Syndapin Oligomers Interconnect the Machineries for Endocytic Vesicle Formation and Actin Polymerization*

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Syndapins were proposed to interconnect the machineries for vesicle formation and actin polymerization, as they interact with dynamin and the Arp2/3 complex activator N-WASP (neural Wiskott-Aldrich syndrome protein). Syndapins, however, have only one Src homology 3 domain mediating both interactions. Here we show that syndapins self-associate via direct syndapin/syndapin interactions, providing a molecular mechanism for the coordinating role of syndapin. Cross-link studies with overexpressed and endogenous syndapins suggest that predominantly dimers form in vivo. Our analyses show that the N-terminal Fes/Cip4 homology domain but not the central coiled-coil domain is sufficient for oligomerization. Additionally, a second interface located further C-terminally mediated interactions with the N terminus. The Src homology 3 domain and the NPF region are not involved and thus available for further interactions interconnecting different syndapin binding partners. Our analyses showed that self-association is crucial for syndapin function. Both syndapin-mediated cytoskeletal rearrangements and endocytosis were disrupted by a self-association-deficient mutant. Consistent with a role of syndapins in linking actin polymerization bursts with endocytic vesicle formation, syndapin-containing complexes had a size of 300–500 kDa in gel filtration analysis and contained both dynamin and N-WASP. The existence of an interconnection of the GTPase dynamin with N-WASP via syndapin oligomers was demonstrated both by coimmunoprecipitations and by reconstitution at membranes in intact cells. The interconnection was disrupted by coexpression of syndapin mutants incapable of self-association. Syndapin oligomers may thus act as multivalent organizers spatially and temporally coordinating vesicle fission with local actin polymerization.

The cortical actin cytoskeleton is crucial for shaping cells and for maintaining specialized morphologies that are directly related to various cellular functions. Cortical cytoskeletal structures can be extremely elaborate and, supported by experimental data in particular for exocytic membrane trafficking events, have therefore mainly been viewed as a barrier for membrane trafficking events (1, 2). For endocytosis, in contrast, mounting evidence suggests that the actin cytoskeleton may also have a supporting role (for reviews, see Refs. 3–6). The formation of vesicles from a donor membrane requires complex protein machinery and additional proteins to control it (7, 8). Among those is the large GTPase dynamin that has been observed to constrict necks of forming endocytic vesicles and is a crucial but mechanistically still not fully understood player in the vesicle fission step (9–12).

We have suggested several possibilities how the actin cytoskeleton may support endocytosis (3). Cytoskeletal components may localize the endocytic machinery to domains of the plasma membrane, either by trapping it or by direct anchoring. This would greatly speed up the assembly of the machinery at locations of receptors and cargo to be internalized. Furthermore, actin polymerization may provide the forces to deform the membrane and to drive vesicle formation and detachment. In line with these hypotheses, short lived actin structures at sites of endocytosis coinciding with dynamin-mediated vesicle release have indeed been observed (13, 14). Most recently, the burst of actin polymerization at clathrin-coated pits has been reported to coincide with a local recruitment of the Arp2/3 complex (15), which promotes actin filament nucleation and polymerization (16, 17). Also the Arp2/3 complex activator N-WASP2 (18, 19) appears transiently at sites of endocytosis (15). The hypothesis that actin polymerization during endocytosis is triggered by N-WASP, and the Arp2/3 complex was substantiated by experiments showing that an interference with N-WASP function in vivo had a strong impact on receptor-mediated endocytosis (20). These data were recently confirmed by the analysis of cells from N-WASP knockout mice that also showed impairments in endocytosis (21, 22).

In order to support and not inhibit endocytic vesicle formation, actin polymerization during endocytosis requires an extremely fine control in time and space. The molecular mechanism that ensures that N-WASP and the Arp2/3 complex trigger local actin polymerization specifically at sites of endocytosis and only during and/or after vesicle fission appears to be the interaction of N-WASP with so-called accessory proteins of the vesicle formation machinery, such as syndapins (23, 24). Syndapins, a family of dynamin-binding proteins also referred to as PACSINs, were suggested to be molecular links between membrane trafficking and cortical cytoskeleton dynamics (reviewed in Ref. 25). Both aspects of syndapin function are supported by in vivo data, the dynamin-binding syndapin Src homology 3 (SH3) domain is a potent inhibitor of receptor-mediated endocytosis, and overexpression of full-length syndapins induced a cortical actin phenotype (24). Dominant-negative effects observed upon overexpression of N-WASP and fragments thereof encompassing the syndapin-binding proline-rich domain and corresponding rescue experiments strongly suggested that N-WASP’s role in endocytosis may involve the syndapin association (20). This hypothesis was further strengthened by the observation that syndapins can recruit N-WASP to cellular membranes and that the reconstitution of such

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The abbreviations used are: N-WASP, neural Wiskott-Aldrich syndrome protein; SH3, Src homology 3; GST, glutathione S-transferase; GFP, green fluorescent protein; MBP, maltose-binding protein; HA, hemagglutinin; PRD, proline-rich domain; HEK293, human embryonic kidney 293; IP, immunoprecipitation; EDC, 1-ethyl-3-(dimethylamino)propyl]carbodiimide; BAR, BIN/amphiphysin/RVS; CC, coiled-coil; FCH, Fes/Cip4 homology.
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protein complexes elicited local actin polymerization in an Arp2/3 complex-Secondary antibodies used in this study include goat anti-mouse peroxidase (Dianova), goat anti-guinea pig peroxidase (ICN Biochemicals), donkey anti-rabbit peroxidase (Dianova), Alexa Fluor™ 568 goat anti-mouse (Molecular Probes, Inc., Eugene, OR), Alexa Fluor™ 350 goat anti-rabbit (Molecular Probes), and Alexa Fluor™ 488 goat anti-mouse (Molecular Probes).

Coprecipitation Assays—In order to test for a direct syndapin I/syndapin I interaction, GST fusion proteins were immobilized on glutathione-Sepharose in the presence of 5% bovine serum albumin and incubated with MBP fusion proteins overnight at 4 °C in phosphate-buffered saline containing 1.25% bovine serum albumin, 300 mM NaCl, and 1% Triton X-100. Proteins coprecipitated with the GST fusion proteins were eluted with glutathione-containing buffer and analyzed by SDS-PAGE and Western blotting with rabbit anti-MBP antibodies.

Gel Filtrations—Rat brain extracts were fractionated on a Superose 6 column (30 × 1 cm). Fractions eluted with buffer A (10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.1 mM MgCl2) were resolved by SDS-PAGE and blotted to nitrocellulose membranes. After Ponceau-S staining, the distribution of syndapin I and of its direct binding partners was detected with antibodies against dynamin 1, N-WASP, and syndapin I.

Preparation of Cell Extracts and Immunoprecipitation—For immunoprecipitations of epitope-tagged proteins, human embryonic kidney 293 (HEK293) cells were transfected with different GFP- and FLAG-tagged constructs using the Lipofectamine PLUS transfection reagent method according to the instructions of the manufacturer (Invitrogen). The cells were grown for an additional 40 h, harvested, and homogenized in immunoprecipitation (IP) buffer (10 mM HEPES, pH 7.4, 1 mM EGTA, 0.1 mM MgCl2, 100 mM NaCl, 1% Triton X-100) for 20 min at 4 °C. Cell lysates were cleared by centrifugation at 14,000 × g for 20 min at 4 °C. 6 μg of monoclonal anti-FLAG antibody M2 (Sigma), 3 μg of mouse anti-GFP antibody 3E6 (Molecular Probes), or nonimmune mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) prebound to protein G-Sepharose was incubated with the high speed supernatants prepared from the lysed HEK cells overnight at 4 °C. The precipitated material was washed and probed for coimmunoprecipitated proteins by SDS-PAGE and immunoblotting using rabbit and mouse monoclonal anti-FLAG antibodies, rabbit and mouse monoclonal anti-GFP antibodies, and rabbit anti-syndapin antibodies.

For coimmunoprecipitations of tertiary complexes of dynamin, syndapin, and N-WASP, HEK293 cells were cotransfected with HA-dynamin 1, with GFP-N-WASP, and, when indicated, with Xpress-syndapin I as described above. Cell lysates adjusted to 30 mM NaCl and 1% Triton X-100 final were incubated with 6 μg of anti-HA antibodies or normal mouse IgG (Santa Cruz Biotechnology) for 2 h at 4 °C and subsequently precipitated with protein A/G-agarose (Santa Cruz Biotechnology). Specifically precipitated material was analyzed for immunoblotting and coprecipitated proteins by SDS-PAGE and immunoblotting using mouse monoclonal anti-HA antibodies, mouse monoclonal anti-GFP antibodies, and rabbit anti-syndapin SH3 antibodies (2521). For competition experiments, HEK293 cells were cotransfected with HA-dynamin 1 and Xpress-syndapin I. Prior to the immunoprecipitation with anti-Xpress antibodies, cell lysates were incubated with GST-N-WASP PRD fusion protein for 10 min at 30 °C as indicated.

For coimmunoprecipitations of syndapin I-containing protein complexes from gel filtration fractions, affinity-purified anti-syndapin I or anti-GST antibodies prebound to protein A/G-agarose (Santa Cruz Biotechnology) were incubated with syndapin I-containing fractions adjusted to 75 mM NaCl and 1% Triton X-100 overnight at 4 °C. After intense washing, the precipitated material was analyzed for the presence of syndapin I, dynamin 1, and N-WASP via immunoblot analyses.

MATERIALS AND METHODS

DNA Constructs and Recombinant Proteins—Constructs coding for glutathione S-transferase (GST) fusion proteins of syndapin I full-length (amino acids 1–441), syndapin I ASH3 (amino acids 1–382), syndapin I SH3 (amino acids 376–441), and the P434L mutant form of the syndapin I SH3 domain were described previously (23, 24). GST-N-WASP PRD (amino acids 313–349) was generated by PCR and inserted into pGEX-5.1. Mammalian expression constructs encoding the corresponding full-length protein or fragments thereof were fused to the C terminus of a green fluorescent protein (GFP), the FLAG epitope, and the Myc epitope were generated by subcloning the corresponding DNA inserts from the pcDNA3.1/His vector (24) into the BamHI-XbaI sites of pEGFP-C1 (Clontech) and the BamHI-EcoRI sites of pCMV-Tag2B (Stratagene) or pRRIK, respectively. Constructs to express maltose-bind-
Cross-linking Experiments—For cross-link studies of endogenous proteins, rat brain extracts were prepared as described (26). Brain extracts at a total concentration of 0.7 mg/ml and a final concentration of 100 mM NaCl were incubated in the presence of increasing amounts of the heterobifunctional cross-linker 1-ethyl-3-[dimethylaminopropyl]carbodiimide (EDC) (Pierce) in 20 mM Hepes, pH 7.4, 0.2 mM MgCl₂, and 2 mM EGTA for 20 min at 30°C. The cross-linking reaction was stopped by the addition of 4× SDS-PAGE sample buffer, and the samples were analyzed by immunoblotting. Extracts from HEK293 cells transfected with different constructs encoding for different tagged syndapin proteins or fragments thereof were prepared as described above. The extracts were incubated with increasing amounts of EDC as indicated for 20 min at 30°C and analyzed by SDS-PAGE on 8% or 5–8% polyacrylamide gels and by subsequent immunoblotting.

In Silico Analyses and Predictions of Protein Structure—Software used for predictions of protein secondary structure (especially of α-helices and loop regions) is available on the World Wide Web at www.ch.embnet.org/cgi-bin/COILS). For mitochondrial targeting experiments, COS-7 cells were seeded in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum.

For mitochondrial targeting experiments, COS-7 cells were seeded onto poly-D-lysine-coated coverslips 1 day prior to transfection. Transfections were performed with Polyfect reagent according to the manufacturer’s instructions (Qiagen). Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, containing 0.9 mM CaCl₂ and 0.5 mM MgCl₂ for 15 min at room temperature and processed for immunofluorescence according to Ref. 27. For mitochondrial staining, cells were incubated with MitoTracker® as described previously (20). Images were recorded digitally using a Leica DMRD fluorescence microscope and a Zeiss Axioplan 2 microscope and processed using Adobe Photoshop software.

Examinations of the actin cytoskeletal phenotype induced by overexpression of syndapin full-length proteins and fragments thereof were performed in HeLa cells and have been described previously (24). F-actin was stained with Alexa568-phalloidin (Molecular Probes). The presence of several or extended lamellipodia areas decorated with filopodial structures and marked by accumulated F-actin signal at the leading edge was used for scoring. The effect of each construct was determined in several independent assays, and 134–528 transfected cells were scored in systematic searches across the coverslips. The assays were conducted as blind studies to ensure that the researcher was unbiased.

Induction of the cortical actin phenotype upon N-WASP overexpression and epidermal growth factor stimulation was performed essentially as established by Miki et al. (28). For quantitative analysis of the phenotype and of changes induced upon cotransfection with syndapin constructs, 316–387 transfected cells from three independent assays were scored in systematic searches across the coverslips and grouped into two categories, smooth and rough/filopodia-decorated appearance of plasma membrane edges, respectively.

Transferrin Uptake Assays—COS-7 cells were subjected to transferrin uptake assays 48 h after transfection as described previously (24, 27). The percentages of transfected cells showing no detectable uptake of transferrin, significantly reduced transferrin signals, and normal levels of internalized transferrin and S.D. values were calculated by scoring and counting cells in at least three independent experiments each.

RESULTS

Syndapins have been suggested to functionally interconnect endocytosis and the actin cytoskeleton by interactions of their C-terminal SH3 domain with dynamin and N-WASP (24). However, syndapins have only one SH3 domain for an interaction with either dynamin or N-WASP. To function as molecular links, syndapins would thus have to build multivalent platforms in a non-SH3 domain-dependent manner.

Analyses of fractions of rat brain cytosol obtained by size exclusion chromatography over a Superose 6 column revealed that the size of syndapin I-containing complexes by far exceeds the calculated size of monomeric syndapin. Syndapin I has a predicted molecular mass of 50.4 kDa, but syndapin-containing complexes were detected in a range between apparent molecular masses of 300 kDa and about 500 kDa, corresponding to fractions 15–17 (Fig. 1A). Complexes with a molecular mass around 400 kDa were especially abundant. The existence of syndapin complexes with even higher molecular mass also remains possible because the analysis was performed with cytosolic extracts cleared from extremely large protein complexes by high speed centrifugation.

Immunoblotting analysis of rat brain cytosol fractionated by gel filtration showed that the N-WASP and dynamin distribution both peak in the syndapin I-containing fractions (Fig. 1A). The apparent molecular weights of syndapin, N-WASP, and dynamin all exceed those of the monomeric proteins and even those of combinations of one syndapin molecule with one interaction partner. Blot overlay analysis with a GST fusion protein of syndapin I as a probe revealed that literally all direct syndapin binding partners run at molecular weights that are much larger than those of the monomeric proteins and thus correspond to protein complexes that partially cofractionate with syndapin I (data not shown).

Coimmunoprecipitations from the gel filtration fractions directly demonstrated that syndapin I is complexed with N-WASP and dynamin in high molecular weight fractions. Both N-WASP and dynamin were specifically coimmunoprecipitated with syndapin I from fraction 16/17 (data not shown) as well as from fraction 15 (Fig. 1B). Based on secondary structure predictions, we have hypothesized earlier that syndapins might have the capability to interact with themselves and form oligomers (23). Since our gel filtration studies (Fig. 1) support such a scenario, we experimentally addressed the existence of syndapin-syndapin interactions by conducting coimmunoprecipitation experiments with differentially tagged syndapin I proteins overexpressed in HEK293 cells (Fig. 2, A–D). Both FLAG-tagged syndapin I and a GFP-tagged syndapin I fusion protein can be expressed and coexpressed in HEK cells (Fig. 2, A and B) and...
were successfully immunoprecipitated by anti-FLAG and anti-GFP antibodies, respectively (Fig. 2C). Analysis of the immunoprecipitates from cells double-transfected with FLAG-syndapin I and GFP-syndapin I demonstrated that FLAG-syndapin I was specifically coimmunoprecipitated with GFP-syndapin I (Fig. 2D). GFP-syndapin did not associate with anti-FLAG antibodies when transfected alone or with nonimmune mouse IgG. Consistently, FLAG-syndapin I was specifically coimmunoprecipitated with GFP-syndapin I but not with GFP alone (Fig. 2D). Control experiments demonstrate that FLAG-syndapin was not precipitated by anti-GFP antibodies upon single transfection of the construct or by unrelated mouse IgG (Fig. 2D). Our coimmunoprecipitation studies thus demonstrate that syndapin I can efficiently form oligomers in vivo.

We next addressed the question of whether the syndapin oligomerization is independent of the SH3 domain, as hypothesized. For this purpose we first coexpressed different GFP-syndapin I constructs in combination with FLAG-syndapin I (Fig. 2, E and F) and conducted immunoprecipitations of the GFP fusion proteins (Fig. 2G). Similar to full-length syndapin I, both GFP-syndapin I with a mutated SH3 domain and GFP-syndapin ΔSH3 effectively and specifically coimmunoprecipitated FLAG-syndapin I (Fig. 2H). In contrast, FLAG-syndapin I was not coimmunoprecipitated together with the GFP-syndapin I SH3 domain (Fig. 2H). These data clearly show that the non-SH3 part of syndapin I is sufficient for oligomerization. This leaves the SH3 domain free for further protein-protein interactions forming larger macromolecular complexes, as seen in Fig. 1.

In order to firmly exclude putative posthomogenization artifacts in our coimmunoprecipitation analyses, we next reconstituted and visualized the syndapin-syndapin interactions that we observed in cellular extracts (Fig. 2, A–H) in intact cells (Fig. 2, I–O). We have developed a mitochondrial targeting system that allows for incorporating syndapin fusion proteins into the outer mitochondrial membranes in a manner...
that ensures that the syndapin part is facing the cytoplasm (20). If syndapin-syndapin interactions are of high affinity and specificity in vivo, GFP-syndapin fusion proteins coexpressed with mito-syndapin constructs should no longer show the rather diffuse localization that they adopt when expressed alone (Fig. 2O) but should instead be recruited to syndapin-coated mitochondria. Our analyses demonstrate that mtosyndapin I was indeed able to effectively recruit GFP-syndapin I (Fig. 2, L–K), as demonstrated by colocalization of the two proteins. Since the cytosol was often depleted for the nonmitochondrially targeted syn-
dapin, the association was apparently so strong that it led to an almost
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situation, we conducted cross-
linking experiments with endogenous syndapin I from brain extracts (Fig. 3). Likewise, we were able to reconstitute the interactions with GST-syndapin I ΔSH3 fusion protein bound to the matrix (Fig. 3). The interaction is thus direct and SH3 domain-indepen-
dent. Consistently, MBP-syndapin SH3 domain was not able to bind to
GST-syndapin I (Fig. 3). Cross-link studies with purified MBP fusion proteins and increasing concentrations of the zero-length cross-linker
EDC also yielded results fully in line with the in vitro reconstitutions (data not shown).

In some of our in vitro reconstitutions, SDS-resistant high molecular weight oligomers were observed (Fig. 3). These complexes could repre-
sent dimers, trimers, and even larger complexes. The sizes of these complexes could not be determined conclusively due to technical limita-
tions of protein standards and SDS-polyacrylamide gels. We therefore
examined the complexes by gel filtration using different chromatogra-
phy materials. These experiments permitted the separation of at least
two high molecular weight species with apparent molecular masses of
270–350 and 530–700 kDa for MBP-syndapin I ΔSH3 and MBP-syndapin I SH3
domain, respectively. Proteins specifically coprecipitated with the GST fusion proteins were eluted with glutathione-containing buffer and analyzed by SDS-PAGE and Western
blotting with anti-MBP antibodies. Detected were MBP-syndapin I full-length (110 kDa), MBP-syndapin I ΔSH3 (105 kDa), and an MBP-containing proteolysis product (80 kDa) as well as SDS-resistant high molecular weight complexes.
Figure 5. The N-terminal FCH domain but not the predicted coiled-coil domain is sufficient to mediate syndapin self-association in vivo. A, secondary structure predictions of coiled-coils and of α-helical structures, as obtained by submitting the syndapin I sequence to the program PAIRCOIL (available on the World Wide Web at paircoil.lcs.mit.edu/cgi-bin/paircoil) and to PHD predictions (www.predictprotein.org), respectively. The probability scores for coiled-coils are displayed graphically. The predictions of α-helical structures are displayed as black bars above the amino acid axis (x axis) and represent the "SUB SEC" output of PHD predictions for all residues with an expected average correlation of >0.69. Note that the white interruptions of the black bars, which represent amino acids for which no strong helix predictions were obtained, are even smaller in other, less stringent secondary structure predictions.
were observed upon incubations with higher EDC concentrations (Fig. 4A). When we overexpressed FLAG-tagged syndapin I to high levels in HEK293 cells, subjected the extracts to cross-link experiments, and performed immunoblot analyses with anti-FLAG antibodies, we obtained similar results. The monomeric form declined, and a band at 100 kDa increased in intensity with rising cross-linker concentrations (Fig. 4B). Myc- and Xpress-tagged fusion proteins yielded similar results (data not shown). The cross-linking studies with overexpressed syndapin I in HEK293 cells support the interpretation that the 100 kDa band observed upon cross-linking endogenous syndapin I in rat brain extracts corresponds to a syndapin dimer and not to a putative 1:1 complex with an unknown brain protein of 50 kDa.

Syndapins contain a stretch of amino acids that is predicted to adopt a helical secondary structure and mediate coiled-coil interactions. It spans amino acids 184–216 (Fig. 5A). This region within the non-SH3 part of syndapin I is well conserved among isoforms and syndapins from species as diverse as flies, humans, mice, frogs, and fish. High PAIRCOIL prediction scores were obtained for a stretch of about 35–40 residues centered on amino acid 200 (data not shown). Based on structural predictions, Peter et al. (30) suggested that the region of syndapins including this stretch might perhaps represent a so-called BIN/amphiphysin/RVS (BAR) domain. BAR domains are strictly centered on amino acid 200 (data not shown). Based on structural predictions, Peter et al. (30) suggested that the region of syndapins including this stretch might perhaps represent a so-called BIN/amphiphysin/RVS (BAR) domain. BAR domains are strictly centered on amino acid 200 (data not shown). Based on structural predictions, Peter et al. (30) suggested that the region of syndapins including this stretch might perhaps represent a so-called BIN/amphiphysin/RVS (BAR) domain. BAR domains are strictly centered on amino acid 200 (data not shown). Based on structural predictions, Peter et al. (30) suggested that the region of syndapins including this stretch might perhaps represent a so-called BIN/amphiphysin/RVS (BAR) domain. BAR domains are strictly around motifs of about 200 amino acids in length that consist of three bundled α-helices connected in part by very sharp and short turns between them. Dimers of BAR domains form a slightly bent, elongated quaternary structure (30).

In order to test this hypothesis experimentally, we performed detailed secondary structure predictions and cloned the coiled-coil (CC) domain of syndapin I as a GFP fusion protein (GFP-syndapin I 124–289 (CC)). We designed the construct in such a manner that it starts with some non-structured residues close to the N terminus of the suggested BAR domain and includes all residues that are predicted to belong to the overall strongly α-helical part of the non-SH3 portion of syndapins (Fig. 5, A–C).

The coiled-coil-encompassing fusion protein GFP-syndapin I 124–289 (CC) was then subjected to a coimmunoprecipitation analysis with full-length syndapin I. Expression of GFP-syndapin 124–289 (CC) gave rise to a band of the correct size of about 40 kDa (Fig. 5D). Although we observed an effective immunoprecipitation of coexpressed full-length syndapin I with anti-FLAG antibodies (Fig. 5D), which completely depleted the cell extracts offered (data not shown), no GFP-syndapin I 124–289 (CC) was coimmunoprecipitated (Fig. 5D). Likewise, we did not detect any self-association in cross-link experiments. Only the monomeric form of GFP-syndapin I 124–289 was detected at all cross-linker concentrations applied (Fig. 5E). Also, we did not detect any cross-link of the coiled-coil domain with coexpressed full-length proteins (data not shown). These data are in disagreement with in vitro protein interaction studies performed with GST fusion proteins of a chicken protein that is considered a syndapin II ortholog (FAP52) in surface plasmon resonance experiments (31). Nikki et al. (31) assigned the self-association capability of FAP52 to the region 146–280. Our data, however, clearly show by two independent methods that self-association of the so-called coiled-coil domain of syndapin I alone does not occur in vivo.

Knowledge of the self-association interfaces within syndapin I should enable us to create both an oligomerization-deficient mutant and a fragment containing exclusively the self-association region and to use them to analyze the importance of self-association for syndapin function in detail. Since we did not identify the syndapin I oligomerization interfaces based on protein structure analyses, we next systematically analyzed them experimentally. Since the ΔSH3 fragments of syndapins are still capable of self-association (Figs. 2 and 3), we generated three further C-terminal truncations that were readily expressed in HEK293 cells (Fig. 5, F and G). Coimmunoprecipitations of these GFP fusion proteins with FLAG-tagged full-length syndapin I revealed that GFP-syndapin I 1–337 (ΔNPF + SH3), GFP-syndapin I 1–289 (i.e. the fragment comprising all of the α-helical N terminus) as well as the shortest GFP fusion protein generated, which lacks the coiled-coil domain and only includes the so-called Fes/Cip4 homology (FCH) domain (GFP-syndapin 11–128) were all coimmunoprecipitated specifically (Fig. 5F).

We next asked whether the FCH domain would be sufficient for oligomerization. Although the FLAG-FCH domain (syndapin I 1–128) was only expressed at very low levels (data not shown), our analyses clearly showed that the GFP-FCH domain (syndapin I 1–128) was indeed specifically coimmunoprecipitated with the FLAG-tagged FCH domain (Fig. 5G).

In cross-link experiments, we aimed to confirm that the N-terminal regions were sufficient for self-association and additionally addressed the question of what type of complexes are assembled by the N-terminal fragments. GFP-syndapin I 1–128 was detected with an apparent molecular mass of about 40 kDa (Fig. 5H). The addition of increasing amounts of the cross-linker EDC led to a strong decline of this monomeric species and gave rise to complexes that were detected at about 80 kDa. The highest EDC concentrations of 15 and 50 mM additionally led to complexes of about twice that size, whereas the 80-kDa species were diminished (Fig. 5H). Control cross-link experiments with GFP alone did not reveal any formation of protein complexes (data not shown). The observed syndapin complex formations were thus mediated by the syndapin I FCH domain part of the fusion protein.

FCH domains are relatively well conserved throughout evolution, but their functions are unknown. FCH domains can be found in about 30 different proteins, many of which control the actin cytoskeleton. Fig. 5I depicts the proteins that are widely present in eukaryotes; proteins exclusively conserved among Ascomycota were omitted. Usually, the FCH domain is positioned near the N termini of the proteins (Fig. 5I). Our data strongly suggest that the FCH domain may be a widely used α-helical oligomerization module.

structure predictions (i.e. the syndapin N terminus is predicted to be highly α-helical), 8 domain structure of syndapin I. C, schematic representation of the different syndapin I constructs used for the interaction examinations in this study. The exact amino acid numbers are indicated. D, coimmunoprecipitation analysis of GFP-coiled-coil domains (CC) of syndapin I in combination with the FLAG-tagged full-length protein. Expression of the constructs was visualized with anti-tag antibodies. Immunoprecipitates were analyzed for successful immunoprecipitation by immunoblotting with polyclonal anti-syndapin antibodies. Immunoblotting of the precipitates with polyclonal anti-FLAG antibodies showed that the coiled-coil domain-encompassing syndapin fusion protein was not coimmunoprecipitated with FLAG-syndapin I full-length. E, cross-link studies of GFP-coiled-coil domain of syndapin I with EDC concentrations from 0 to 50 mM final also showed no formation of syndapin coiled-coil domain complexes. F, coimmunoprecipitation analyses revealed that different C-terminally truncated GFP-syndapin I fusion proteins associate with FLAG-tagged syndapin I full-length. Lysates of cotransfected HEK293 cells and immunoprecipitated proteins were analyzed by immunoblotting with anti-FLAG (not shown) and anti-GFP antibodies, respectively. For the analysis of coimmunoprecipitated GFP fusion proteins polyclonal anti-GFP antibodies were used. IP, immunoprecipitation; Det, detected by. G, coimmunoprecipitation of GFP-syndapin I 1–128 with FLAG-tagged syndapin I 1–128 by anti-FLAG antibodies but not control IgGs shows that the N terminus of syndapin I is capable of and sufficient for self-association. H, cross-link studies with GFP-syndapin 11–128 with rising EDC concentrations (0–50 mM) analyzed by immunoblotting the resulting protein conjugates with anti-GFP antibodies confirmed the self-association capabilities of the FCH domain. I, FCH domain-containing proteins, which are conserved among different eukaryotic species, as revealed by conducting a HomoloGene search (available on the World Wide Web at www.ncbi.nlm.nih.gov). Conserved proteins that were found in Ascomycota only, such as Rgd1p, Rgd2p, Hop1p/Cdc15p, and Syp1p, are not depicted. SH2, Src homology 2; Rho-GAP, Rho-GTPase activating protein; HR1, protein kinase C-related homology region I; C1, protein kinase C conserved region I; WW, two conserved tryptophans domain.
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FIGURE 6. Identification of a second oligomerization interface interacting with the syndapin N terminus. A, coimmunoprecipitation analysis with FLAG-syndapin I full-length and different truncated GFP-syndapin I fusion proteins coexpressed in HEK293 cells. Lysates and coimmunoprecipitated proteins were immunoblotted with anti-GFP antibodies. Polyclonal anti-GFP antibodies were used for analysis of the coimmunoprecipitates except for lane 6, where a monoclonal anti-GFP antibody was used and the secondary antibody-horseradish peroxidase conjugate thus detected the IgG heavy chains (marked by an asterisk). Note that there is coimmunoprecipitation of pieces comprising amino acids 124–337 but not of the region 289–337 alone. IP, immunoprecipitation; Det., detected by. B, cross-link studies with GFP-syndapin I 124–337 with rising EDC concentrations. Immunoblotting analysis of the resulting protein conjugates with anti-GFP antibodies reveal only the presence of the monomeric protein, demonstrating that syndapin 124–337 is incapable to self-associate. C, coimmunoprecipitation of GFP-syndapin I 124–337 with FLAG-tagged syndapin I 1–128 by anti-FLAG antibodies shows that the second, more central oligomerization interface specifically associates with the N-terminal FCH domain.

After having demonstrated that the N terminus of syndapin I is sufficient for oligomerization, we next analyzed whether this interface would also be crucial for syndapin self-association. We thus deleted the N-terminal 124 amino acids from GFP-syndapin I ASH3 and tested the resulting truncated protein in coimmunoprecipitation experiments with FLAG-tagged full-length proteins. Surprisingly, we observed that GFP-syndapin I 124–386 was still specifically coimmunoprecipitated with anti-FLAG antibodies (Fig. 6A). These data suggest that a second region involved in oligomerization exists and that it is located somewhere between the FCH and the SH3 domain.

In order to identify this region, we systematically truncated the fragment 124–386 further. We subjected both GFP-syndapin I 124–337, a fragment additionally lacking the NPF region, and the NPF region alone (GFP-syndapin I 336–386) to coimmunoprecipitation analyses with FLAG-tagged full-length protein. Whereas GFP-syndapin I 124–337 was still specifically coimmunoprecipitated, the NPF region of syndapin I alone (GFP-syndapin I 336–386) was not detected in the anti-FLAG coimmunoprecipitates (Fig. 6A). Thus, the SH3 domain, the NPF region, and the central coiled-coil domain (residues 124–289) (Fig. 5) are incapable of mediating syndapin self-association, whereas amino acids 124–337 do interact with full-length syndapin I.

In coimmunoprecipitates from experiments with GFP-syndapin I 289–337 and FLAG-full-length syndapin I, no coimmunoprecipitated GFP-syndapin I 289–337 at a size of 30 kDa was detectable using monoclonal anti-GFP antibodies (Fig. 6A). Only the heavy chains of the anti-FLAG antibodies (Fig. 6A), which had quantitatively immunoprecipitated the FLAG-tagged full length (data not shown), were strongly detected. It can thus be concluded that both the peptide 289–337 alone and the coiled-coil domain 124–289 (Fig. 5) alone are incapable of interacting with full-length syndapin in vivo (Fig. 6, A and B) but together form the second self-association module.

To gain further insight into the topology of syndapin superstructures, we next addressed the question of whether this second syndapin/syndapin interaction module has the capability to self-assemble or whether it associates with other parts of the syndapin molecule. Cross-link studies clearly demonstrated that GFP-syndapin I 124–337, visible as a band of 45–50 kDa, has no tendency to form protein complexes with itself (Fig. 6B). Theoretically, GFP-syndapin I 124–337 should be able to associate with endogenous syndapin, but such complexes are not seen due to the low amount of endogenous syndapin present in HEK293 cells. Since we also demonstrated that the NPF region and the SH3 are not involved in self-association (Figs. 2 and 6), this left only the N-terminal FCH domain as the binding interface for the central self-association region. Indeed, GFP-syndapin I 124–337 coexpressed with FLAG-tagged syndapin I 1–128 was successfully and specifically coimmunoprecipitated with anti-FLAG antibodies (Fig. 6C). These results are consistent with cross-link studies showing an association of coexpressed protein fragments comprising the N terminus with fragments containing the second, more central interaction region (data not shown).

As a conclusion of our analyses, we designed a mutant of syndapin I lacking both syndapin/syndapin interaction interfaces we identified, the N-terminal region of amino acids 1–123 comprising the FCH domain and additionally amino acids 290–335, which were crucial for syndapin/syndapin association via the central domain. This mutant of syndapin I (124–289 + 336–441) was successfully coexpressed with FLAG-syndapin I but was indeed not coimmunoprecipitated by anti-FLAG antibodies (Fig. 7A). Consistently, the self-association-incompetent mutant fused to GFP was also not coimmunoprecipitated with the FLAG-tagged version of it (data not shown). Likewise, we did not detect any higher molecular weight species of the self-association-incompetent mutant in cross-link studies (data not shown).

GFP-syndapin I 124–289 + 336–441 is self-association-deficient but still contains the protein interaction modules revealed to be important for syndapin protein interactions with components of membrane trafficking machineries and of the actin cytoskeleton (23, 32). We therefore used the self-association-incompetent mutant to analyze the importance of syndapin self-association for both syndapin-mediated cortical actin cytoskeleton rearrangements and endocytosis. Overexpression of syndapins leads to the formation of filopodial structures protruding from lamellipodial sheets. This phenotype is dependent on the Arp2/3 complex and its activation by molecules, such as N-WASP bound by the syndapin SH3 domain. Consistently, this phenotype was dependent on a functional syndapin SH3 domain, which however needed to be embedded into the rest of the
domain interactions may be the molecular mechanism for syndapin-mediated cortical cytoskeleton rearrangements. In contrast to overexpression of wild-type GFP-syndapin I (Fig. 7, B and C), the self-association-incompetent mutant failed to elicit the syndapin-mediated cortical actin rearrangements (Fig. 7E); nor did the fragment lacking the entire α-helical self-association domain (Fig. 7D). Overexpression of the wild-type protein resulted in extended lamellipodial areas, which were marked by accumulated F-actin staining at the leading edge and decorated to a variable extent with filopodial structures (Fig. 7, B and C). Due to the decoration with membrane folds and filopodia-like protrusions of different length, the leading edges usually appeared rough or very thorny (see especially Fig. 7B). This observation was consistent with previous observations with Xpress-tagged syndapin I (24). The described morphological features were used for quantitative analyses in blind studies, which highlighted the phenotype induction by GFP-syndapin I (Fig. 7G). Whereas about 60% of the cells show a robust phenotype upon overexpression of full-length GFP-syndapin I, the self-association-incompetent mutant 124–289 + 336–441 and the fragment lacking the entire N-terminal region of syndapin I involved in self-association (GFP-syndapin I 336–441) both yielded only 25–30% of cells with actin-rich lamellipodia decorated with filopodia (Fig. 7G). These values represented only a slight increase in comparison with the values of 10–15% obtained for the SH3 domain and for syndapin ΔSH3, which were indistinguishable from control (Fig. 7G). Interestingly, overexpression of the self-association region of syndapin I alone (residues 1–337) led to a suppression of dynamic actin structures at the cell cortex below control levels (Fig. 7, F and G). Taken together, this suggests that for the induction of cortical actin cytoskeletal rearrangements, both the syndapin I SH3 domain and the protein interfaces mediating syndapin self-association are required.

Since self-association-incompetent syndapin mutants failed to support syndapin-mediated cortical actin polymerization, they represent a tool for addressing whether syndapin-mediated actin polymerization during vesicle formation contributes to the receptor uptake mechanism, as previously hypothesized (24). Indeed, overexpression of the self-association-incompetent syndapin I 124–289 + 336–441 mutant inhibited the receptor-mediated uptake of transferrin (Fig. 8, A and B). About 70% of the cells were significantly affected, and about 40% showed a complete block of transferrin uptake (Fig. 8E). The defect caused by just deleting the self-association interfaces was indistinguishable from those caused by overexpressing a construct lacking the entire α-helical self-association region from syndapin I (Fig. 8, C and D) and by overexpression of the N-WASP and dynamin-binding SH3 domain alone. Overexpression of wild type full-length syndapin I, in contrast, had only a very moderate effect on endocytic uptake (Fig. 8E). Thus, solely deleting the self-association interfaces from syndapin I is sufficient to disrupt syndapin functions and to transform it into a dominant-negative effector.

We have previously demonstrated that syndapin-mediated actin polymerization involves an Arp2/3 complex activator bound by the syndapin SH3 domain and can be suppressed by the N-WASP C terminus, which aberrantly activates the Arp2/3 complex in the cytosol (24). We have also shown that reconstitution of syndapin-N-WASP complexes in vivo led to local actin polymerization, which was dependent on the syndapin SH3 domain, the N-WASP PRD, and the Arp2/3 complex (20). To address the role of syndapin self-association in N-WASP-mediated cytoskeletal processes, we made use of the cortical phenotype that Miki et al. described in COS-7 cells upon N-WASP overexpression and epidermal growth factor stimulation (28). In line with the literature, the majority of the N-WASP-transfected cells were marked by filopo-
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FIGURE 8. Deletion of solely the two self-association interfaces of syndapin I is sufficient to create a mutant with dominant negative effects on receptor-mediated endocytosis. A–D, receptor-mediated endocytosis of Alexa Fluor 488-transferrin conjugates (B and D) is impaired in COS-7 cells overexpressing epitope-tagged syndapin I 124–289 + 336–441 (A self-association interfaces) (B) and syndapin I 336–441 lacking the entire self-association region (C). The crosses indicate the positions of transiently transfected cells not visible in the transferrin images. Bar, 20 μm. E, quantitation of the results by assessing the percentages of cells lacking transferrin signal (blockade of endocytosis; dark gray), displaying significantly reduced levels of uptake (light gray), and showing endocytosis capabilities similar to untransfected cells (white). The error bars represent S.D. from the means of several independent assays: untransfected cells, 86.5 ± 5.5% normal, 85 ± 3.6% reduced, 5.0 ± 2.4% block (n = 333); syndapin I SH3 domain, 24.3 ± 2.5% normal, 31.7 ± 3.1% reduced, 43.9 ± 3.3% block (n = 379); syndapin I 336–441, 29.9 ± 5.9% normal, 24.8 ± 4.8% reduced, 45.3 ± 9.4% block (n = 420); syndapin I 124–289 + 336–441, 30.0 ± 5.0% normal, 30.3 ± 3.5% reduced, 39.8 ± 6.3% block (n = 436); syndapin I full-length, 67.3 ± 10.1% normal, 22.6 ± 6.0% reduced, 10.1 ± 5.6% block (n = 381).

dia-like structures that either extended in all three dimensions (Fig. 9A) or mainly decorated the leading edges of the cells (Fig. 9B). In order to be able to compare the different effects, we quantified all of our results (Fig. 9C–E). The cortical phenotype described by Miki et al. (28) was observed in two-thirds of all N-WASP-transfected COS-7 cells. Since only 18% of the untransfected and 24% of the GFP-transfected control cells showed comparable appearances, albeit usually less pronounced, N-WASP overexpression caused a significant change in morphology (Fig. 9D).

We have shown previously that the syndapin SH3 domain alone was sufficient to elicit N-WASP-Arp2/3 complex-dependent actin polymerization in vivo (33). We therefore asked whether the N-WASP phenotype would be suppressed by a syndapin fragment solely comprising the self-association domain. Since this tool cannot interact with N-WASP, it is expected to leave associations of endogenous syndapin with N-WASP unaffected but to interfere solely with the self-association of endogenous syndapins. Overexpression of Xpress-syndapin I 1–337 led to a strong suppression of the N-WASP-induced phenotype, and double-transfected cells had smooth cell borders very similar to untransfected cells (Fig. 9, C–E). This effect was apparently dose-dependent. Cells coexpressing only extremely low amounts of syndapin I 1–337 still showed the N-WASP phenotype (Fig. 9, F–H, cell marked with black asterisk), whereas cells expressing syndapin I 11–337 at higher levels lacked the N-WASP phenotype (Fig. 9, F–H, cell marked with white asterisk). With only 34% of the cells showing some N-WASP-like cortical phenotype, the suppression by syndapin I 1–337 was drastic. In contrast, a slight increase of positively scored cells was observed by cooverexpression of fully functional syndapin I, which alone had only moderate effects in COS-7 cells (31% (Fig. 9D), Xpress-syndapin I full-length cooverexpression led to 75% of the cells showing the N-WASP-induced microspike formation (Fig. 9E). These data together with our previous observations (20, 24, 33) confirm that syndapin-N-WASP-Arp complexes play an important role in actin dynamics.

Direct syndapin/syndapin self-association may thus be a molecular mechanism by which the actin polymerization burst observed in tight coordination with endocytic vesicle formation in time and space (13) is brought about. Syndapin oligomers could theoretically interconnect different syndapin SH3 interaction partners, such as dynamin controlling the fission reaction and N-WASP activating the Arp2/3 complex, in one complex. To directly address this experimentally, we conducted coimmunoprecipitation experiments with lysates of HEK293 cells transfected with HA-dynamin I, Xpress-syndapin I, and GFP-N-WASP (Fig. 10). GFP-N-WASP was specifically immunoprecipitated with anti-GFP antibodies (Fig. 10B). Both Xpress-syndapin I (Fig. 10B) and HA-dynamin (Fig. 10A) were specifically coimmunoprecipitated with N-WASP. In the corresponding experiments, HA-dynamin I, specifically immunoprecipitated with the anti-HA antibodies (Fig. 10A), coimmunoprecipitated both Xpress-syndapin (Fig. 10B) and GFP-N-WASP (Fig. 10C), but not GFP alone.3 These data indicate that dynamin, syndapin, and N-WASP indeed exist in one complex in vivo. Interestingly, omitting Xpress-syndapin I did not disrupt the interconnection of dynamin with N-WASP. Analysis of the immunoprecipitates by immunoblotting with pan-syndapin antibodies revealed two syndapin bands, one at about 56 kDa representing Xpress-syndapin I and an additional 65 kDa band (Fig. 10B). This 65 kDa signal corresponds to the ubiquitously expressed syndapin II, since it was not detected with antibodies specific for syndapin I (data not shown). Interestingly, the syndapin II signal increased in coimmunoprecipitates from lysates of cells transfected with only HA-dynamin and GFP-N-WASP (Fig. 10B). Thus, endogenous syndapin II did apparently contribute to the tertiary complexes formed and was furthermore able to substitute for syndapin I. This is in line with our findings that also syndapin II self-associates and can furthermore form heterodimers with syndapin I.4

In order to formally demonstrate that N-WASP and dynamin effec-

3 M. M. Kessels and B. Qualmann, unpublished observations.
4 M. M. Kessels and B. Qualmann, manuscript in preparation.
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FIGURE 9. Suppression of the N-WASP overexpression phenotype by cooverexpression of the syndapin self-association region alone. A and B, COS-7 cells were transfected with GFP-N-WASP (transfected cells are marked by asterisks) and stimulated with epidermal growth factor according to Ref. 28 and stained for F-actin using phalloidin-Alexa568, visualizing the induction of filopodia-like structures. C–H, coexpression of the Xpress-tagged syndapin I self-association region (residues 1–337) (i.e. of a construct lacking the SH3 domain associating with N-WASP (D and G) with GFP-N-WASP (C and F) strongly suppresses the N-WASP phenotype. Note that this suppression is dose-dependent, since GFP-N-WASP-expressing cells (C and F) with little syndapin 1–337 coexpressed (black asterisk in F and H) still show the N-WASP phenotype, whereas in cells expressing more readily detectable levels of syndapin 1–337 (white asterisk in G and H), the suppression of the phenotype is very effective. I, quantitative analyses of microspike induction in COS-7 cells transfected with GFP, GFP-N-WASP, syndapin I 1–337, and full-length syndapin I alone as well as in combination. A cortical phenotype as described by Miki et al. (28) for N-WASP overexpression was observed for 17.7 \( \pm \) 3.0% of the untransfected cells (337 cells scored), 24.0 \( \pm \) 3.1% of the GFP-transfected cells (331 cells scored), 65.4 \( \pm \) 0.9% of the N-WASP-transfected cells (387 cells scored), 33.8 \( \pm \) 2.0% of the N-WASP and syndapin I 1–337-cotransfected cells (313 cells scored), 74.5 \( \pm \) 7.8% of the N-WASP and syndapin I-cotransfected cells (348 cells scored), and 30.9 \( \pm \) 7.7% of the syndapin I-transfected cells (316 cells scored). Errors are S.D.

We designed a mitochondrially targeted N-WASP, which was successfully targeted to mitochondria in COS-7 cells, as demonstrated by a colocalization with MitoTracker (data not shown). We thus cotransfected this construct together with syndapin I and dynamin (Fig. 11). In cells expressing mitochondrially targeted N-WASP (Fig. 11A), Xpress-tagged full-length syndapin I (Fig. 11B) was successfully recruited to mitochondria, as evidenced by the fact that syndapin adopted a distribution pattern similar to mito-GFP-N-WASP in these cells. Importantly, coexpressed HA-dynamin 1 (Fig. 11C) also displayed a pattern very similar to that of syndapin I and mito-GFP-N-WASP in triple-transfected cells (Fig. 11, A–C). We observed both individual mitochondria carrying N-WASP-dynamin complexes (Fig. 11, A–C, arrows) as well as aggregates of mitochondria that were positive for all three proteins (Fig. 11, A–C, arrowheads). The formation of such clusters of mitochondria is probably due to the interconnecting properties of the recruited proteins, since we did not see such an effect in cells transfected with mito-GFP N-WASP alone (data not shown).

The fact that HA-dynamin 1 (Fig. 11F) is neither recruited to wild-type mitochondria (not shown) nor to mitochondria coated with GFP (Fig. 11D) when overexpressed alone (not shown) or together with syndapin (Fig. 11E) shows that both the dynamin and the syndapin enrichment observed in our triple expression experiments (Fig. 11, A–C) is specifically dependent on N-WASP targeted to mitochondria.

In line with syndapin oligomers acting as a clamp between N-WASP and dynamin, cooverexpression of the self-association-incompetent...
syndapin I mutant (124–289 + 336–441) (Fig. 11I) suppressed the mitochondrial accumulation of HA-dynamin (Fig. 11J) at N-WASP-coated mitochondria (Fig. 11J) and led to a relatively diffuse localization of dynamin (Fig. 11, H–K).

Consistent with previous examinations that showed that syndapins directly bind to the PRD of N-WASP (20), offering this domain at mitochondrial membranes was sufficient to recruit syndapin and dynamin. Despite the technical limitation that mito-GFP-N-WASP PRD expression was very low and the mitochondrial targeting was not very robust, a colocalization with both the direct binding partner syndapin and with the direct binding partner dynamin was seen (Fig. 11, L–Q). Merging all three fluorescence signals (Fig. 11Q) as well as merging only mito-GFP-N-WASP PRD and dynamin signals (Fig. 11P) clearly revealed similar localization patterns of the complex components.

**DISCUSSION**

Syndapins participate in both receptor-mediated endocytosis and actin organization, probably represented by the syndapin interactions with (i) the large GTPase dynamin and (ii) the Arp2/3 complex activator N-WASP. Syndapins would therefore be able to coordinate vesicle formation with the observed local actin polymerization at sites of endocytosis (13). The syndapin interactions with both machineries, however, are mediated by the same binding module, the single C-terminal SH3 domain domain (23, 24). Only by building macromolecular complexes with multiple SH3 domains free for further interactions would syndapins be able to directly couple different cellular functions mediated by SH3 domain interactions. In this study, we experimentally show this property of syndapins and demonstrate its importance for both syndapin-mediated cortical actin reorganizations and endocytic vesicle formation.

Our gel filtration analyses of rat brain high speed supernatants are in line with the hypothesized formation of such complexes. A large portion of syndapin I is present in protein complexes with molecular weights higher than expected for complexes composed of only a syndapin and one SH3 domain interaction partner, such as dynamin or N-WASP. That syndapins within such large complexes are indeed complexed with dynamin and N-WASP and do not just cofractionate with these proteins was demonstrated by coimmunoprecipitations.

In order to reveal the molecular mechanisms by which syndapins may play such a connecting role, we experimentally addressed our earlier hypothesis of a potential oligomerization of syndapins (23). Our examinations applying a set of different methods indeed demonstrate that syndapin I forms oligomers both in vitro and in vivo. Putative postsolubilization artifacts in the cross-link and coimmunoprecipitation experiments performed can be firmly excluded, because we were also able to reconstitute the syndapin self-association at mitochondrial membranes of intact cells. The robust coimmunoprecipitations and in vivo recruitment observations suggest that the underlying syndapin/syndapin protein interactions are of high affinity. The reconstitutions with purified proteins clearly demonstrate that the syndapin/syndapin interaction is direct. Direct syndapin/syndapin interactions are also strongly supported by the efficient chemical cross-link of syndapin I and fragments thereof by a zero-length cross-linker, because only molecules in intimate contact are connectable by such cross-linkers. Importantly, our examinations also revealed that these interactions are SH3 domain-independent. Syndapin oligomers with several SH3 domains free for further interactions could create multimeric complexes interconnecting proline-rich domain-containing proteins, such as dynamin and N-WASP. We have therefore experimentally demonstrated that syndapins indeed fulfill a prerequisite for physically linking different cellular machineries, such as those for actin polymerization and endocytic vesicle formation, by SH3 domain interactions.

Also, other SH3 domain-containing proteins that were identified as components of the endocytic machinery are multivalent, either via dimerization (34, 35) or via the presence of multiple SH3 domains (36, 37). In endophilins and amphiphysins, homo- and heterodimerizations appears to be mediated through a common structural motif, the so-called BAR domain (30) (reviewed in Ref. 38). Our analyses indicated that syndapins contain a central α-helical structure that probably forms a helix bundle (23) and may show some similarity to a portion of BAR domains, as defined by Peter et al. (30). However, coimmunoprecipita-
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FIGURE 11. Reconstitution of complexes composed of N-WASP, syndapin, and dynamin at mitochondrial membranes. A–C, mito-GFP-N-WASP (A) recruits Xpress-syndapin I (detected by anti-syndapin antibodies) (B) and HA-dynamin 1 (detected by anti-HA antibodies) (C). The arrowheads point to aggregates of mitochondria immuno-positive for all three proteins, and arrows highlight single mitochondria covered with N-WASP–syndapin–dynamin complexes. D–G, GFP-coated mitochondria (D) accumulate neither Xpress-syndapin I (E) nor HA-dynamin 1 (F). Instead, syndapin and dynamin show a localization pattern that is similar to each other’s but distinct from the mitochondrial pattern, as also well seen in the merged image (G; mito-GFP in green, syndapin in blue, and dynamin in red). H–K, syndapin I124–289 + 336–441 (Δself-association interfaces), as detected by polyclonal antibodies directed against the syndapin SH3 domain (L), fails to recruit coexpressed HA-dynamin 1 (J) to GFP-N-WASP-enriched mitochondria (H), as also seen in the merged image (K; GFP-N-WASP in green, syndapin in blue, and dynamin in red). L–Q, the mitochondrially targeted PRD of N-WASP (L) is also able to recruit Xpress-syndapin I (M) and HA-dynamin (G). N, merged image of GFP-N-WASP PRD in green and syndapin in blue; overlap appears turquoise. O, merged image of GFP-N-WASP PRD in green and dynamin in red; overlap appears yellow. Q, merged image of GFP-N-WASP PRD in green, syndapin in blue, and dynamin in red; overlap appears white. Bars for A–C and D–G, 10 µm. Bars for H–K and L–Q, 5 µm.

Construction experiments and cross-link studies both consistently demonstrate that the α-helical region encompassing the predicted coiled-coil stretch within syndapin I is not sufficient to mediate syndapin/syndapin self-association. Thus, although syndapin contains an α-helical structure that may show some similarity to a portion of BAR domains, our analyses clearly show that this central domain of syndapin I is unable to form a dimer.

Our extensive coimmunoprecipitation and cross-link analyses allowed us to pinpoint the molecular interface for the syndapin/syndapin interaction within the α-helical part of syndapin I to the first 128 amino acids. This part of syndapin corresponds to the N-terminal FCH domain. FCH domains occur in about 30 further proteins, which are highly conserved throughout evolution. Many of these proteins control the actin cytoskeleton, but until now the role of the FCH domain within these proteins remained rather obscure. Both our coimmunoprecipitation and cross-link analyses clearly show that the FCH domain is an α-helical self-association module.

In addition to the FCH domain, we identified a second self-assembly interface in the central region of syndapin that, in contrast to the FCH domain, is not able to associate with itself but binds to the N terminus, as demonstrated in cross-link and coimmunoprecipitation analyses. Related analyses for syndapin II-1 and syndapin II-2 led to similar findings. This is well in line with high sequence conservation among syndapins in the N-terminal, α-helical part. In the second self-association interface, that spans the syndapin I amino acids 124–337, the residues 289–337 turned out crucial but not sufficient for oligomerization.

This identification of two self-association modules within syndapins is interesting, because it would permit the formation of larger syndapin lattices formed upon combination of two dimerization reactions. Additionally, we have shown by cross-links that the FCH domain alone is capable of forming higher oligomers. In line with the putative formation of larger syndapin lattices, high molecular weight complexes of defined sizes formed by different purified recombinant syndapin fusion proteins were indeed observed in our gel filtration analyses.

Functionally, syndapin oligomers seem to be of special importance at the cell cortex. The self-association-incompetent mutant of syndapin I was unable to elicit the syndapin-mediated cortical actin phenotype, and a fragment capable of self-association but lacking the NPF and SH3 domains even had a negative effect. These findings are in line with our observation that the syndapin-mediated actin phenotype relies on a successful targeting of the Arp2/3 complex to the plasma membrane. This targeting can be disrupted by cooverexpression of the C-terminal WA domain of N-WASP. This fragment lacks the domains that mediate the interactions with molecules that control N-WASP activity and may target N-WASP to the cell cortex (including the syndapin binding interface). It can thus be hypothesized that the N-terminal part of syndapin I (i.e., the region that self-assembles) may also mediate transient membrane associations. The observation that syndapins accumulate in membrane folds in vivo and that syndapins distribute into different membrane fractions may relate to this. Such a targeting to the cell cortex could either be brought about by a direct membrane association of a syndapin oligomer or an SH3 domain- or NPF-mediated interconnection via a second syndapin molecule complexed with the N-WASP-interacting one. Consistently, our analyses show that the...
function of the syndapin N terminus is crucial for the N-WASP-induced cortical actin rearrangements.

A combination of membrane- and self-association properties within one protein domain is reminiscent of the proposed features of BAR domains. BAR domains can dimerize and were suggested to be involved in membrane association, although they often seem not to associate with membranes themselves but work in conjunction with other established lipid-interacting domains (30). Therefore, and due to their banana shaped appearance, dimers of two BAR domains were hypothesized to just sense the curvature of lipid layers rather than bind them tightly (30). While this manuscript was in revision, the FCH domain of the name-giving protein CIP4 and of the related protein FBP17 was suggested to be part of a structure spanning about 260 amino acids, which secondary structure predictions suggested to be similar to BAR domains (30) composed of three α-helices and which was thus called F-BAR (39). This domain of FBP17 and CIP4 bound to lipids. Interestingly, the full-length proteins FBP17 and CIP4 do not only bind lipids in vitro but also tubulate the plasma membrane in vivo (39, 40). Itoh et al. (39) suggested that the F-BAR is responsible for this property and that the underlying function similarly to BAR domains is membrane curvature induction. The entire stretch shows a low but maybe functionally relevant sequence similarity to syndapin I, and syndapin 1 full-length indeed binds to lipids in vitro as well (39). It has, however, to be noted that syndapin overexpression did not tubulate the plasma membrane (40). Consistently, in FBP17 the so-called F-BAR was not sufficient for tubulation but further C-terminal regions were required, which at least include a proline-rich stretch (40) not conserved in syndapin I.

Syndapin oligomers that are formed in a non-SH3 domain-dependent manner and are targeted to the plasma membrane by some yet to be identified mechanism would allow syndapins to physically connect several of the SH3 domain interaction partners (e.g. dynamin and N-WASP) at the cell cortex. They may thereby mediate the tight spatial and temporal coordination of endocytic vesicle formation with the actin polymerization bursts (13), which involve the Arp2/3 complex and the syndapin binding partner N-WASP (15). Our studies indeed experimentally demonstrated the existence of dynamin, N-WASP, and syndapin in one complex by two independent means in vivo. We reconstituted such complexes at membranes in living cells and isolated them by immunoprecipitation. The specific coimmunoprecipitation of both the direct dynamin binding partner syndapin I and the direct syndapin I binding partner N-WASP with immunoprecipitated dynamin and the corresponding immunosialation of N-WASP-, syndapin-, and dynamin-containing complexes upon immunoprecipitation of N-WASP strongly argues for the existence of multimeric complexes, in which dynamin and N-WASP are both bound by the SH3 domains of a syndapin dimer that acts as a multivalent interconnector. This dependence on syndapin self-association was highlighted by the fact that coexpression of a self-association-incompetent mutant suppressed the recruitment of dynamin to sites of high N-WASP abundance in vivo.

During endocytosis, the interaction with the dynamin-associated syndapin would allow for a specific recruitment of N-WASP to the neck of coated pits and thereby induce a polarity of actin polymerization and a directed movement of a newly formed vesicle away from the plasma membrane by creating actin structures with the appropriate localization, timing, and polarity for detaching and moving vesicles. The detection of N-WASP at the interface of endo- and lysosomes and actin tails in Xenopus extracts (41) and our finding that overexpression of all syndapin fusion proteins that are not capable to oligomerize but contain the dynamin- and N-WASP-binding SH3 domain have a strong dominant-negative effect on receptor-mediated endocytosis support such a scenario. Such syndapin fragments will compete with endogenous proteins for association with dynamin and N-WASP but then fail to make the obviously crucial interconnections between syndapin SH3 domain interaction partners. We thus propose that syndapins belong to an emerging meshwork of adaptor or scaffolding molecules containing multiple protein interaction modules that appear to play an important role in the structural organization and/or functional coordination of the endocytic process and that this might allow syndapins to mediate contributions of the N-WASP-Arp2/3 complex machinery for actin nucleation at different stages of the endocytic process.

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