Endogenous plasma membrane t-SNARE syntaxin 4 is present in rab11 positive endosomal membranes and associates with cortical actin cytoskeleton

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Abstract **Membrane fusion requires the formation of a complex between a vesicle protein (v-SNARE) and the target membrane proteins (t-SNAREs). Syntaxin 4 is a t-SNARE that, according to previous overexpression studies, is predominantly localized at the plasma membrane. In the present study endogenous syntaxin 4 was found in intracellular vesicular structures in addition to regions of the plasma membrane. In these vesicular structures syntaxin 4 colocalized with rab11, a marker of recycling endosomes. Furthermore, syntaxin 4 colocalized with actin at the dynamic regions of the plasma membrane. Treatment with *N*-ethylmaleimide, the membrane transport inhibitor, caused an increased accumulation of syntaxin 4/rab11 positive vesicles in actin filament-like structures. Finally, purified recombinant syntaxin 4 but not syntaxin 2 or 3 cosedimented with actin filaments in vitro, suggesting direct interaction between these two proteins. Taken together, these data suggest that syntaxin 4 regulates secretion at the actin-rich areas of the plasma membrane and may be recycled through rab11 positive intracellular membranes.**

Key words: Syntaxin 4; Actin; rab11

1. Introduction

Membrane traffic is needed for the synthesis and processing of proteins and lipids as well as the maintenance of the compartmentalization of the cell. Trafficking of intracellular membranes involves the budding of vesicles from the donor membrane and the fusion of vesicles with their respective target membranes. Several proteins are involved in membrane fusion events, including the *N*-ethylmaleimide (NEM)-sensitive factor (NSF), soluble NSF attachment proteins (SNAPs) and SNAP receptors (SNAREs). The pairing of target SNARE (t-SNARE) with the vesicle SNARE (v-SNARE) pulls the membranes together and is possibly the driving force in the mixing of the lipid bilayers. After membrane fusion the disassembly of the SNARE complexes is mediated by SNAPs, and the ATPase activity of NSF [1,2]. Released v-SNAREs are recycled back to their membrane of origin [3].

A unique set of SNAREs is located in distinct intracellular compartments. Therefore it was originally thought that this would provide the specificity of the vesicle targeting with the target membrane. However, it was later found that a SNARE can be involved in several different fusion steps mediated by variable sets of SNAREs [4,5]. In vitro experiments have demonstrated that SNAREs do not show high specificity in forming complexes with each other [6]. Clearly other proteins are needed to mediate the specificity of vesicle targeting and fusion. Those proteins include inter alia small Rab GTPases, Sec1 proteins, and complexins [7–9].

Syntaxins belong to a t-SNARE family of which over a dozen have already been cloned [10]. Overexpression studies have suggested that syntaxins 1, 2, 3, and 4 are located predominantly at the plasma membrane. Syntaxin 1 is predominantly expressed in brain tissue and is thought to function specifically in neurotransmitter release, whereas syntaxins 2, 3, and 4 have a wider tissue distribution [11].

In this study, we investigated subcellular localization of endogenous syntaxin 4 in normal rat kidney (NRK) cells. In addition to regions of the plasma membrane, syntaxin 4 was found to localize in rab11 positive intracellular vesicular structures. At the plasma membrane syntaxin 4 was associated with cortical actin cytoskeleton.

2. Materials and methods

2.1. Materials

NEM, cytochalasin D and monoclonal mouse anti-α-tubulin were from Sigma (St. Louis, MO, USA) and Oregon Green 488 phalloidin from Molecular Probes (Eugene, OR, USA). Mouse monoclonal early endosome antigen 1 (EEA1) antibody was from Transduction Laboratories (Lexington, KY, USA). Lyso-bis-phosphatidic acid antibody was a gift from Professor Jean Gruenberg (University of Geneve, Switzerland). An affinity-purified goat polyclonal rab11 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lissamine rhodamine (LRSC)-conjugated, rhodamine Red™-X-conjugated and fluorescein (FITC)-conjugated secondary antibodies were purchased from Jackson Immuno Research (West Grove, PA,

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Abbreviations: ADF, actin monomer binding protein cofilin/actin depolymerizing factor; NEM, *N*-ethylmaleimide; EEA1, early endosome antigen 1; NRK, normal rat kidney; NSF, *N*-ethylmaleimide-sensitive fusion factor; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; TGN, trans-Golgi network

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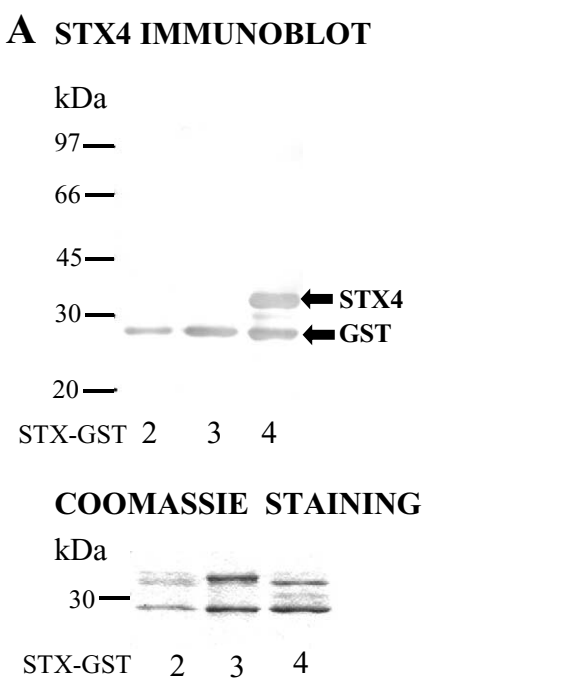

Fig. 1. Characterization of the syntaxin 4 antibodies. A: Syntaxin 4 antiserum specifically recognized the cytosolic domain of syntaxin 4 (33 kDa) but not the cytosolic domain of syntaxin 2 or syntaxin 3 (both 33 kDa). Equal amounts of syntaxin 2, 3, and 4 cytosolic domain GST fusion proteins were incubated with thrombin to cleave off the GST (27 kDa, indicated with arrow) and analyzed by SDS–PAGE, transferred to nitrocellulose filters and probed with affinity-purified syntaxin 4 antiserum followed by alkaline phosphatase-conjugated secondary antibodies (upper panel). Coomassie blue staining of the thrombin cleaved recombinant proteins (lower panel). B: Recombinant syntaxin 4 blocked efficiently labelling in Western blotting with syntaxin 4 antiserum. The Western blotting of NRK cell extract with affinity-purified syntaxin 4 antiserum and horseradish peroxidase-conjugated secondary antibodies (lane 1). Antiserum was preincubated with recombinant syntaxin 2 (lane 2), syntaxin 3 (lane 3) or syntaxin 4 (lane 4). C: Specificity of syntaxin 4 antiserum in immunofluorescence. The cells were fixed with lysine-periodate-paraformaldehyde and permeabilized with 0.05% saponin. Syntaxin 4 was visualized using affinity-purified syntaxin 4 antiserum and LRSC-conjugated goat anti-rabbit IgG. Preincubation of the antiserum with recombinant syntaxin 4 abolished the immunofluorescence staining. Bars, 10 μm.

USA). All other reagents were of analytical grade and were obtained from commercial sources.

2.2. Production of fusion proteins and antibodies

The cytosolic domains of mouse syntaxin 2 (1–265), rat syntaxin 3 (1–263) and rat syntaxin 4 (1–272) were cloned into the pGEX 2T vector (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and purification of glutathione-*S*-transferase (GST) fusion proteins was performed according to the manufacturer's instructions (Amersham Pharmacia Biotech AB). The antiserum for syntaxin 4-GST protein was produced in New Zealand White rabbits. Actin monomer binding protein cofilin/actin depolymerizing factor (ADF) antibody was obtained by immunizing two guinea pigs with purified recombinant mouse ADF [12].

2.3. Immunocytochemistry

NRK cells were grown as confluent monolayers on coverslips in Dulbecco's modified Eagle's (DME) medium supplemented with 2 mM L-glutamine, 100 U of penicillin, 100 mg of streptomycin sulfate

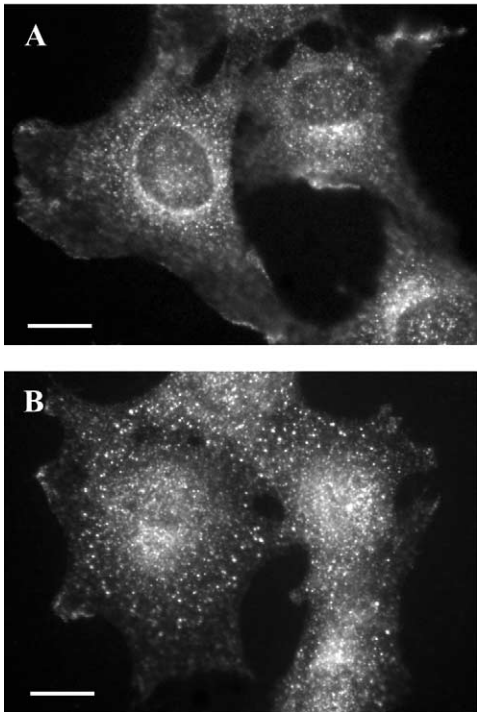

Fig. 2. The intracellular syntaxin 4 labelling does not represent newly synthesized syntaxin 4 protein. The NRK cells were treated with 50 μg/ml cycloheximide for 2 h (B). A shows control untreated NRK cells. The cells were fixed with lysine-periodate-paraformaldehyde and permeabilized with 0.05% saponin. Syntaxin 4 was visualized using affinity-purified syntaxin 4 antiserum and LRSC-conjugated goat anti-rabbit IgG. Bars, 10 μm.

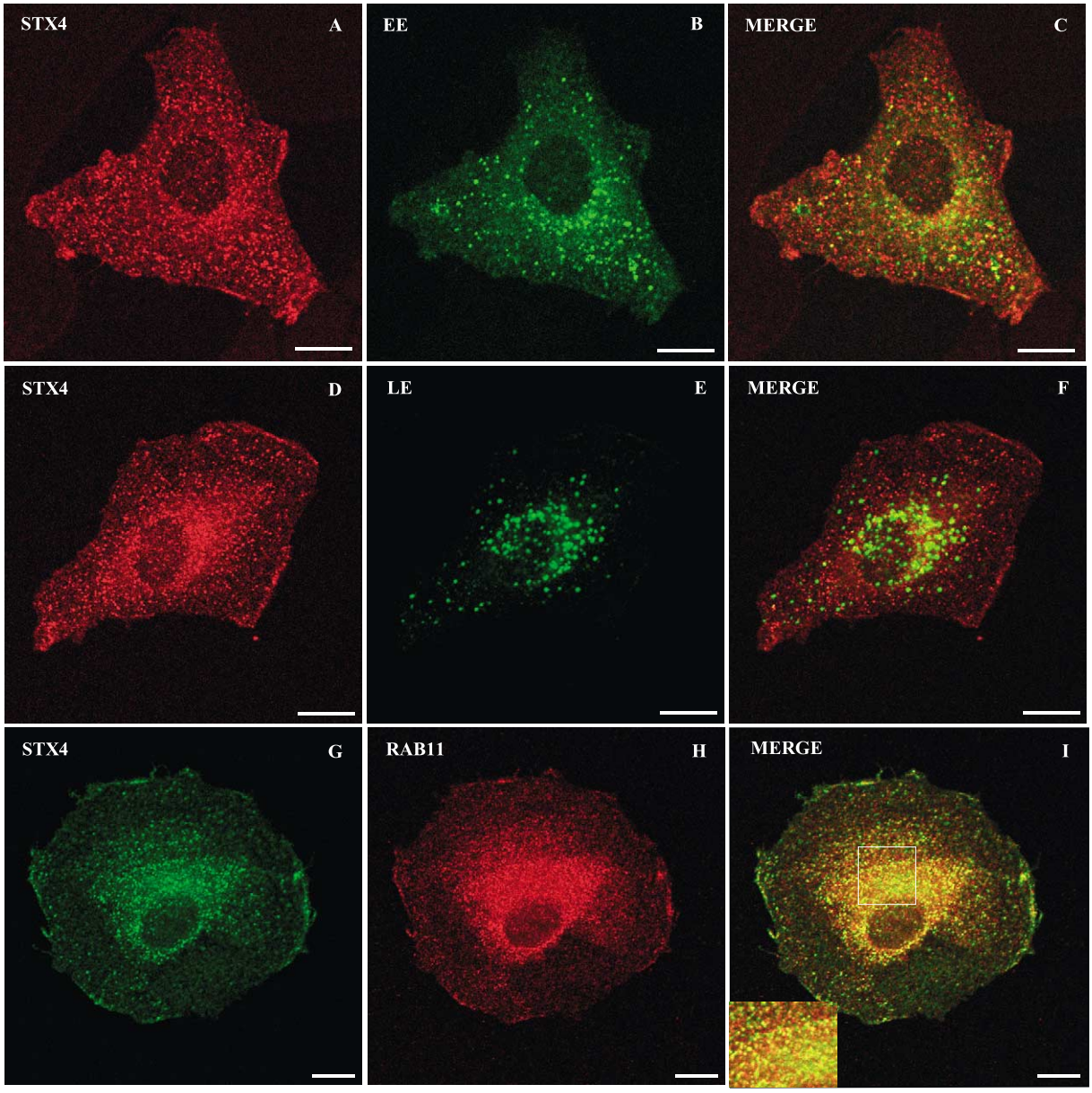

Fig. 3. Syntaxin 4 positive vesicular structures do not colocalize with an early endosome marker, EEA1, or a late endosome marker, lyso-bisphosphatidic acid, but did colocalize with a recycling endosome marker, rab11. Syntaxin 4 was visualized using affinity-purified syntaxin 4 antiserum and LRSC- or FITC-conjugated goat anti-rabbit IgG (A, D, G). EEA1 was visualized using mouse monoclonal antibody and FITC-conjugated goat anti-mouse IgG (B). Late endosomes (LE) were visualized using mouse monoclonal antibody and FITC-conjugated goat anti-mouse IgG (E). Rab11 was visualized using goat polyclonal IgG and rhodamine Red™-X-conjugated donkey anti-goat IgG (H). The yellow color in merged images (C, F, I) releaves the colocalization. Confocal fluorescence images were viewed using Leica SP1 microscope system. Bars, 10 μm.

per ml, and 10% (v/v) fetal calf serum (Biological Industries, Beit Haemek, Israel). The cells were fixed with lysine-periodate-paraformaldehyde [13] and permeabilized with 0.05% saponin. Conventional fluorescence images were viewed using an Olympus AX70 fluorescence microscope 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 images were recorded using a laser scanning Leica SP1 confocal microscope.

2.4. Actin filament cosedimentation assays

The assay was performed as previously described [12]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out by using the buffer system of Laemmli [14]. Rabbit muscle actin was prepared from acetone powder as described in Pardee and Spudich [15].

3. Results

3.1. Endogenous syntaxin 4 colocalizes with rab11 positive membranes

Previously, overexpression studies have suggested that syntaxins 1, 2, 3, and 4 are primarily localized at the plasma membrane [11]. However, the subcellular localization of endogenous syntaxins has not been reported so far. In this study we investigated the localization of endogenous syntaxin 4. Since syntaxins 2, 3 and 4 are highly homologous to each other, we first determined the specificity of the antiserum. The affinity-purified syntaxin 4 antiserum recognized the cytosolic domain of syntaxin 4 but did not cross-react with the

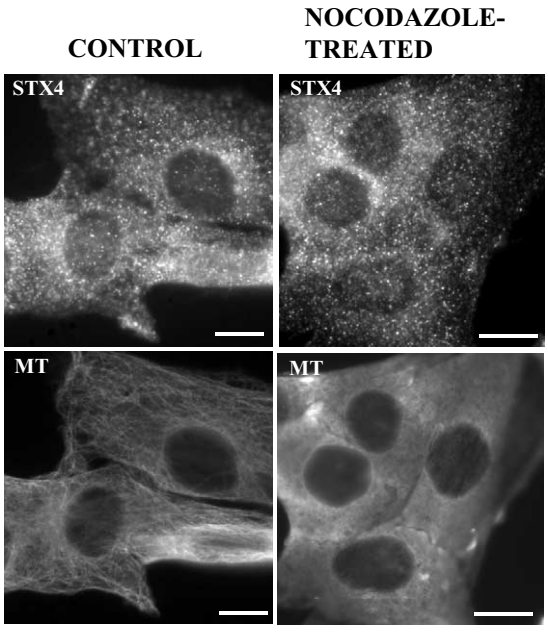

Fig. 4. Syntaxin 4 positive membranes do not codistribute with microtubules. Microtubules (MT) were depolymerized by incubating NRK cells on ice for 5 min and repolymerization was prevented with 10 μM nocodazole. The cells were fixed, permeabilized and then stained with affinity-purified syntaxin 4 antiserum and LRSC-conjugated goat anti-rabbit IgG as well as mouse monoclonal anti-α-tubulin and FITC-conjugated goat anti-mouse IgG. Bars, 10 μm.

cytosolic domain of syntaxin 2 or 3 in Western blotting (Fig. 1A). The immunoblotting of NRK cell extract with anti-syntaxin 4 antibodies produced a single band migrating at the molecular mass of 33 kDa corresponding well with the calculated molecular mass of 34 kDa (Fig. 1B). When syntaxin 4 antiserum was preincubated with recombinant syntaxin 2, 3 or 4 protein only syntaxin 4 blocked 33 kDa band. This confirms that our antiserum specifically recognizes endogenous syntaxin 4. Affinity-purified syntaxin 4 antiserum stained intracellular vesicular structures and regions of the plasma membrane in NRK cells (Fig. 1C). Immunofluorescence staining was greatly attenuated when antiserum was preincubated with recombinant syntaxin 4 but was unaffected by preincubation with recombinant syntaxin 2 or 3 (Fig. 1C).

Since syntaxin 4 had been previously localized at the plasma membrane it was considered possible that the syntaxin 4 positive intracellular vesicles were constitutive exocytic vesicles carrying syntaxin 4 as cargo. To study this, we treated cells with cycloheximide for 2 h to deplete newly synthesized proteins from the intracellular membranes (Fig. 2B). This treatment did not abolish the intracellular vesicular syntaxin 4 labelling, demonstrating that the syntaxin 4 labelling in intracellular membranes did not represent the newly synthesized syntaxin 4 on its way to the plasma membrane.

We further characterized these vesicles by using indirect immunofluorescence microscopy. The syntaxin 4 vesicles did not colocalize with an early endosome marker, EEA1, or a late endosome marker, lyso-bis-phosphatidic acid (Fig. 3A–F). However, syntaxin 4 colocalized extensively with recycling endosome marker rab11 both in the intracellular vesicles and at the plasma membrane (Fig. 3G–I).

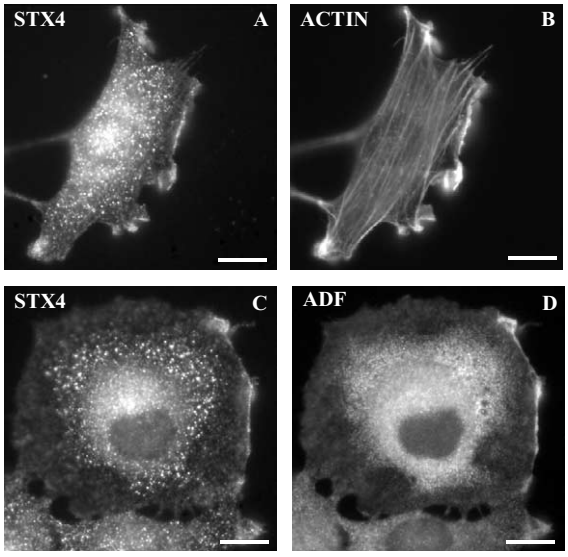

Fig. 5. Syntaxin 4 staining coincides with actin at cortical regions. The cells were fixed, permeabilized and then double stained with affinity-purified syntaxin 4 antiserum and LRSC-conjugated goat anti-rabbit IgG (A, C) as well as Oregon Green phalloidin (B) or guinea pig anti-α-ADF and FITC-conjugated donkey anti-guinea pig IgG (D). Bars, 10 μm.

3.2. *Syntaxin 4 associates with the cortical actin cytoskeleton*

Since rab11 positive membranes are a part of the endosomal compartment and constitutive exocytosis and since these compartments are maintained by microtubule-mediated membrane traffic [16], we investigated the role of microtubules in syntaxin 4 localization. Microtubules were depolymerized with cold treatment and repolymerization was prevented with nocodazole. The disruption of microtubules did not affect the localization of endogenous syntaxin 4, suggesting that interactions with microtubules do not contribute to the subcellular localization of syntaxin 4 vesicles (Fig. 4).

Little is known about the role of the actin cytoskeleton in exocytosis in animal cells. However, actin cables are essential in polarized growth and transport in yeast [17]. Actin is also a component of an exocytic complex, the exocyst, that has recently been suggested to be involved in vesicle targeting in mammalian cells as well [18]. We therefore investigated the distribution of actin filaments and syntaxin 4 in NRK cells. Syntaxin 4 colocalized with actin filament structures at the plasma membrane (Fig. 5A, B). Similarly, syntaxin 4 and

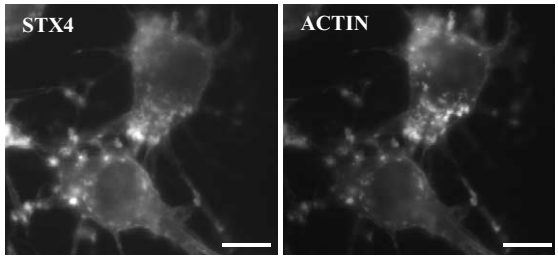

Fig. 6. Syntaxin 4 staining is colocalized with actin containing aggregates after depolymerization of actin filaments. The NRK cells were treated with 10 μM cytochalasin D for 30 min to disrupt the actin filaments. The cells were fixed, permeabilized and then stained with syntaxin 4 antiserum and LRSC-conjugated goat anti-rabbit IgG as well as Oregon Green phalloidin. Bars, 10 μm.

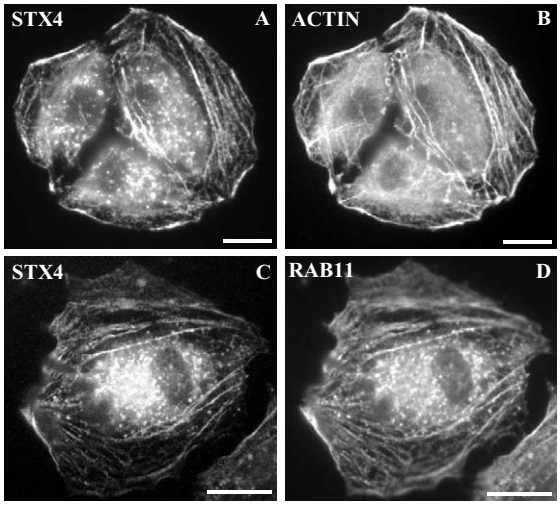

Fig. 7. Syntaxin 4 staining colocalized with actin filaments and rab11 staining after treatment with NEM. NRK cells were treated with 1 mM NEM for 15 min and then further incubated for 2 h without NEM. The cells were fixed, permeabilized and then double stained with affinity-purified syntaxin 4 antiserum and LRSC-conjugated goat anti-rabbit IgG (A, C) as well as Oregon Green phalloidin (B) or goat polyclonal rab11 antibody and rhodamine Red™-X-conjugated donkey anti-goat IgG (D). Bars, 10 μm.

ADF colocalized at the cortical actin cytoskeleton (Fig. 5C, D). ADF is a ubiquitous actin filament depolymerizing protein that localizes to the highly dynamic regions of the actin cytoskeleton [19]. The association of syntaxin 4 with actin filaments was further studied by disrupting the actin cytoskeleton with cytochalasin D, a fungal drug that prevents actin polymerization. The cytochalasin D treatment resulted in the disassembly of actin stress fibers and the accumulation of aberrant actin filament structures (Fig. 6). Syntaxin 4 colocalized with these actin filament structures, further suggesting that syntaxin 4 vesicles interact with actin filaments in NRK cells.

3.3. *Syntaxin 4 vesicles accumulate on actin filaments in the presence of NEM*

Next we investigated how the inhibition of the SNARE complex disassembly with NEM affects the localization of syntaxin 4 positive vesicles. NEM is a sulfhydryl alkylating reagent, which has been reported to inactivate the membrane fusion component NSF [20]. We have previously shown that NEM treatment stopped membrane transport from the trans-Golgi network (TGN) to the plasma membrane [21]. Indirect immunofluorescence microscopy showed that after NEM treatment the majority of the syntaxin 4 positive staining had moved to actin filament-like structures. Indeed, the double staining experiments showed that these structures colocalized with actin filaments (Fig. 7A, B). This suggests that syntaxin 4 positive vesicles associate with actin filaments in NEM-treated cells. Since syntaxin 4 positive vesicles in untreated cells contain rab11 (Fig. 3), we studied whether the rab11 staining would also relocate to the actin filaments together with syntaxin 4 in the presence of NEM. Interestingly, syntaxin 4 and rab11 still colocalized in the presence of NEM, both markers being now associated with actin filaments (Fig. 7C, D).

Our experiments suggest that syntaxin 4 associates with actin filaments either directly or indirectly. Therefore, interaction of purified recombinant syntaxin 4 with actin filaments was examined using a cosedimentation assay. The cytosolic domain of syntaxin 4, but not the cytosolic domains of syntaxin 2 or 3, cosedimented with actin filaments in vitro (Fig. 8A). Whereas approximately 10% of the recombinant syntaxin 4 sedimented under these conditions in the absence of actin, approximately 40% of the syntaxin 4 cosedimented with 2 μM actin filaments (Fig. 8B). However, it is important to note that the amount of cosedimented syntaxin 4 did not increase with higher actin concentrations. This suggests that the recombinant syntaxin 4 perhaps exists in two different conformations [22], and only approximately 40–50% of the syntaxin 4 used is in a conformation that is able to bind to actin.

4. Discussion

In the present study we have shown that endogenous plasma membrane t-SNARE syntaxin 4 is not predominantly localized in the plasma membrane. In addition to the plasma membrane localization, syntaxin 4 is associated with the rab11 positive membranes, which in other cells are shown to include the TGN, the recycling endosome, exocytic vesicles, the plasma membrane and H^+-K^+-ATPase-rich tubulovesicular compartment that underlies the plasma membrane [23–25]. It is therefore possible that syntaxin 4 cycles between the plasma membrane and the recycling endosomes.

We also found that syntaxin 4 is associated with actin filaments at the cell cortex since syntaxin 4 staining colocalized with actin and ADF staining at the plasma membrane. Our in vitro actin cosedimentation experiments suggested that syntaxin 4 binds directly to actin, whereas the homologous syntaxins 2 and 3 did not associate with actin filaments in vitro. Furthermore, when actin filaments were disassembled with cytochalasin D, syntaxin 4 accumulated with actin into characteristic aggregates. After NEM treatment, syntaxin 4 and

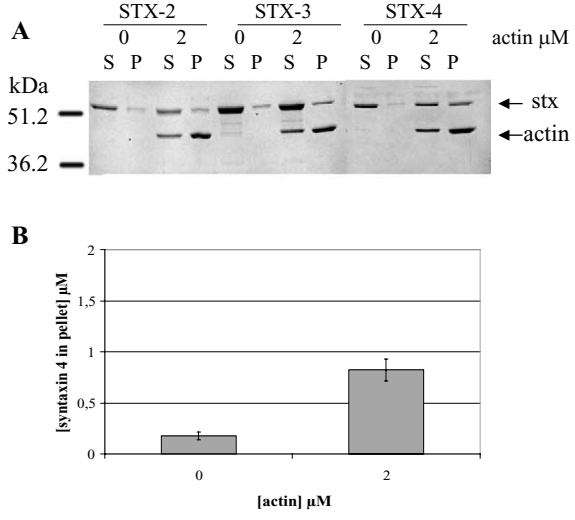

Fig. 8. Association of syntaxin 4 with actin filaments in vitro. A: Syntaxins (2 μM) were mixed with 0 or 2 μM actin at pH 7.5 and actin filaments were sedimented by centrifugation. Equal proportions of supernatants and pellets were loaded on SDS–PAGE gels. A significant proportion of the syntaxin 4 sediments with actin filaments whereas syntaxins 2 and 3 do not. B: The amount of syntaxin 4 in the pellet fraction in the presence of 0 or 2 μM actin was quantified from three independent experiments.

rab11 staining accumulated in the actin filament bundles (Fig. 7). However, these actin bundles are morphologically distinct from stress fibers and represent abnormal actin filament structures. It is important to note that NEM is known to modify actin in vitro [26], and therefore the abnormal actin filament structures seen in NEM-treated cells may result from altered assembly kinetics of actin filaments.

Actin cables are essential for polarized growth and secretion in yeast [17]. In animal cells, on the other hand, microtubules mediate long-range transport to the cell surface and short-range transport is mediated by actin [27,28]. In yeast, an actin binding macromolecular complex, the exocyst, has been suggested to play a role in the targeting of secretory vesicles to the plasma membrane, especially during the budding of the daughter cell [29]. Recently, the exocyst complex has been found to function in animal cells as well. The exocyst complex contains eight subunits and its components are conserved from yeast to mammals. Similarly to yeast, in neuronal cells exocyst complex promotes neurite outgrowth by targeting the secretory vesicles to the specific domains of the plasma membrane [18]. In polarized epithelial cells, targeting to the basolateral plasma membrane is possibly mediated by the exocyst complex [30]. Syntaxin 4 has been found to localize in the basolateral membrane [31] and therefore it has been speculated that syntaxin 4 together with the exocyst complex might mediate basolateral transport [32]. In addition to this, α-fodrin, which forms meshworks beneath the plasma membrane with F-actin, has been reported to associate with syntaxins 1, 3 and 4 [33]. This would suggest that syntaxin 4 may regulate traffic to the membrane ruffles or other highly dynamic areas at the plasma membrane.

In addition to exocytosis actin has been reported to be involved in endocytosis. The actin cytoskeleton promotes internalization of ligands and trafficking along endosomal pathway [34,35]. There is also increasing evidence that actin cytoskeleton has a role in the movement of membrane elements such as lysosomes [36]. It has been suggested that actin connected movement is a result from dynamic of the actin cytoskeleton or function of actin-associated molecular motors [37–40].

The association of t-SNARE, syntaxin 4 with actin filaments is a novel finding. Although at first sight this is a surprising result another SNARE, SNAP-23, has been reported to be associated with vimentin intermediate filaments [41]. The vimentin-associated pool of SNAP-23 was reported to be a reservoir, which supplies SNAP-23 to the plasma membrane fusion machinery. In the presence of NEM, SNAP-23 was shown to dissociate from the vimentin filament and form a complex with syntaxin 4 [41]. In our study, in contrast, NEM treatment was found to promote the actin-associated pool of syntaxin 4. However, some of the syntaxin 4 stayed at the plasma membrane after NEM treatment. Therefore syntaxin 4 and SNAP-23 can form a complex at the plasma membrane where it has been previously reported to reside [42]. How these two pools of SNAREs are regulated is an interesting subject of future studies.

In conclusion, our results indicate that syntaxin 4 is located both at the plasma membrane and the recycling endosomes. Furthermore, our data demonstrate that syntaxin 4 is associated with the cortical actin cytoskeleton, and may promote membrane traffic to the highly dynamic regions of the plasma membrane.

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