Sorting nexin 4 and amphiphysin 2, a new partnership between endocytosis and intracellular trafficking

Corinne Leprince[1,*], Erwan Le Scolan[1], Brigitte Meunier[1], Vincent Fraisier[2], Nathalie Brandon[1], Jean De Gunzburg[1] and Jacques Camonis[1]

[1]INSERM U528, [2]CNRS UMR144, Institut Curie Section de Recherche, 26 rue d'Ulm, 75248 Paris Cedex 05, France

*Author for correspondence (e-mail: Corinne.Leprince@curie.fr)

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Summary

Endocytosis is a regulated physiological process by which membrane receptors and their extracellular ligands are internalized. After internalization, they enter the endosomal trafficking pathway for sorting and processing. Amphiphysins consist of a family of proteins conserved throughout evolution that are crucial elements of the endocytosis machinery in mammalian cells. They act as adaptors for a series of proteins important for the endocytic process, such as dynamin. In order to improve our knowledge of amphiphysin function, we performed a two-hybrid screen with the N-terminal part of murine amphiphysin 2 (residues 1-304). One of the interacting clones corresponded to sorting nexin 4 (SNX4), a member of the SNX family of proteins which are suspected to regulate vesicular trafficking. This interaction was confirmed in vivo by co-immunoprecipitation. Immunofluorescence analysis revealed that amphiphysin 2 might bind reticulo-vesicular structures present throughout the cell body and be associated with SNX4 on these structures. In an endocytosis assay, overexpressed C-terminal or full-length SNX4 was able to inhibit transferrin receptor endocytosis as efficiently as the SH3 domain of amphiphysin 2. At lower levels of expression, SNX4 colocalized with transferrin-containing vesicles, some of which were also positive for amphiphysin 2. These results indicate that SNX4 may be part of the endocytic machinery or, alternatively, that SNX4 may associate with key elements of endocytosis such as amphiphysin 2 and sequester them when overexpressed. The presence of amphiphysin 2 on intracellular vesicles and its interplay with SNX4, which is likely to take part in intracellular trafficking, suggest that amphiphysin 2 is not only a regulator of the early steps of endocytosis. It could also play a role at the surface of the endocytic vesicle that has just been formed and of the future endosomes, in order to regulate intracellular trafficking.

Key words: Amphiphysin, Sorting nexin, Endocytosis, Trafficking, Endosome

Introduction

Amphiphysins are molecules known to control the early steps of endocytosis in mammalian cells (Wigge and McMahon, 1998; Slepnev and De Camilli, 2000). In humans, they are encoded by at least two genes: *AMP1* on chromosome 7 (Yamamoto et al., 1995; Lichte et al., 1992) and *AMP2* on chromosome 2 (Leprince et al., 1997). The two proteins are 41% identical in their amino acid sequence and share a number of structural features such as a C-terminal SH3 domain and N-terminal coiled-coil regions (Wigge and McMahon, 1998). Amphiphysin 1 is expressed mostly in brain (Lichte et al., 1992; De Camilli et al., 1993). Amphiphysin 2 is more broadly distributed with high levels of mRNA splicing variations giving rise to an extended family of amphiphysin 2 isoforms (Leprince et al., 1997; Butler et al., 1997; Sakamuro et al., 1996; Ramjaun et al., 1997; Sparks et al., 1996; Gold et al., 2000; Tsutsui et al., 1997). The central region of the protein is the main site of variations between isoforms. The longest amphiphysin 2 (named Amp2a, Bramp2 for 'Brain amphiphysin 2′, or N-Amp2 for neuronal Amp2) contains a long central insert that interacts with clathrin and endophilin, two key molecules for the formation of endocytic vesicles (Ramjaun and McPherson, 1998; Micheva et al., 1997). Amphiphysin 1 also binds the heavy chain of clathrin, as well as the alpha subunit of the AP2 adaptor complex via DPF/W motifs (MacMahon et al., 1997; Owen et al., 1999). Through their N-terminal coiled-coil domains, amphiphysins can oligomerize in homo- or hetero-dimers (Ramjaun et al., 1999) and an even more distal N-terminal site is supposed to act on the curvature of lipid membranes (Takei et al., 1999; Farsad et al., 2001). Thus, amphiphysins are major partners for a number of molecules involved in clathrin-dependent endocytosis.

Another crucial element of the endocytosis machinery, dynamin, should be added to the list of amphiphysin-interacting molecules. In this latter case, the binding is dependent on the SH3 domain of amphiphysins and a proline-rich region of dynamin (David et al., 1996; Grabs et al., 1997; Owen et al., 1998). Even though the biochemical basis of dynamin function needs to be clarified, dynamin is essential for the membrane fission reaction leading to the formation of an individualized endocytic vesicle. In agreement with this model, microinjection of the SH3 domain of amphiphysin 1 into the lamprey synapse (Shupliakov et al., 1997) or transient transfection of the SH3 domain of amphiphysin 1 (Wigge et

al., 1997) or amphiphysin 2 (Owen et al., 1998) into fibroblasts inhibited clathrin-dependent endocytosis of membrane receptors by sequestration of dynamin. The same SH3 domain is responsible for the binding of amphiphysin 2 to the proline-rich domain of synaptojanins (synaptojanin 1, 2B1 and 2B2) (Nemoto et al., 2001), which are phosphoinositide phosphatases crucial for clathrin uncoating of the vesicles. Thus, amphiphysins provide a link between the clathrin-coated pits that precede clathrin-dependent endocytosis and the newly formed endocytic vesicle, by influencing either directly or indirectly, membrane curvature, membrane fission and/or vesicle uncoating.

Following endocytosis, membrane proteins and their ligands are transported to early endosomes and sorted in three possible directions: directly recycling to the cell surface, transported to perinuclear recycling endosomes, or transported to lysosomes via late endosomes (Kirchausen, 2000). The sorting and transport processes within the endosomal compartment are highly regulated and rely on a molecular machinery that still needs to be fully defined.

Sorting nexins (SNX) are a family of proteins present in a number of organisms from human to *Caenorhabditis elegans* and yeast. Human SNX1 was first characterized as an epidermal growth factor (EGF) receptor interacting molecule in a two-hybrid screen with a cytoplasmic portion of the receptor (Kurten et al., 1996). Later reports characterized other SNX family members by homology with SNX1, and showed that they interact with different kinds of membrane receptors: tyrosine kinase receptors, serine-threonine kinase receptors of the TGFβ family, transferrin receptor and leptin receptor (Haft et al., 1998; Parks et al., 2001). The binding capacities for these different kinds of receptors vary from one SNX to another. Presently, the SNX family is still expanding (more than 20 members in humans), essentially through searches in sequence databases.

A number of SNX molecules have coiled-coil domains in the C-terminal part of the molecule but the most obvious structural signature is the presence of a 'Phox homology' (PX) domain, which was initially defined in the $P47^{phox}$ and $p40^{phox}$ subunits of NADPH oxidase (Prehoda et al., 2001). PX domains were recently shown to bind phosphoinositides, with different PX domains having different phosphoinositide specificities. Point mutations in the PX domain that affect phosphoinositide binding also affect membrane attachment of SNX (Prehoda and Lim, 2001; Xu et al., 2001; Kanai et al., 2001). Thus, PX domains join the family of phosphoinositide-binding modules, including PH domains, FYVE domains and ENTH domains, that participate in membrane anchorage. In addition, it remains possible that PX domains have a general low affinity SH3-binding capacity by a conserved poly-proline motif (Hiroaki et al., 2001).

The function of SNX molecules is more documented in yeast cells than in mammalian cells. The SNX1 yeast orthologue, Vps5p, is essential for the correct targeting of carboxypeptidase Y from the trans-Golgi network (TGN) to a pre-vacuolar/endosomal compartment. Vps5p is a component of a multimeric complex containing other subunits – Vps26p, Vps29p, Vps35p and Vps17p. This complex called 'retromer' is believed to act as a membrane coat in which SNX molecules can either recruit cargo proteins or participate in the formation of the vesicle (Horazdovsky et al., 1997). It remains to be established if similar roles can be fulfilled by mammalian SNX molecules (Haft et al., 2000).

In this work, we present data showing that amphiphysin 2 and SNX4 interact, both in a yeast two-hybrid assay and in vivo in mammalian cells where they associate in the cell cytosol and on cytoplasmic vesicular structures. Overexpression of SNX4 inhibits the endocytosis of the transferrin receptor as efficiently as the SH3 domain of amphiphysin 2. At lower levels of expression, SNX4 colocalizes with transferring-containing vesicles, some of which are also amphiphysin 2 positive. Even though it is possible that SNX4 plays a role at the endocytosis step, we propose that the amphiphysin 2/SNX4 partnership is important for the control of the endosome fate, after the formation of the endocytic vesicle.

Materials and methods

Cells

Cells were grown at 37°C in a 5% CO_2 incubator, in DMEM (Life Technologies) supplemented with 10% fetal calf serum (HeLa cells) or 10% calf serum (3T3 cells). The culture medium was also supplemented with penicillin, streptomycin and glutamine (all from Life Technologies).

Antibodies

The antibodies used in this study were a mouse monoclonal anti-myc (clone 9E10; Santa Cruz, CA and Roche Meylan, France), a rabbit polyclonal anti-myc antibody (Upstate Biotechnology), mouse monoclonal anti-EEA1, anti-CD63, anti-LAMP1 and anti-calnexin antibodies (all from Becton Dickinson Transduction Laboratories, San Diego, CA), and a mouse monoclonal anti-BIN1 antibody (Upstate Biotechnology, Lake Placid, NY). The rabbit polyclonal anti-Rab11 antibody was a kind gift from J. Salamero (CNRS UMR144, Institut Curie, Paris). A previously generated rabbit polyclonal antiserum against a C-terminal part of murine Bramp2 (Leprince et al., 1997) was proven to be mouse-specific.

cDNA constructs

Different portions of mouse amphiphysin 2 cDNA were cloned by PCR in yeast expression vectors. Briefly, full length Amp2m previously described in macrophages (Gold et al., 2000) or partial cDNAs corresponding to residues 1-146, 147-410 and 1-304 of Amp2m were cloned in the pLex10 vector producing a N-terminal fusion protein with the DNA-binding domain of LexA. The full sequence of mouse Amp2m is registered in GenBank under the accession number AF068915.

Full length human SNX4 cDNA (Kurten et al., 1996) and partial cDNAs corresponding to residues 1-367 and 1-404 of SNX4 were cloned in the pGAD1318 vector, producing a N-terminal fusion protein with the activation domain of GAL4.

Full length cDNAs of mouse Amp2m, mouse Bramp2 (Leprince et al., 1997), human SNX4 and a partial cDNA corresponding to residues 368-450 of SNX4 were cloned by PCR in different vectors containing a CMV promoter for mammalian cell expression, pRK5 or pRK5myc. All constructs were verified by DNA sequencing.

Rab5 cDNA and Rab11 cDNA cloned in a GFP-fusion expression vector were kind gifts from P. Chavrier (CNRS UMR144, Institut Curie, Paris) and RGS14 cDNA cloned in pRK5myc was kindly given by S. Traver (Traver et al., 2000).

Two-hybrid screening

Two-hybrid screening was performed in the L40 yeast strain. An N-

terminal portion of murine amphiphysin 2 (residues 1-304) expressed in fusion with the LexA DNA-binding domain was used as a bait to screen a Jurkat cell two-hybrid cDNA library (Leprince et al., 1997; Vojtek et al., 1993). Yeast cells co-transformed with pLex and pGAD vectors are able to grow on leucine-and tryptophan-deficient media. Yeast clones producing proteins that interact with amphiphysin 2 were selected on media deficient in leucine, tryptophan and histidine. Interaction between the bait and a potential partner was confirmed in a beta-galactosidase assay since LacZ expression in L40 is dependent on the reconstitution of a functional LexA/GAL4 transcription factor. For each selected yeast clone, the pGAD plasmid was purified, reamplified in DH5alpha bacteria and the insert was sequenced.

Two-hybrid assays were also used for testing selected protein-protein interactions with full length or partial mouse amphiphysin 2 and human SNX cloned in pLex and pGAD vectors, respectively.

Cell line transient transfections

3T3 cells were transiently transfected with SNX4 in pRK5myc using Lipofectamine Plus according to the manufacturer's instructions (Life Technologies). For biochemistry experiments, cells were plated at 2×10^6 per dish one day before transfection, transfected with 0.4 µg of DNA and cultured for an additional 24 hours. For immunofluorescence analysis, cells were plated on coverslips at 6×10^4 per well in a 24 well-plate, transfected one day later and cultured for an additional 24 hours.

HeLa cells were transiently transfected by using either the calcium phosphate method for immunoprecipitations experiments or Exgen 500 (Upstate Biotechnology) for immunofluorescence. Briefly, for subsequent immunoprecipitation experiments, cells were plated at 1×10^6 per dish in fresh medium. One day later, cells were added with a mix of 5-10 µg DNA in calcium-containing buffer, cultured for 16 hours, washed with fresh medium and cultured for an additional 24 hours before lyses and biochemical experiments. For immunofluorescence analysis, cells were plated on coverslips at 3×10^4 per well in a 24 well-plate. DNA was mixed with Exgen 500 (Upstate Biotechnology) according to the manufacturer's instructions and added to cells. HeLa cells were cultured for additional 16-24 hours before immunofluorescence assay.

Immunoprecipitations and western blotting assays

Transfected cells were washed in cold PBS and resuspended by scrapping in cold hypotonic buffer (10 mM HEPES pH 7.5, 10 mM NaCl, 1 mM EDTA) containing a protease inhibitor cocktail (Roche) supplemented with 1 µg/ml pepstatin, 1 mM Na_3VO_4 and 50 mM NaF (all from Sigma-Aldrich). Cells were disrupted in a dounce homogeneiser and cell extracts were centrifuged at 1500 ***g*** to remove nuclei, intact cells and debris. The resulting supernatants (PNS: post nuclear supernatant) were ultracentrifuged for 30 minutes at 100,000 ***g***. The pellets containing cell membranes were washed once in hypotonic buffer and resuspended in 10 mM Tris pH 7.5, 150 mM NaCl, and 1% NP40 (all from Sigma-Aldrich). The membranes were resuspended by pipetting, and rotated for 30 minutes at 4°C in order to achieve maximum solubilization. The unsolubilized material was removed by centrifugation at 10,000 ***g*** for 30 minutes. The supernatants of the 100,000 ***g*** ultracentrifugations (cytosolic fractions) were supplemented with Tris, NaCl and NP40 in order to reach the concentrations mentioned above.

The membrane and cytosolic fractions were submitted to immunoprecipitations by incubation with anti-myc or anti-amphiphysin 2 antibody for 2 hours at 4°C. Immune complexes were recovered by using Protein A-Sepharose beads (Roche) and washed 3 times in 10 mM Tris pH 7.5, 150 mM NaCl, 1% NP40. Immunoprecipitation products were run on SDS-PAGE and transferred to nitrocellulose (Hybond ECL, Amersham-Pharmacia, Orsay, France). After saturation in 10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.05% Tween 20 containing 5% non-fat powdered milk, membranes were blotted with relevant antibodies, followed by a secondary HRPO-conjugated antibody. Immunoreactive bands were revealed by ECL (Amersham-Pharmacia).

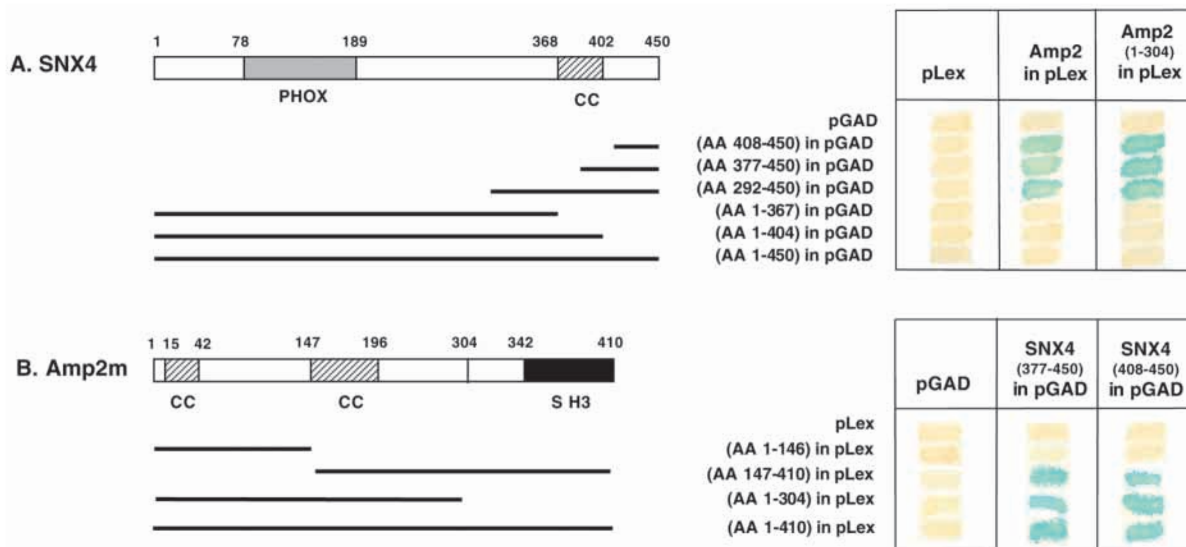


Fig. 1. Two-hybrid analysis of the amphiphysin 2/SNX4 interaction. (A) Different portions of human SNX4 cDNA cloned in the pGAD vector were co-expressed in the L40 yeast strain with full length or partial Amp2m (residues 1-304) cloned in the pLex vector. The three upper fragments correspond to clones selected in the two-hybrid screen when the three lower fragments were cloned secondarily. (B) Different portions of mouse Amp2m cDNA cloned in the pLex vector were co-expressed in the L40 yeast strain with a C-terminal part of human SNX4 (residues 377-450 or 408-450) cloned in the pGAD vector. In both cases, yeast cells were selected on medium deficient in tryptophan and leucine. Protein interaction was analyzed in a beta-galactosidase assay.

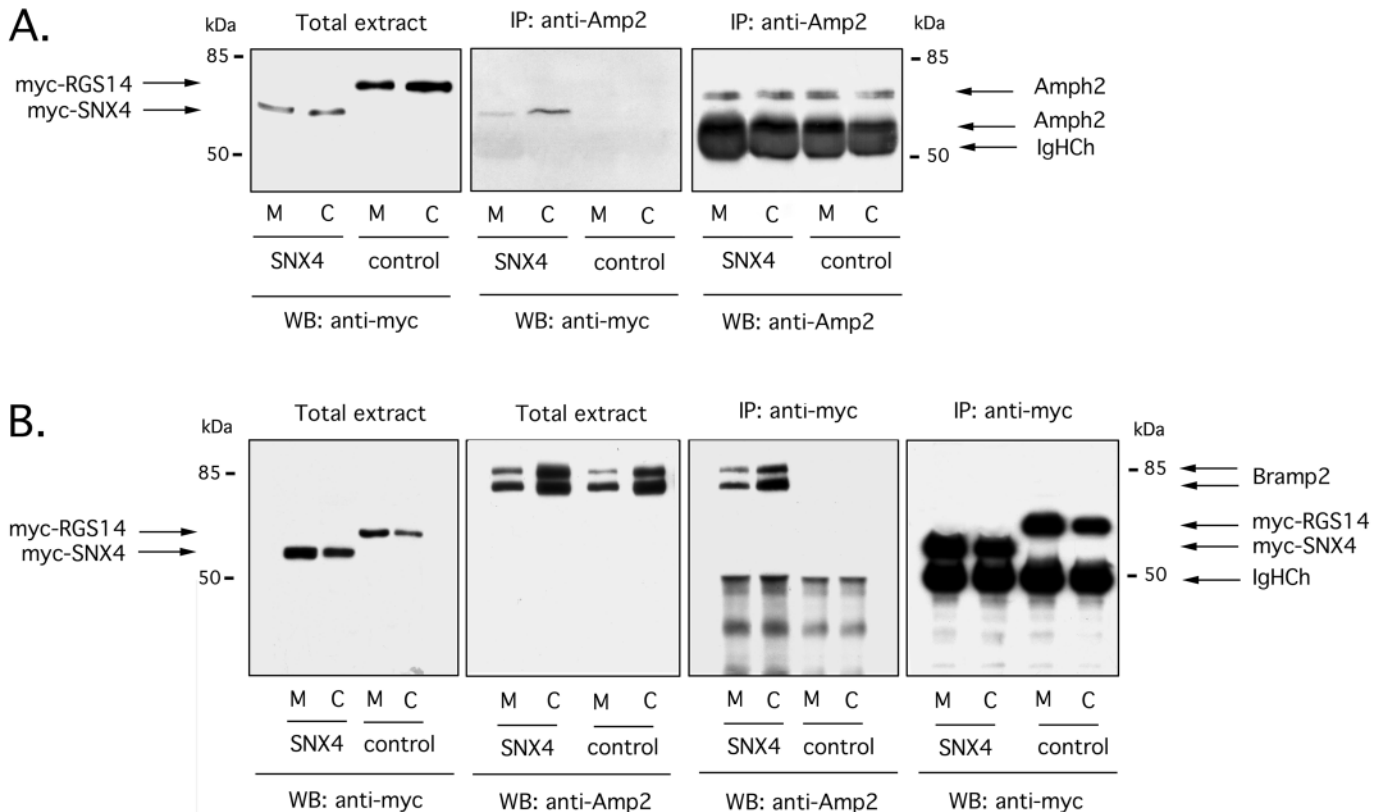


Fig. 2. Co-immunoprecipitation between amphiphysin 2 and SNX4. (A) 3T3 cells were transfected with SNX4 or control RGS14 in the mammalian expression vector pRK5myc. After 24 hours in culture, cells were submitted to hypotonic lysis and membrane/cytosol fractionation. Immunoprecipitations of the endogenous amphiphysin 2 were performed on cytosolic (C) and membrane (M) fractions. Total extracts and anti-amphiphysin 2 immunoprecipitations (IP) were run on SDS-PAGE gels, transferred to nitrocellulose membranes and blotted with relevant antibodies (WB: anti-myc or anti-Amp2). (B) HeLa cells were co-transfected with Bramp2 in pRK5 and SNX4 or control RGS14 in pRK5myc. After 40 hours in culture, cells were submitted to hypotonic lysis and membrane/cytosol fractionation. Anti-myc immunoprecipitations were performed on cytosolic (C) and membrane (M) fractions. Total extracts and anti-myc immunoprecipitations were run on SDS-PAGE gels, transferred to nitrocellulose membranes and blotted with relevant antibodies (WB: anti-Amp2 or anti-myc). Reactive bands were revealed by ECL.

Immunofluorescence analysis

HeLa or 3T3 cells were transiently transfected with various cDNAs. After 24 hours of transfection, cells were washed in cold PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 30 minutes at 4°C, unless they are pre-permeabilized. In this latter case, cells were incubated for 5 minutes at 4°C in 80 mM PIPES pH 6.8, 5 mM EGTA, 1 mM $MgCl_2$ containing 0.1% bovine serum albumin (BSA fraction V, Sigma-Aldrich) and 0.01% saponin (Sigma-Aldrich). After 3 washes in cold PBS, cells were fixed in 4% paraformaldehyde for 30 minutes.

Thereafter, pre-permeabilized or not, cells were washed three times in cold PBS, incubated for 15 minutes in PBS containing 50 mM NH_4Cl, washed again and incubated for 10 minutes with 0.1% saponin. Incubation with primary antibodies diluted in immunofluorescence buffer (IF-buffer: PBS containing 1% BSA and 0.1% saponin) was performed for 30 minutes. After three washes in IF-buffer, cells were labeled for 30 minutes with secondary antibodies coupled to the relevant fluorochrome (Alexa 488-coupled antibodies from Molecular Probes, or Cy-3- and Cy-5-coupled antibodies from Jackson Laboratories). Coverslips were washed in IF buffer, then in PBS and mounted in Mowiol (Sigma-Aldrich).

Cell images were acquired by confocal microscopy with a Leica SP2. Digital monochrome images were collected for each appropriate channel and pseudo-colored with Metamorph (Universal Imaging).

Endocytosis assays

For transferrin receptor endocytosis assay, cells were preincubated for 1 hour in serum-free DMEM containing 20 mM HEPES pH 7.5 at 37°C. Endocytosis of Alexa 488-conjugated transferrin (Molecular Probes) was performed at 37°C for 15 minutes in endocytosis medium (DMEM, 20 mM Hepes pH 7.5, 1 mg/ml BSA) containing 50 μg/ml Alexa 488-conjugated transferrin. Cells were rapidly cooled at 4°C, washed twice in cold PBS, and fixed with 4% paraformaldehyde for 3 hours at 4°C. Then, cells were processed for indirect immunofluorescence as described above.

Results

SNX4 is an amphiphysin 2 interacting protein in a two-hybrid assay

Most of the known amphiphysin 2 partners that play a role in endocytosis interact with the central or C-terminal part of the molecule. A more complete knowledge of the biological role of amphiphysin 2 could result from the identification of additional partners, such as proteins interacting with the N-terminal half of amphiphysin 2, which is only known so far as a dimerization region and a lipid interacting region. To this aim, we performed a two-hybrid screen using the

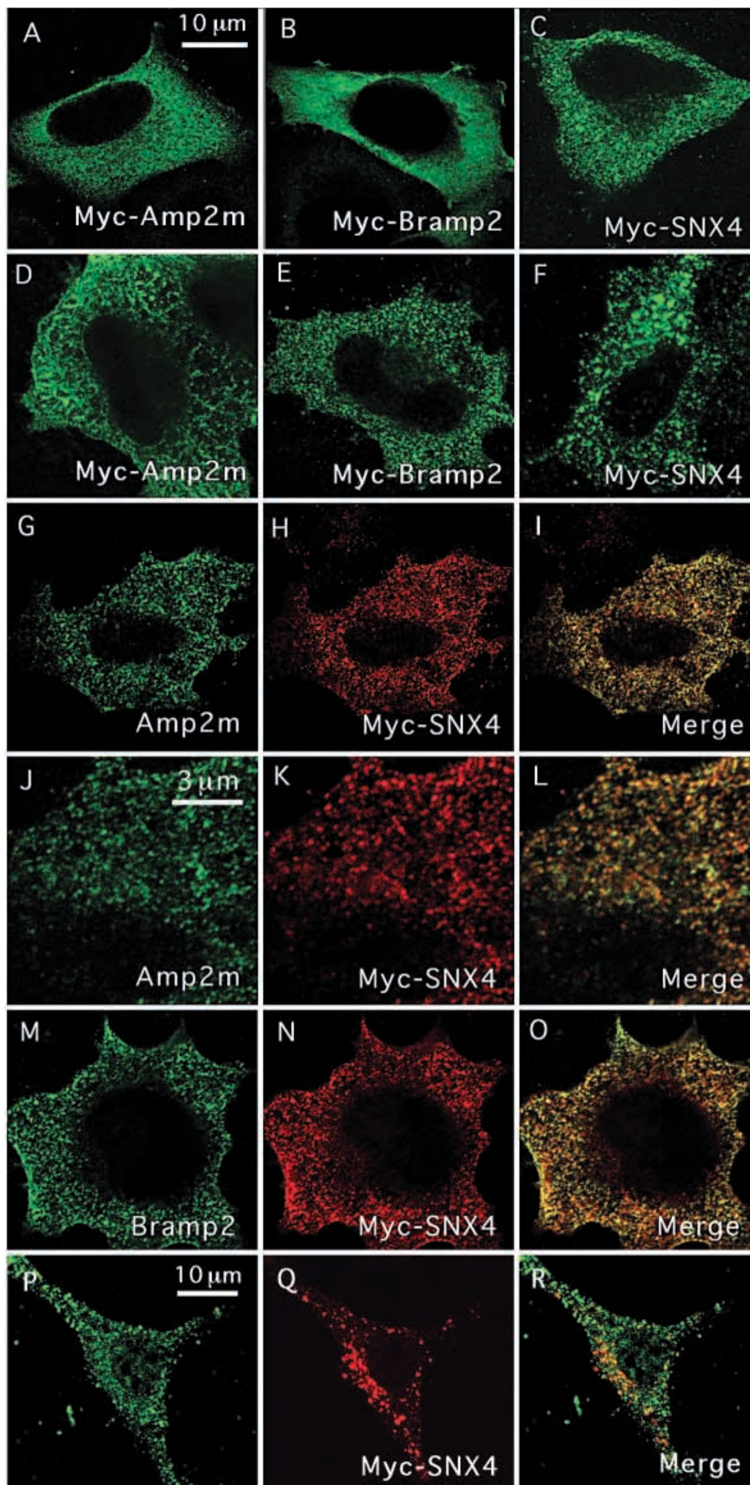


Fig. 3. Intracellular distribution of amphiphysin 2 and SNX4. (A-O) HeLa cells were transiently transfected (A,D) with Amp2m in pRK5myc, (B,E) with Bramp2 in pRK5myc or (C,F) with SNX4 in pRK5myc. They were (A-C) directly fixed with PFA, stained with anti-myc antibody and fluorochrome-conjugated secondary antibodies or (D-F) first pre-permeabilized with 0.01% saponin before fixation in PFA and staining as above. (G-L) HeLa cells were co-transfected with Amp2m in pRK5 plus SNX4 in pRK5myc, pre-permeabilized with 0.01% saponin before co-staining with anti-BIN1 mouse monoclonal antibody and anti-myc rabbit polyclonal antibody, followed by species-specific secondary antibody. (M-O) Hela cells were co-transfected with Bramp2m in pRK5 plus SNX4 in pRK5myc, pre-permeabilized with 0.01% saponin before co-staining. (J,K,L) panels are magnifications of the (G,H,I) panels, respectively. (P) native 3T3 cells. (Q-R) 3T3 cells were transiently transfected with SNX4 in pRK5myc. (P-R) cells were pre-permeabilized in 0,01% saponin before fixation and staining with anti-myc plus rabbit anti-amphiphysin 2 antibody, followed by fluorochrome conjugated secondary antibodies.

Images were acquired on a confocal microscope and pseudo-colored with Metamorph. Co-localization appears in yellow.

N-terminal half of mouse amphiphysin 2 (residues 1-304) as bait and a Jurkat cell cDNA library. The N-terminal part of the molecule is common to six of the 12 amphiphysin 2 isoforms found by our comparison of human, mouse and rat sequences presently available in databases (data not shown).

Among all the interacting clones selected in the two-hybrid screen, we identified several partial cDNAs coding for the same protein, sorting nexin 4 (SNX4). Three of them (residues 408-450, 377-450 and 292-450) are presented in Fig. 1. They code for C-terminal portions of SNX4, which also interacted with full-length amphiphysin 2.

SNX4 was initially characterized as a SNX1 homologue able to interact with membrane receptors such as EGF, PDGF, insulin or leptin receptors (Haft et al., 1998). As with many other members of the SNX family, SNX4 presents a N-terminal Phox homology (PX) domain (Prehoda and Lim, 2001) and a C-terminal coiled-coil region. The shortest clone isolated in the screen (residues 408-450) defined a C-terminal 42 amino-acid region, just after the SNX4 coiled-coil domain, as the domain interacting with amphiphysin 2. Longer C-terminal parts of SNX4 (residues 377-450 and 292-450) including the coiled-coil domain kept their binding capacities. However, N-terminal parts of SNX4 (residues 1-367 and 1-404) and full length SNX4 were deficient in interacting with amphiphysin 2. Thus, full length SNX4 – even though well expressed in yeast (data not shown) – has a general conformation that does not allow interaction with amphiphysin 2 in the conditions of the two-hybrid assay.

In order to define the region of amphiphysin 2 involved in the interaction, we cloned different portions of amphiphysin 2 in yeast expression vectors. As shown in Fig. 2B, the partial amphiphysin 2 construct (residues 147-410) codes for a polypeptide that interacted with C-terminal SNX4, as well as the partial amphiphysin 2 used in the screen (residues 1-304). This suggests that the internal region of amphiphysin 2 (Amp2m), starting at the second coiled-coil (residue 147) and ending at residue 304, is important for the interaction with SNX4. The absence of interaction obtained with the N-terminal part (residues 1-146)

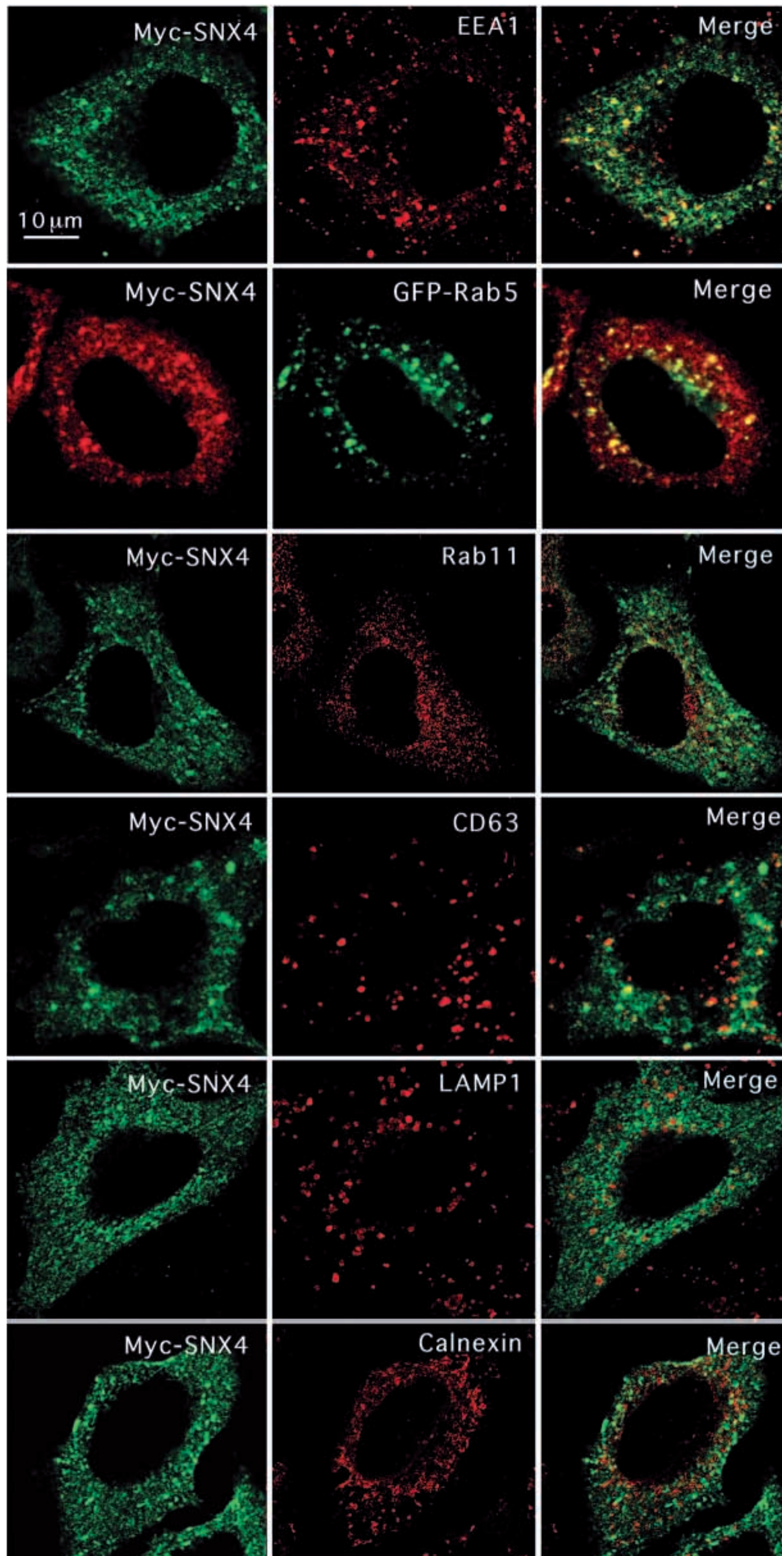


Fig. 4. Comparison of the distribution of SNX4 with markers of intracellular organelles. HeLa cells were transiently transfected with SNX4 in pRK5myc, fixed with PFA and stained with relevant antibodies: the anti-myc mouse monoclonal antibody and antibodies specific for markers of intracellular organelles, anti-EEA1 (early endosomes), anti-Rab11 (recycling endosomes), anti-CD63 (late endosomes/lysosomes), anti-LAMP1 (lysosomes), and anti-calnexin (endoplasmic reticulum). In the case of GFP-Rab5, cells were co-transfected with SNX4 in pRK5myc and GFP-Rab5 before staining with anti-myc antibody. Images were acquired on a confocal microscope and pseudo-colored with Metamorph. Co-localization appears in yellow.

suggests that this part of the molecule is not necessary for binding to SNX4. Because the longer isoform of amphiphysin 2 (Bramp2/Amp2a highly expressed in brain) gave a high background in two-hybrid assays (data not shown), the interaction of SNX4 with Bramp2 was not addressed by this method.

SNX4 and amphiphysin 2 can be co-immunoprecipitated in vivo

The interaction between amphiphysin 2 and SNX4 was confirmed by using a GST-amphiphysin 2 (Amp2m) and in vitro ^{35}S-labeled SNX4 (residues 368-450) (data not shown).

In order to assess whether this interaction existed also in mammalian cells, immunoprecipitations were performed first with murine 3T3 cells (Fig. 2A) and second with human HeLa cells (Fig. 2B). Good anti-SNX4 antibodies were not available for such experiments, inciting us to overexpress myc-SNX4 and a control myc-RGS14 (Traver et al., 2000) by cell transfection. In membrane (M) and cytosol (C) total extracts, myc-SNX4 and myc-RGS14 were detected at 55-60 kDa and 65-70 kDa, respectively (Fig. 2A, left panel). A mouse amphiphysin 2 specific antibody was able to immunoprecipitate the endogenous murine amphiphysin 2 as a 60-70 kDa doublet (Fig. 2A, right panel). The immunoprecipitated amphiphysin 2 was associated with myc-SNX4 in the cytosol fraction and to a lesser extent in the membrane fraction. Control myc-RGS14 was not immunoprecipitated (Fig. 2A, central panel).

Such physical association between amphiphysin 2 and SNX4 was confirmed in HeLa cells that have been transfected with Bramp2/Amp2a and myc-SNX4 (Fig. 2B). Membrane and cytosol total extracts contained the 55-60 kDa myc-SNX4, the 65-70 kDa myc-RGS14 and a 80-90 kDa doublet of Bramp2. Overexpressed amphiphysin 2/Bramp2 was easily detected in the immunoprecipitations of myc-SNX4 from both cytosolic and membrane fractions, but not in the immunoprecipitations of the control myc-RGS14.

Taken together, these results indicate that the endogenous as well as the overexpressed amphiphysin 2 can indeed interact with full length SNX4 in the context of mammalian cells.

Intracellular localization of SNX4 and amphiphysin 2

To acquire further insight into the capacity of SNX4 and amphiphysin 2 to meet and to cooperate in entire cells, we compared their respective subcellular localizations. HeLa cells

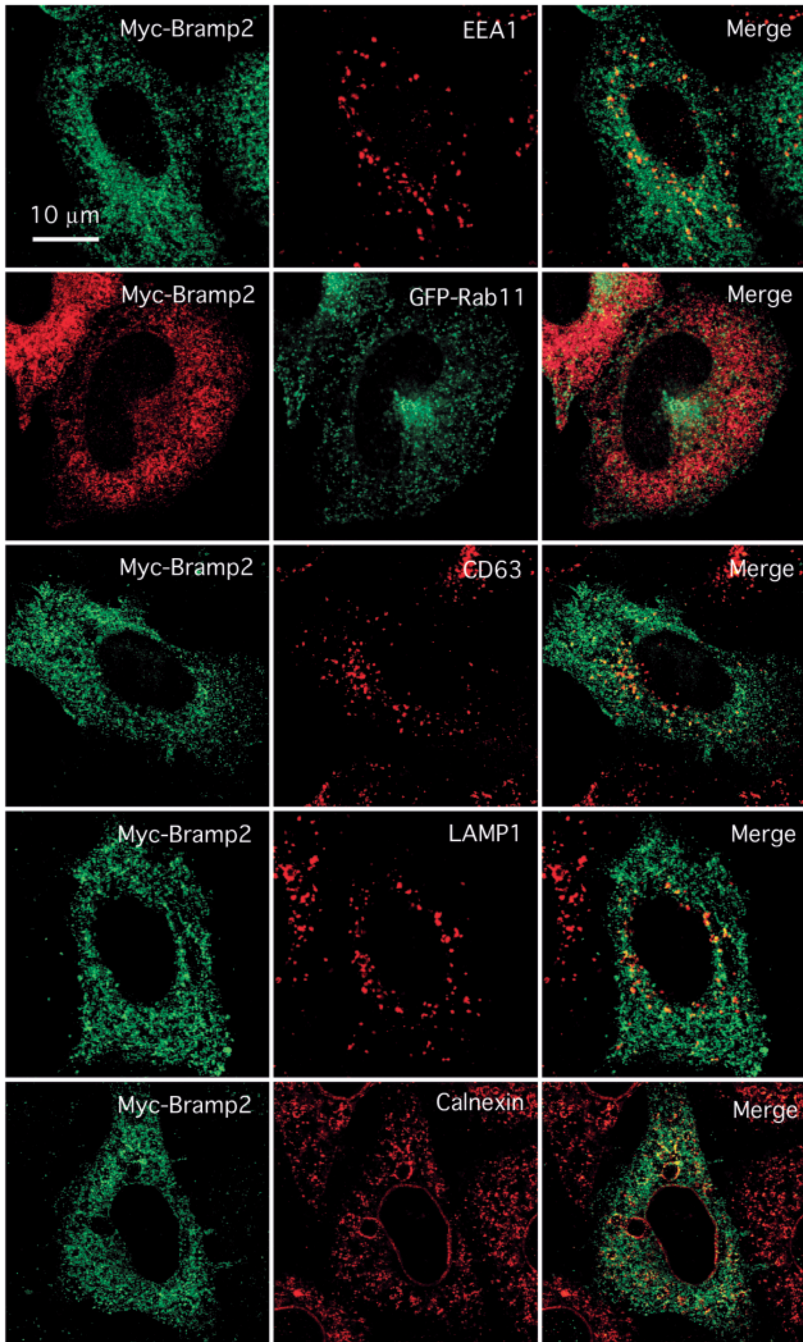


Fig. 5. Comparison of the distribution of amphiphysin 2 with markers of intracellular organelles. HeLa cells were transiently transfected with Bramp2 in pRK5myc, pre-permeabilized in 0.01% saponin and fixed with PFA. Then, they were stained for myc expression and organelle-specific markers: anti-EEA1 (early endosomes), anti-CD63 (late endosomes/lysosomes), anti-LAMP1 (lysosomes), and anti-calnexin (endoplasmic reticulum). In the case of GFP-Rab11, cells were co-transfected with Bramp2 in pRK5myc and GFP-Rab11 before staining with anti-myc antibody. Images were acquired on a confocal microscope and pseudo-colored with Metamorph. Colocalization appears in yellow.

were transiently transfected with myc-amphiphysin 2 (mouse Amp2m or Bramp2) or myc-SNX4 and then labeled with anti-myc antibody. As shown in Fig. 3A,B transfected amphiphysin 2 was highly expressed throughout the cell and exhibited an important cytosolic staining. The same images were obtained with cells transfected with non-tagged amphiphysin 2 revealed with a specific anti-BIN1 antibody or our polyclonal rabbit anti-Bramp2 (data not shown). Both antibodies were unable to detect endogenous amphiphysin 2 in HeLa cells, the latter one because of its mouse specificity. SNX4 showed a more discrete localization characterized by a major punctate distribution, probably on vesicular structures, and a minor cytosolic diffuse staining (Fig. 3C). In conditions of pre-permeabilization with 0.01% saponin, which removes a great part of the cytosol, amphiphysin 2 (Amp2m and Bramp2) also presented a punctate distribution, reflecting the membrane-associated localization of the remaining amphiphysin 2 (Fig. 3D,E). These latter images were similar to the images obtained with myc-SNX4 transfected cells (Fig. 3F) even though SNX4 staining displayed bigger patches of fluorescence. When both labeling images were analyzed in parallel, in the same pre-permeabilized and co-transfected HeLa cells, it was evident that myc-SNX4 and amphiphysin 2 (Amp2m or Bramp2) colocalized to a great extent (Fig. 3G-O). The same conclusion was drawn from 3T3 cells in which our polyclonal anti-amphiphysin 2 antibody was able to recognize the endogenous amphiphysin 2 (at least the 60 and 70 kDa isoforms seen in immunoprecipitations). As for HeLa cells, amphiphysin 2 was clearly distributed throughout the cytoplasm and pre-permeabilization with 0.01% saponin helped to visualize a punctate staining due to a vesicular amphiphysin 2. In 3T3 cells, transfected myc-SNX4 colocalized at least in part with endogenous amphiphysin 2 (Fig. 3P-R).

In a second step, we tried to characterize the subcellular structures containing the SNX4 staining on one hand and the amphiphysin 2 staining on the other hand. Myc-SNX4 transfected HeLa cells were labeled with anti-myc antibody in parallel with specific antibodies for the endogenous markers EEA1, Rab11, CD63, LAMP1, calnexin, or in parallel with GFP-Rab5. As shown in Fig. 4, myc-SNX4 colocalized with the early endosomal markers, EEA1 and Rab5. CD63, a marker for late endosomes/lysosomes, and even less Rab 11, a marker for recycling endosomes, were marginally present on SNX4 positive structures. Other markers such as LAMP1 for lysosomes, calnexin for the endoplasmic reticulum or a Golgi specific marker (data not shown) did not colocalize with SNX4. Thus, SNX4 can be added to the growing list of SNX family members presenting an early endosomal localization (Teasdale et al., 2001; Kurten et al., 2001). Yet this localization does not account for all of the SNX4 positive vesicular structures, as was the case for SNX1 (Zhong et al., 2002).

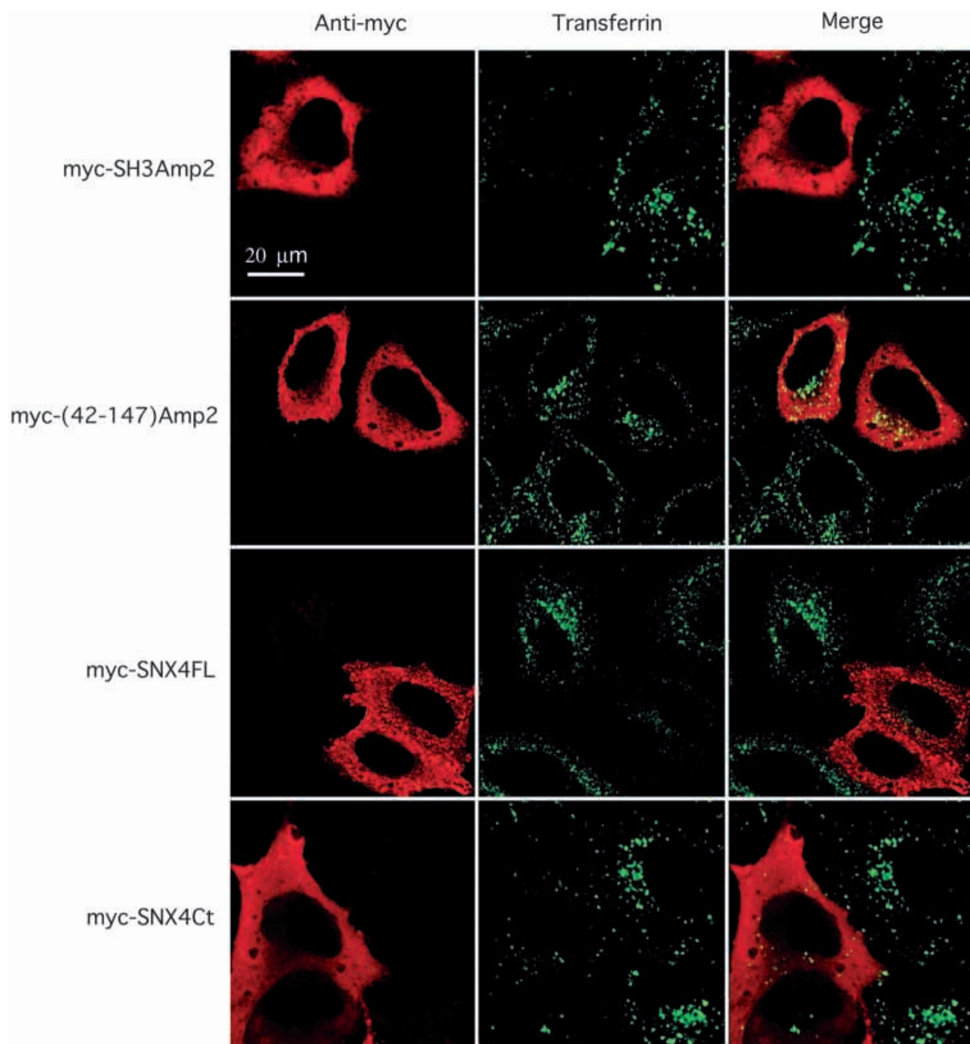


Fig. 6. Effect of SNX4 overexpression on transferrin receptor endocytosis. HeLa cells were transfected with SH3-Amp2 in pRK5myc, with a fragment of Amp2 (residues 42-147) in pRK5myc, with full length SNX4 in pRK5myc or with C-terminal SNX4 in pRK5myc (residues 368-450). Cells were incubated with Alexa 488-transferrin for 15 minutes at 37°C before PFA fixation and staining with anti-myc antibody followed by Cy3-coupled anti-mouse antibody. Images were acquired on a confocal microscope and pseudo-colored with Metamorph.

In Fig. 5, amphiphysin 2 distribution was analyzed after transfection of myc-Bramp2/Amp2a in parallel with the same endogenous markers as above: EEA1, CD63, LAMP1 were detected by antibodies and Rab11 was detected by GFP-Rab11. Bramp2 gave a reticulo-vesicular staining pattern, which was less clustered than SNX4, and partially colocalized with EEA1, CD63, LAMP1 and calnexin. This suggests that amphiphysin 2, which is largely distributed throughout the cell body, can be associated with different types of endosomal structures, from early to late endosomes/lysosomes and with endoplasmic reticulum. Amphiphysin 2/SNX4 interaction may take place on part of these vesicles, for example on early endosomes as could be seen in a three-color analysis with anti-EEA1 antibody (data not shown).

Inhibition of transferrin receptor endocytosis after SNX4 overexpression

Amphiphysin 2 helps to recruit key elements of the endocytosis machinery such as dynamin. Previous studies have showed that overexpression of the SH3 domain of amphiphysins inhibits the early steps of endocytosis by sequestration of dynamin. We tested whether partial or full length SNX4 was able to interfere with endocytosis. As expected, the SH3 domain of amphiphysin 2 was a very potent inhibitor of transferrin receptor endocytosis (Fig. 6), whereas an internal domain of amphiphysin 2 (residues 47-142 between the two coiled-coil regions) had no effect. In the same experimental conditions, full length SNX4 and a C-terminal part of SNX4 (residues 368-450) – both tagged with a myc epitope – were also very efficient in inhibiting transferrin receptor endocytosis (Fig. 6, lower panels). Such an interference of SNX4 with the endocytic process could be due to a vesicular trafficking block but no particular accumulation of transferrin could be seen in a juxta-plasma membrane vesicular compartment. Another possibility is that SNX4 might have functional relationships with key elements of the endocytic machinery, acting itself on endocytosis or by sequestration of key elements of endocytosis, in the wrong subcellular location and/or in a non-functional state. One of such elements is likely to be amphiphysin 2.

In conditions of lower expression, myc-SNX4 was no longer inhibitory for transferrin receptor endocytosis. Fig. 7A (upper panels) gives a good representation of three different myc-SNX4 expression levels (high, intermediate and very low/null) giving no, intermediate or very good transferrin receptor endocytosis, respectively. The lower panels (Fig. 7A) are magnifications of an intermediate state. Transferrin containing vesicles are clearly SNX4 positive. A fraction of them are early

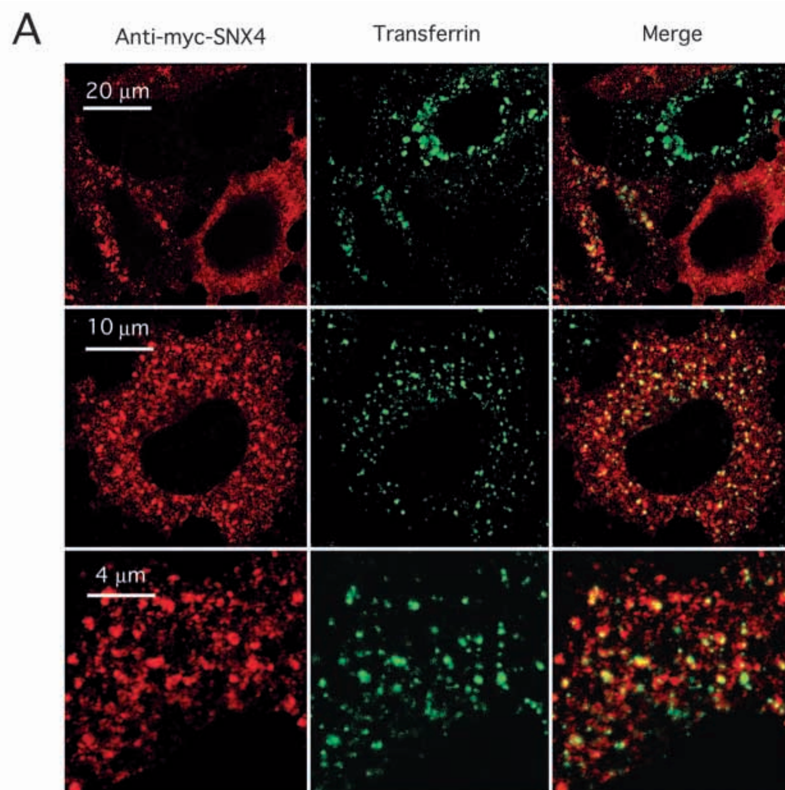
endosomes labeled with EEA1 (data not shown). But most importantly, a fraction of these transferrin-containing vesicles are double positive for SNX4 and amphiphysin 2 as can be shown in a three-color experiment (Fig. 7B, white dots). This image is an instantaneous representation of a dynamic process in which transferring-containing vesicles that are recycling back to the plasma membrane are the site of interaction between amphiphysin 2 and SNX4. Taken together, these results suggest that amphiphysin 2 is playing a role from endocytosis to endosomal trafficking. One of its regulatory roles may involve its partner SNX4.

Discussion

In this study, we report the interaction of amphiphysin 2 with SNX4, a partnership that might be biologically relevant between endocytosis and endosomal trafficking. On one hand, amphiphysin 2 has been shown to be important for the early steps of endocytosis in mammalian cells (Wigge and McMahon, 1998). It is now well established that amphiphysin 2 helps to recruit dynamin via its C-terminal SH3 domain, at the site of clathrin-dependent endocytosis. It may also control membrane curvature either directly or indirectly through endophilin and the uncoating of clathrin-coated vesicles through synaptojanins. On the other hand, SNX4 belongs to a family of molecules initially characterized for their ability to bind membrane receptors such as EGF, PDGF, insulin or leptin receptors (Kurten et al., 1996; Haft et al., 1998). SNX molecules have been conserved throughout evolution and exist in yeast where Vps5p is the ortholog of human SNX1 (Horazdovsky, 1997). In yeast cells, Vps5p associates with other molecules in a multimeric complex called ‘retromer’ that is seen as a new kind of coat surrounding a certain type of intracellular organelles. Vps5p mutants are deficient in intracellular transport between the late Golgi and vacuolar/endosomal compartments. A current hypothesis is that inside the retromer complex, SNX molecules control either recruitment of cargo proteins or a process of vesicule budding/fusion that is essential to trafficking. It was recently shown that SNX1 and SNX2 associate with the human orthologs of Vps26p, Vps29p and Vps35p, other components of the retromer (Haft et al., 2000). Therefore, it is likely that SNX molecules are also part of a retromer coat that would control intracellular trafficking in mammalian cells.

The association between amphiphysin 2 and SNX4 has been identified in a yeast two-hybrid screen with the N-terminal half of amphiphysin 2 (Amp2m, residues 1-304) and a Jurkat oligodT cDNA library. The minimal region of interaction that we were able to define for SNX4 is a C-terminal 42 amino acid region, just after the coiled-coil domain. For amphiphysin 2, a central portion starting at the second coiled-coil domain and ending at residue 304 seems to be required.

SNX4 is the only member of the SNX family that we isolated in our two-hybrid



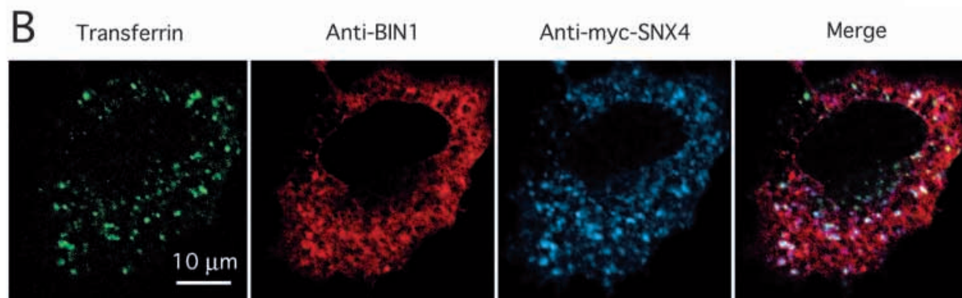


Fig. 7. Comparison of the distribution of SNX4 and amphiphysin 2 with endocytosed transferrin receptor. (A) HeLa cells were transfected with full length SNX4 in pRK5myc. Before PFA fixation, cells were starved and incubated with Alexa 488-transferrin for 15 minutes at 37°C. Then, they were stained with anti-myc antibody followed by Cy3-coupled anti-mouse antibody. (B) HeLa cells were co-transfected with SNX4 in pRK5myc and Bramp2 in pRK5. Before PFA fixation, they were starved and incubated with Alexa 488-transferrin for 15 minutes at 37°C. Then, they were stained with anti-myc and anti-BIN1 antibodies, followed by Cy3 and Cy5 conjugated secondary antibodies. Images were acquired on a confocal microscope and pseudo-colored with Metamorph. Co-localization of the 3 fluorochromes appears in white.

screen, raising the question of the specificity of its interaction with amphiphysin 2. In preliminary experiments, C-terminal parts of human SNX1 (including or not 1, 2 or 3 coiled-coil domains) were unable to bind amphiphysin 2 in two-hybrid assays (data not shown). Even though a more extensive interaction study would be necessary with the numerous members of the SNX family, this result is consistent with the very poor amino acid similarity between the SNX4 C-terminal region (42 residues) and other SNX sequences. In any case, as SNX molecules have the ability to form homo- and hetero-oligomers, amphiphysin 2 might associate with different SNX even indirectly within such an oligomeric complex. The question of interaction specificity is also important for amphiphysin 2, which is expressed as multiple molecular isoforms. Co-immunoprecipitation experiments showed that SNX4 associates with the long Bramp2/Amp2a overexpressed in HeLa cells, and also with shorter isoforms naturally expressed in 3T3 cells as a 60-70 kDa doublet.

In 3T3 and HeLa cells, myc-tagged SNX4 was present primarily on reticulo-vesicular structures and secondarily in the cytosol. Part of the SNX4 positive vesicles are early endosomes, in agreement with previous works showing that the intracellular distributions of a number of SNX molecules show colocalization with early endosomal markers (Teasdale et al., 2001; Barr et al., 2000). But SNX4 is distributed beyond the early endosomal compartment, on a greater population of vesicles, a characteristic that was also pointed out for SNX1 (Zhong et al., 2002).

In the same two cell types, amphiphysin 2 was highly expressed in the cytosol and to a lesser extent in association with membranes, as documented by biochemical and immunofluorescence analysis. The same kinds of images were obtained with HeLa cells expressing moderate levels of myc-tagged amphiphysin 2 and 3T3 cells expressing a native amphiphysin 2. In an immunofluorescence assay including a pre-permeabilization step, amphiphysin 2 exhibited a punctate staining on reticulo-vesicular structures and a partial colocalization with early to late endosomes/lysosomes and with endoplasmic reticulum. The endosomal localization of amphiphysin 2 is consistent with previous studies demonstrating that amphiphysin 2 could associate with early phagosomes in macrophages (Gold et al., 2000). The limited aspect of the co-staining between amphiphysin 2 and organelle markers is due to their very different distributions: throughout the cell body for amphiphysin 2 and in restricted cell compartments for the organelle markers.

The presence of amphiphysin 2 on vesicles that are far from the plasma membrane and are far from being strict endocytic vesicles, combined with its colocalization with SNX4 suspected to regulate intracellular trafficking, suggests that amphiphysin 2 may have a post-endocytic role on the endocytic vesicle and on the following endosome.

Besides being an adaptor for components of the endocytosis machinery that assemble at the neck of the nascent endocytic vesicle, amphiphysin 2 has been previously implicated in a number of other processes such as tumor progression or cytoskeleton organization. BIN1, one of the amphiphysin 2 isoforms with a nuclear localization signal, was shown to interact with myc and suppress its tumor promoting effect (Sakamuro et al., 1996). Other studies in different cell types have documented an interaction of amphiphysin with the actin cytoskeleton. First, mutations in *RVS167* or *RVS161* genes – two yeast homologs of amphiphysin – produced defects in the peripheral actin cytoskeleton, whereas normal Rvs167p localized to actin rich cortical patches (Sivadon et al., 1995; Balguerie et al., 1999). Second, in muscle cells, the shortest amphiphysin 2 isoforms were shown to be highly expressed (Butler et al., 1997) and localize around the submembranous cytoskeleton of T-tubules. These data were emphasized by recent studies in *Drosophila* where a unique gene, *amph*, is responsible for the expression of amphiphysin. Mutant flies do not show any particular deficiency in the endocytosis of synaptic vesicles but they have a muscle cell defect with reduced and mislocalized T-tubule projections (Razzaq et al., 2001; Zelhof et al., 2001; Leventis et al., 2001). In mammalian cells, an amphiphysin 2 isoform displaying an additional short sequence (encoded by exon 10) was also shown to induce tubular membrane invaginations, particularly critical for muscle cell morphology (Lee et al., 2002). Looking at an extended series of cell types, drosophila studies further suggest that amphiphysin is always present in membrane domains that undergo great changes in curvature and surface area, for example in the apical membrane of epithelial cells. Thus, amphiphysin seems to be essential for a series of membrane movements and this biological effect may rely on its connection with the actin cytoskeleton, its ability to tubulate lipids and/or its docking potential for a number of proteins (Zhang et al., 2002).

This new aspect of amphiphysin function reinforces the importance of the interaction that we describe herein between amphiphysin 2 and SNX4, a molecule that is suspected to play a role in vesicular trafficking. If amphiphysin is able to regulate membrane morphology and movements, it is conceivable that the amphiphysin 2 present around an endosome could regulate the budding events that are crucial for intracellular trafficking. Its partnership with SNX4 can be seen as another level of regulation with the same goal of vesicular trafficking. SNX molecules have been shown to associate with membrane receptors such as EGF, PDGF, insulin, transferring and leptin receptors. In this regard, the interference of SNX4 with transferrin receptor endocytosis that we documented herein may be due to a direct effect of SNX4 on the endocytic process. But another possibility is that the endocytosis inhibition is just a sequestration of key elements of endocytosis. One of these elements may be amphiphysin 2 and the inhibition is rendered possible by amphiphysin 2 pleiotropic functions, playing a role in endocytosis, as well as vesicular trafficking. Inside a vesicular coat, SNX4 is suspected to act either on the selection of cargo proteins or on the membrane budding/fusion processes. We documented above the simultaneous presence of SNX4 and amphiphysin 2 on transferring-containing vesicles that are on their way for recycling. This is an instantaneous picture of a dynamic process that involves not less dynamic molecular interactions. At the surface of the endocytic vesicle or of the endosome, it is conceivable that the interaction between SNX4 and amphiphysin modifies SNX4 conformation and, consequently, either its membrane anchorage, its interaction with other components of the coat, or its functional effect on membrane movements that still need to be well defined. Reciprocally, the same molecular interaction may modify amphiphysin ability to act on membranes dynamics, i.e. its connection with the actin cytoskeleton, its ability to

tubulate lipids or its adaptor potential in the aim to regulate vesicular trafficking. Further studies will be necessary to define the exact contribution of amphiphysin 2 and SNX4 – taken individually or in association – in the molecular machinery underlying vesicular trafficking.

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