and 20 μM cytochalasin B. They were washed twice in PBS at 4°C and once in KHM buffer (78 mM KCl, 50 mM HEPES KOH, pH 7.0, 4 mM $MgCl_2$, 10 mM EGTA, 8.37 mM $CaCl_2$, 1 mM DTT, 20 μM cytochalasin B). Cells were resuspended in KHM buffer and homogenized in a tight-fitting Dounce homogenizer. The homogenate was put onto grids and incubated for 3 min. The grids were fixed in 3.7% paraformaldehyde for 10 min and quenched in 50 mM PBS/glycine for 10 min.

Immunoisolation

The cells were broken as described for electron microscopy. Subsequently, anti-vimentin-coated Dynabeads were incubated with the homogenate. The beads were washed three times in PBS and proteins were solubilized by incubating in SDS-Laemmli buffer. The samples were separated on a SDS-PAGE, transferred onto Immobilon-membrane. Vimentin and SNAP23 were revealed by chemiluminiscence and quantified by densitometry by using Bio1D software (Vilbert-Lourmat, Marne-La-Vallée, France).

RESULTS

In primary culture of human fibroblasts, SNAP23 immunoreactivity recognized by a rabbit antibody raised against the recombinant human protein produced in *Escherichia coli* (TG7) appeared as spots associated with filamentous structures in confocal microscopy. These structures were also vimentin-positive (Figure 1A) but not tubulin-, keratin-, or actin-positive (Figure 2 and our unpublished data). The same structures were observed using two anti-peptide antibodies directed against SNAP23, the anti-Nter peptide (1–17) (Figure 1A) and the anti-peptide [196–211] (our unpublished data). SNAP23 was the only SNARE found on vimentin filaments among the tested ones (cellubrevin, TI-VAMP/VAMP7, endobrevin/VAMP8, syntaxin 3,4). In particular, syntaxin 4, a main t-SNARE partner of SNAP23, localized to punctate structures not aligned along cytoskeletal structures (Figure 1A). Vimentin association of SNAP23 was intriguing because SNAP23 immunoreactivity was restricted to the apical and lateral plasma membrane in CaCo-2 cells (Galli *et al.*, 1998). However, it should be noted that these cells are free of vimentin intermediate filaments. Interestingly, in HeLa cells, which produce vimentin and keratin intermediate filament networks, SNAP23 localized to vimentin filaments as well as to the plasma membrane (Figure 1B) but not to keratin filaments (our unpublished data). We wondered whether the vimentin-bound pool of SNAP23 could correspond to vesicular structures anchored to intermediate filaments or to a direct protein–protein association.

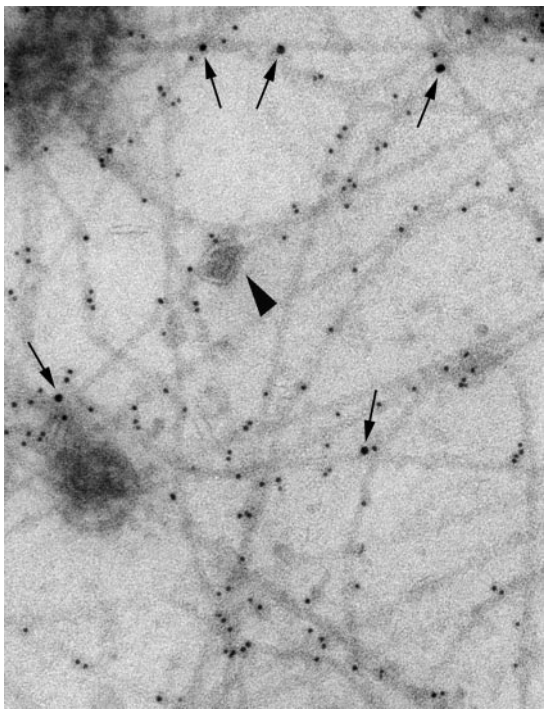


Figure 3. Direct association of SNAP23 on vimentin filaments. Human fibroblast were homogenized in a cell cracker, treated with cytochalasin B and nocodazole, and deposited on electron microscopy grids. The sample was double-stained for SNAP23 (10-nm gold particle, arrows) and vimentin (5-nm gold particle). The arrowhead points to a vimentin-bound vesicle, which is SNAP23-negative.

To investigate this question, we prepared extracts from human fibroblasts by passing the cells through a cell cracker followed by combined cytochalasin B and nocodazole treatments to disrupt microfilaments and microtubules. This preparation was then processed for immunogold labeling of SNAP23 and vimentin followed by electron microscopy observation (Maison *et al.*, 1993). All of the intermediate filaments remaining in the preparation were positive for vimentin. SNAP23 immunoreactivity associated directly with vimentin filaments and not with membranes attached to them (Figure 3).

This prompted us to investigate the effect of vimentin filament disruption on SNAP23 intracellular distribution. First, we treated human fibroblasts in primary culture with acrylamide, a poison of intermediate filament organization (Eckert, 1985; Olink-Coux *et al.*, 1992). Acrylamide induced massive, but incomplete, aggregation of vimentin filaments and a significant redistribution of SNAP23 to the plasma membrane (Figure 4). Second, we studied the subcellular localization of syntaxin 4 and SNAP23 in primary culture fibroblasts derived from wild-type (V+) and vimentin knockout embryonic mice (V−) (Colucci-Guyon *et al.*, 1994; Gillard *et al.*, 1998). Both cell types expressed equal amounts of syntaxin 4 and SNAP23, whereas vimentin expression was totally obliterated in V− cells (Figure 5A). The anti-SNAP23 antibody recognized a single band corresponding to the expected molecular weight for SNAP23 in Western blot of cell extracts. We did not observe any cross-reactivity with any other antigen, including vimentin, a major protein in V+ cell extracts (Figure 5A). Confocal sections of V+ fibroblast cells in primary culture showed that SNAP23 immunoreactivity appeared as spots associated with filamentous vimentin (Figure 5B). Strikingly, SNAP23 immunoreactivity was concentrated in spots partially associated or in proximity with the plasma membrane in V− cells (Figure 5B). This distribution was similar to that of syntaxin 4 in human (Figure 1), V− and V+ mouse (our unpublished data) fibroblasts in primary culture. Permeabilization of cells with Triton X-100 before fixation (Kreis, 1987) induced the loss of SNAP23 immunoreactivity in V−, but not in V+ cells (Figure 6). We conclude the following: 1) SNAP23 association with vimentin filaments was dependent upon their integrity; 2) in cells producing vimentin filaments, intracellular pools of SNAP23 are observed; and 3) the vimentin-associated pool of SNAP23 is Triton X-100 insoluble in contrast to the plasma membrane pool.

Altogether, these results suggested that the vimentin-associated pool of SNAP23 could constitute a reservoir of the plasma membrane pool of SNAP23. To test this hypothesis, we treated human fibroblasts with NEM to inactivate NSF (Beckers *et al.*, 1989). As expected, the SNAP23–syntaxin 4 complex immunoprecipitated from the Triton X-100 soluble fraction was more abundant in NEM-treated cells than in control (NEM + DTT-treated) cells (Figure 7A, lanes 5–8). Hence, inactivation of NSF leads to the stabilization of plasma membrane SNARE complexes as shown previously (Sogaard *et al.*, 1994; Banerjee *et al.*, 1996; Galli *et al.*, 1998). If the pool of SNAP23 assembling into SNARE complexes was mobilized from the vimentin-associated pool, then the latter should decrease upon NEM treatment. Indeed, NEM treatment decreased the concentration of SNAP23 in the Triton X-100 insoluble fraction (Figure 7A, compare lane 1 and 2). No difference was observed in the case of syntaxin 4, a minor pool of this SNARE being found in the Triton X-100 insoluble fraction in both cases (Figure 7A). Accordingly, NEM treatment of human fibroblasts in primary culture decreased the vimentin-associated pool of SNAP23, as observed by confocal microscopy (Figure 7B). Next, we immunoisolated vimentin filaments, in the absence of Triton X-100, from cell homogenates treated with nocodazole and cytochalasin B (Maison *et al.*, 1993), as in our study by electron microscopy shown in Figure 3. We used magnetic beads coated with anti-vimentin antibodies and the amount of SNAP23 in vimentin immunoisolates was measured by Western blot. Treatment with NEM induced a 70% decrease in the amount of SNAP23 associated with vimentin (Figure 7C). Syntaxin 4 was not found in vimentin immunoisolates (our unpublished data). Altogether, these results show that stabilization of SNARE complexes by inactivation of NSF decrease the vimentin-associated pool of SNAP23, but increase the syntaxin 4- and most likely plasma membrane-associated pool of SNAP23.

DISCUSSION

Our results showing mobilization of SNAP23 from a reservoir pool on vimentin filaments to functional SNARE complexes at the plasma membrane suggests a new role of vimentin in the regulation of SNAP23 function and maybe

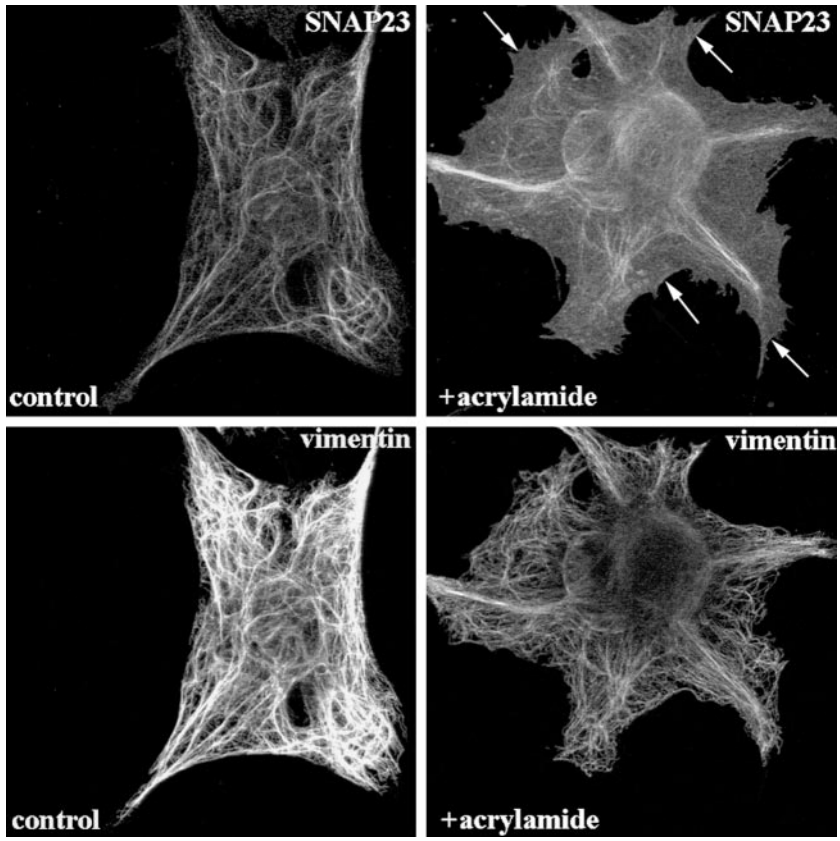


Figure 4. Vimentin alone targets SNAP23. Confocal sections of human fibroblasts showing redistribution of SNAP23 following acrylamide-induced vimentin disruption. SNAP23 immunoreactivity redistributes in large vimentin-positive structures and is found at the plasma membrane. Arrows point to plasma membrane localization of SNAP23.

other membrane proteins in fibroblasts. Vimentin has been implicated in the formation of aggresomes, a pericentriolar structure accumulating misfolded proteins, including membrane proteins such as cystic fibrosis transmembrane conductance regulator, which have not been degraded by the proteasome (Johnston *et al.*, 1998), and ubiquitin-related proteins have been found to regulate the interaction of vimentin filaments with the plasma membrane (Wu *et al.*, 1999)

Thus, vimentin could be involved in sequestering proteins, such as SNAP23, along their biosynthesis or degradation pathways. How could SNAP23 move from the vimentin-associated pool to the plasma membrane? Targeting of SNAP23 to vimentin filaments could be direct or it could involve partners such as a recently identified SNAP25-interacting protein (SNIP) that partitions entirely in Triton X-100 insoluble fractions (Chin *et al.*, 2000). Targeting to the plasma membrane likely involves syntaxins (Cabaniols *et al.*, 1999; Veit, 2000). If vimentin-associated SNAP23 were palmytoylated, then its targeting to the plasma membrane could depend on proteins similar to GDP dissociation inhibitor, which solubilize and target small GTPases to membranes (Soldati *et al.*, 1994; Ullrich *et al.*, 1994). If vimentin-associated SNAP23 were not palmytoylated, depalmytoylation/palmytoylation cycles, as for the α subunits of heterotrimeric G proteins (Levis and Bourne, 1992), would occur in the course of SNAP23 cycling between vimentin filaments and the plasma membrane. Phosphorylation of SNAP23 by SNAP23 kinase decreases its degradation and increases the kinetics of t-SNARE assembly (Cabaniols *et al.*, 1999) so it could regulate SNAP23 targeting to the plasma membrane.

In this study, we propose that transfer of SNAP23 from the vimentin-associated reservoir to the functional plasma membrane pool may modulate availability of SNAP23 to form SNARE complexes in fibroblasts. How could the vimentin-associated reservoir of SNAP23 play a role in membrane traffic? Although the role of microtubules and the actin cytoskeleton in membrane traffic has been well documented (Goodson *et al.*, 1997), only few reports examine the role of intermediate filaments in membrane traffic. Recently, the group of Marcus has found a decreased synthesis of glycosphingolipids (GSL) in fibroblast cells derived from vimentin knockout mice (Gillard *et al.*, 1998). This result led the authors to postulate a possible implication of vimentin in intracellular membrane trafficking, in particular in the recycling of GSL between the Golgi apparatus and the endosomes, a pathway responsible for the incorporation of sugars into GSL. Intermediate filaments reorganization is coupled to granule secretion activation in neutrophils (Pryzwansky and Merricks, 1998) but a direct involvement of vimentin in exocytosis has not been demonstrated. SNAP23 is involved in transferring receptor recycling in Madin-Darby canine kidney cells (Leung *et al.*, 1998). Thus, we have measured the rate of endocytosis and recycling to the plasma membrane of iodinated mouse transferrin in primary cultures of V+ and V− fibroblasts. We found that

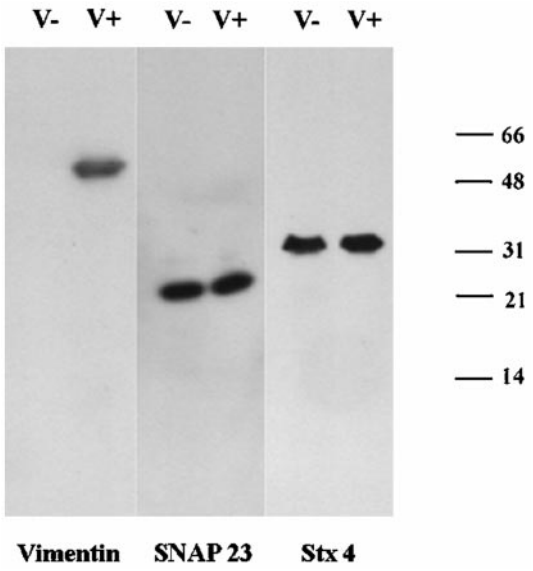
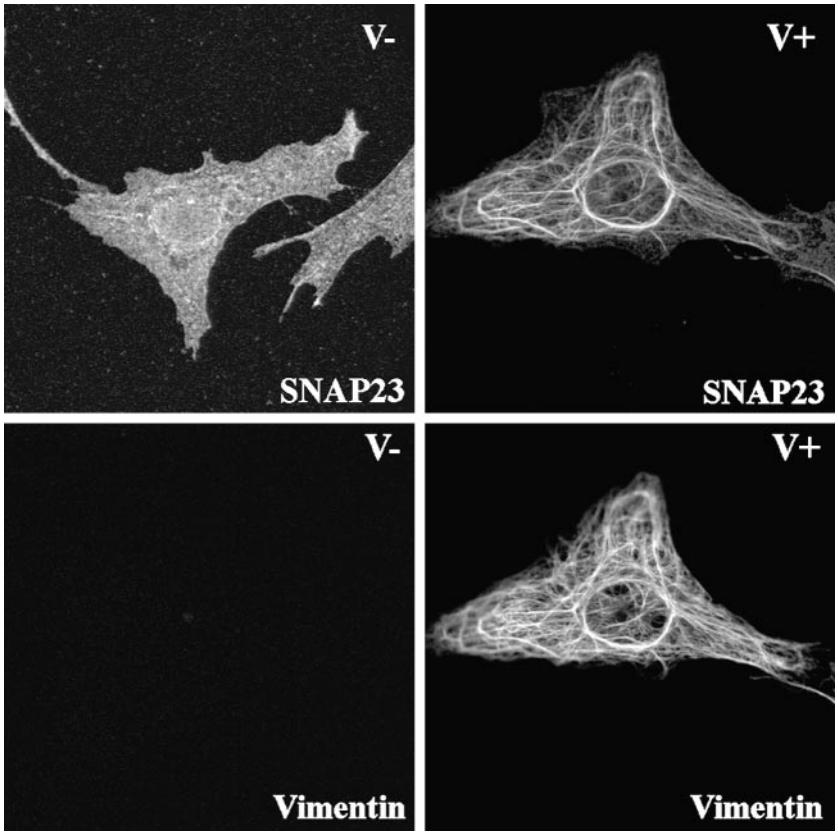


Figure 5. (A) Western blot analysis of extracts of fibroblasts from wild-type (V+) and vimentin knockout (V−) mice. Vimentin is expressed only by V+ fibroblasts, whereas SNAP23 and syntaxin 4 (Stx4) equally expressed by V+ and V− cells. Note the high specificity of the anti-SNAP23 (TG7) antibody. (B) SNAP23 localizes at the plasma membrane in V− fibroblasts. V+ and V− fibroblasts were double-stained for SNAP23 and vimentin. SNAP23 colocalizes with vimentin in V+ cells but is found in punctate structures mostly at the plasma membrane in V− cells.



endocytosis of holo-transferrin and exocytosis of recycled apo-transferrin were identical in both cell types (our unpublished data). This may indicate that a low amount of SNAP23 at the plasma membrane is enough to sustain normal exocytosis of transferrin receptor in V+ cells. Nevertheless, our observations could suggest that targeting of SNAP23 to vimentin may affect other membrane transport pathways. The latter could include release of Golgi secretory vesicles, including secretory granules that depend on compound exocytosis (Guo *et al.*, 1998; Chen *et*

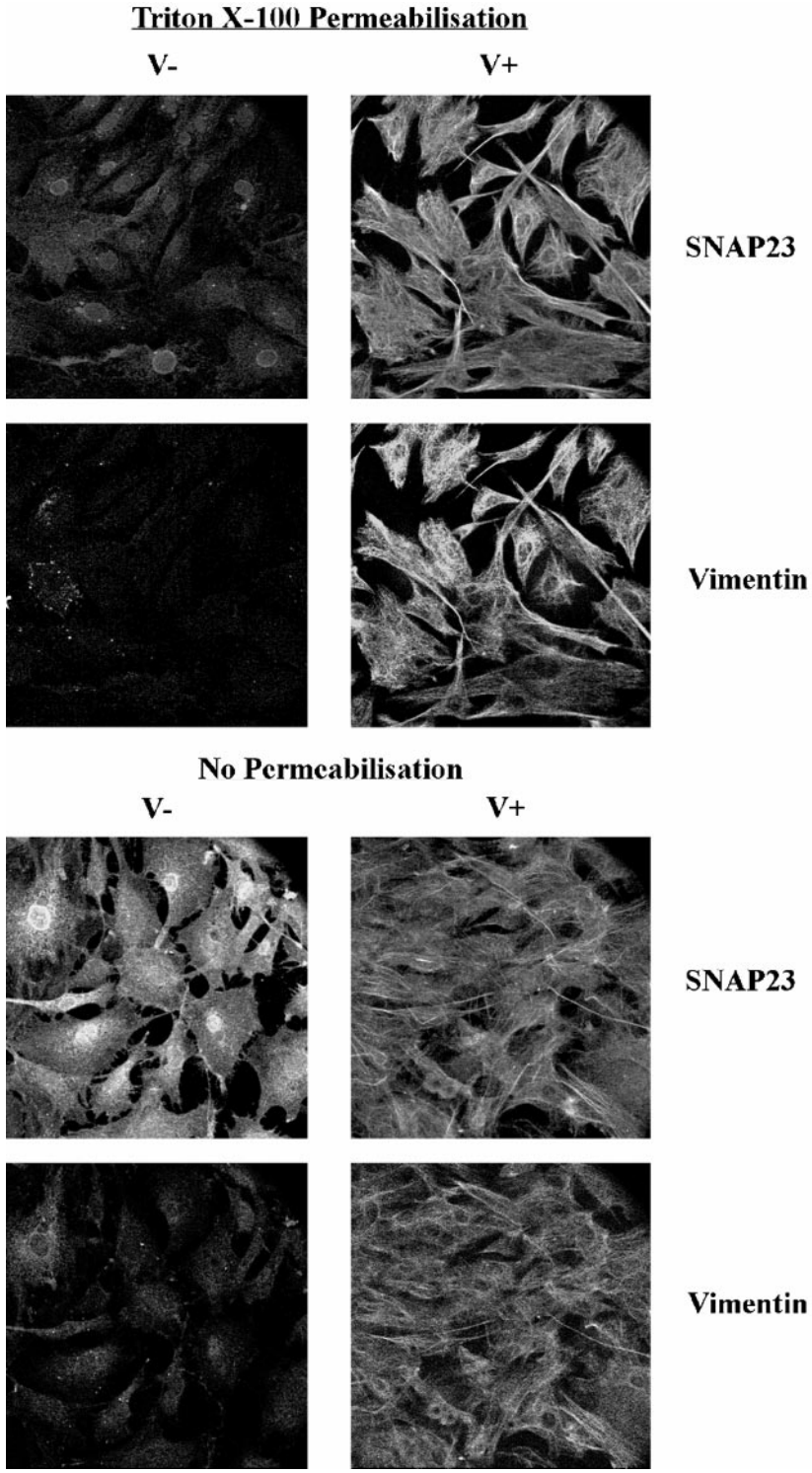


Figure 6. Vimentin-associated SNAP23 is Triton X-100 insoluble. V+ and V− fibroblasts were permeabilized with Triton X-100 (Triton X-100 prepermeabilization) or not (no prepermabilization) before methanol fixation, double-stained for SNAP23 and vimentin, and observed on the confocal microscope by using the same parameters in all cases. The images were then treated equally. SNAP23 immunoreactivity is extracted by Triton X-100 prepermeabilization in V− cells but not in V+ cells.

al., 2000). Alternatively, the vimentin-associated reservoir of SNAP23 may regulate membrane traffic only in a yet uncovered signal transduction or cell-specific functional context. Indeed, vimentin together with actin are present in podosomes, a specialized type of focal adhesions found in macrophages (Correia *et al.*, 1999). Interestingly, dynamin and endophilin, two proteins involved in endocytosis, are also found in podosomes (Ochoa *et al.*, 2000).

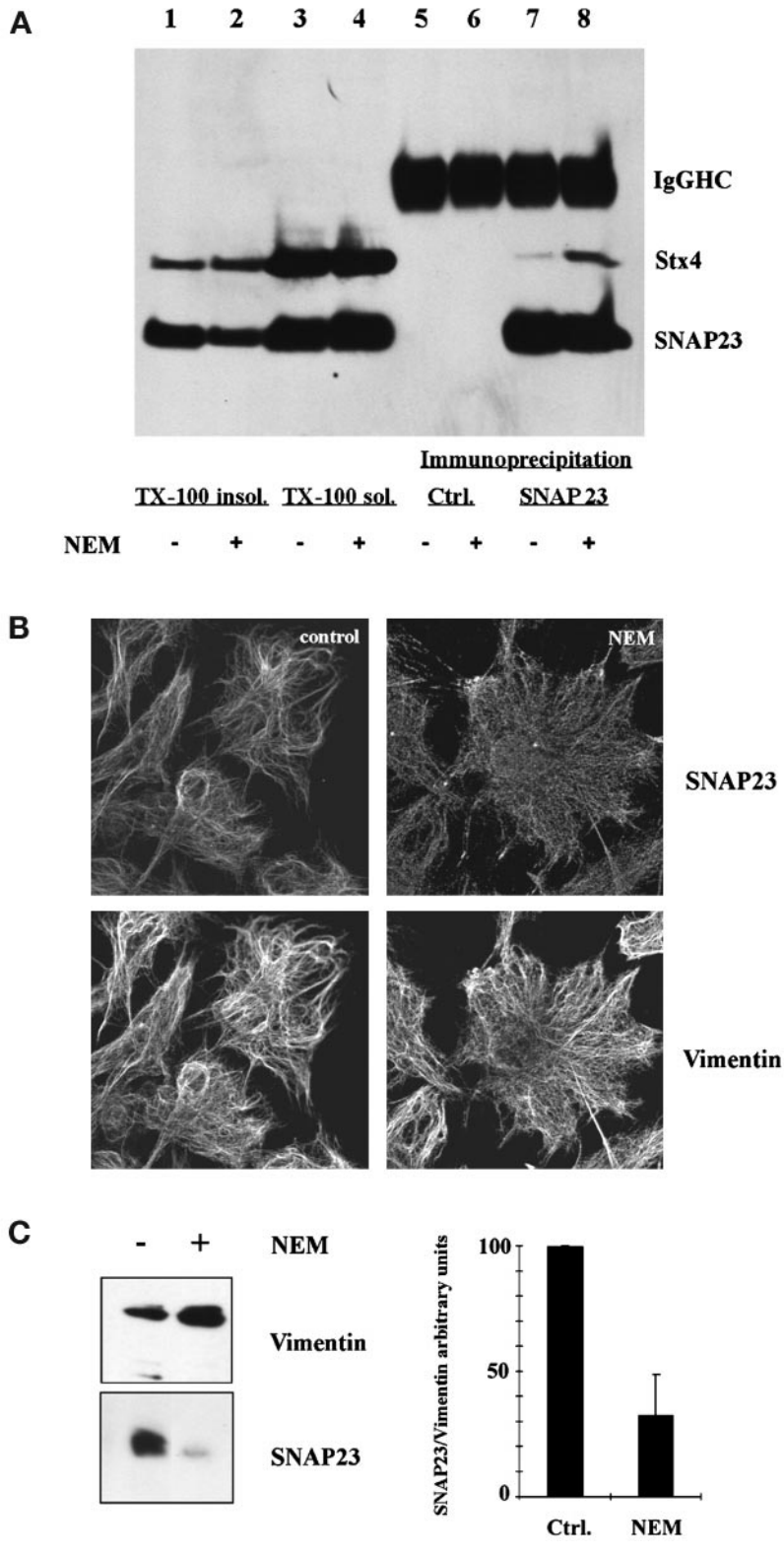


Figure 7. NEM induces the redistribution of SNAP23. (A) NEM treatment of human fibroblasts leads to a decrease of the Triton X-100 insoluble (TX-100 insol.) pool of SNAP23 and an increased recovery of syntaxin 4 (Stx4) in SNAP23 immunoprecipitates. Immunoprecipitation was carried out from the Triton X-100 soluble (TX-100 sol.) fraction with sheep anti-rabbit magnetic beads coated with control rabbit immunoglobulins (Ctrl.) or anti-SNAP23 antibodies. The amounts loaded in each condition correspond to an equivalent cell number. Control rabbit immunoglobulins did not immunoprecipitate SNAP23 or Stx4. (Immunoglobulin heavy chains, IgG HC.) (B) SNAP23 localizes to the plasma membrane following NEM treatment. Human fibroblasts were treated (NEM) or not (Ctrl.) with NEM and double-stained for SNAP23 and vimentin. (C) NEM treatment decreases the vimentin-associated pool of SNAP23. Human fibroblasts were homogenized with a cell cracker and vimentin filaments were immunoisolated. Bars represent the average of three independent experiments. In each experiment, the intensity of the vimentin and SNAP23 bands was quantified. The amount of SNAP23 was expressed as a ratio of the amount of vimentin recovered in each sample. In each experiment, the SNAP23/vimentin ratio was set to 100 arbitrary units for control cells.

The association of a pool of t-SNARE to cytoskeletal structures is not specific to SNAP23. Indeed, SNAP25 is also partially Triton X-100 insoluble in neuronal cells (Chin *et al.*, 2000). In these cells, SNAP25 and SNIP, partially colocalize with cortical actin but Chin *et al.* (2000) have not compared the localization of SNAP25 with that of intermediate filaments and have not demonstrated direct association of SNAP25 to actin. Thus, it is not yet clear whether the insoluble pool of SNAP25, like that of SNAP23, is associated with intermediate filaments. Future studies should clarify this point and address the functional relevance of the cytoskeletal pools of SNAP23 and SNAP25.

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