II Materials and Methods

2.1 Materials

2.1.1 Enzymes and antibodies

Enzymes

- Restriction enzymes and T4 DNA ligase were obtained from (Amersham Life Science, Freiburg, Germany) or from (Promega, Mannheim, Germany)
- Taq DNA polymerase was product of (InViTek, Berlin-Buch, Germany)

Antibodies

- Antibodies to α-adaptin, β-adaptin, γ-adaptin, clathrin heavy chain, amphiphysin I, AP180, EEA1, EPS15, p47A, syntaxin 6 and LAMP-1 were obtained in form of the "coated vesicle sampler antibody kit" and "organelles sampler kit" (Transduction Laboratoties, Germany).
- Rabbit IgG against syndapin I and syndapin II was a gift from Dr. B. Qualmann (Leibnitz-Institut für Neurobiologie, Magdeburg, Germany)
- Rabbit anti-serum against human pacsin II (syndapin 2) was kindly provided by Dr. M. Plomann (Institut für Biochemie II, Medizinische Fakultät, Universität Köln, Germany)
- A monoclonal antibody to rat dynamin II which recognizes an epitope located between 274 to 555 was purchased from Transduction Laboratory.
- A polyclonal antibody (IgG) to dynamin II was generated by immunization of rabbits with the C-terminal peptide of human dynamin II (TIIRPAEPSLDD) covalently linked to keyhole limpet hemocyanin (Maier et al., 1996).
- The antibody to TGN38 was obtained by the same procedure above using the C-terminal peptide (FALEGKRSKVTRRPKASDYQRLNLKL).
- The antibody to bacterial GST was raised in goat (Rockland, Gilbertsville, PA).
- Goat IgG against rabbit IgG conjugated with Cy3 and goat IgG against mouse IgG conjugated with Cy2 were from DIANOVA (Hamburg, Germany)

Miscellaneous

- Protease inhibitor tablets (Boehringer Mannheim GmbH, Germany)

- $[S^{35}]$-sulfate, specific activity 1325Ci/mm(49025GBq/mm) was from NEN™ Life Science (Boston, USA)
- PCR Kit and PCR purification Kit (InViTek)
- Plasmid and DNA gel purification kits, Ni-NTA-agarose, (QIAGEN, Hilden, Germany)
- S-protein agarose, (Novagen, Germany)
- Glutathione-sepharose 4B beads (Pharmingen Europe, Germany)
- Mitochondria were stained with MitoTracker Red CMXRos (Molecular Probes, Göttingen, Germany)
- Scintillator: 0.4% PPO, 0.005% POPOP, dissolved in toluene.

2.1.2 Vectors and cDNAs

- pTrcHis2 and pcDNA4/TO/*myc*-His vectors were purchased from Invitrogen (Groningen, The Netherlands)
- pET30a(+) was from Novagen (Bad Soden, Germany)
- pEGFP-C1 was purchased from Clontech (Heidelberg, Germany)
- pGEX-2T-amph-SH3 (Ramjaun et al., 1997) was a gift from Dr. P. McPherson (Department of Neurology and Neurosurgery, Monteral Neurological Institute, McGill University, Canada)
- cDNA of human dynamin II, spliced variant (bb) was obtained from Dr. C. Diatloff-Zito (Institut Curie-Biologie, Paris, France) (Diatloff et al., 1995)

2.1.3 Proteins

SH3 domains of syndapin-1, syndapin-2- and of the P434L mutant of syndapin-1 were obtained from Dr. B. Qualmann (Leibnitz Institut for Neurology, Magdeburg, Germany) as GST fusion proteins.

2.1.4 Lipids

Lipids type X extracted from bovine brain with acetone were purchased from Sigma (Germany)

2.1.5 *E.coli* strains

E.coli Top-10 (Genotype: F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G) was purchased from Invitrogen.

E.coli strain HMS174 (F^- *rec*A1 *hsd*R (r_{K12}^- m_{K12}^+)Rif^R) and *E.coli* strain BL21(DE3) pLysS [F^- *omp*T *hsd*S_B(rB-mB-) *gal dcm* (DE3) pLysS (Cm^R)] were from Novagen.

2.1.6 Cell lines, media and antibiotics

The epithelial cell line, HepG2, derived from human hepatoblastoma, HeLa cells derived from human cervical adenocarcinoma, and COS-7 cells (kidney fibroblasts of the african green monkey) were from ATCC (USA). T-REx^{TM}293 cells, a human embryonic kidney cell line that constitutively expresses the tetracycline repressor protein from pcDNA6/TR plasmid, was from Invitrogen.

DMEM, sulfate-free DMEM, FCS and tetracycline-free FCS were from Biochrom KG (Berlin,Germany).

Blasticidin, Zeocin and tetracycline were products of Invitrogen.

2.1.7 Solutions and buffers

HCB 20: 20 mM NaCl, 20 mM HEPES, pH7.2, 1 mM DTT, 2 mM EGTA, 1 mM $MgCl_2$, PIT

HCB 150: HCB 0 + 150 mM NaCl

HME: 10 mM HEPES, pH 7.2, 1 mM $MgCl_2$, 0.1 mM EDTA

LB medium: 1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl, pH 7.0. For preparaton of LB agar plates, add 1.5% of agar to the LB medium.

M9E medium:

Composition of M9E medium:

M9 salts*	1x
D-(+)glucose	1%
$MgSO_4$(1 M)	0.2%
$CaCl_2$(1 M)	0.01%
Kanamycin	50µg/ml
Chloramphenicol	15µg/ml

* 20 X M9 salts:

$Na_2HPO_4.7HO_2$	256g
KH_2PO_4	60g
NaCl	10g
NH_4Cl	20g
Distilled H_2O	1000ml

Adjusted to pH 7.2 and sterilized by autoclave

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PBS: 0.8% NaCl, 0.02% KCl, 0.268% $Na_2HPO_4.7H_2O$, 0.024% KH_2PO_4, pH 7.4

SDS-sample buffer: 50 mM Tris-HCl, 10% glycerol, 2% SDS, 0.0025% bromophenol blue, 100 mM DTT, pH 6.8

Sucrose gradient solutions: (0.25 M, 0.8 M, 1.18 M, 1.2 M and 1.8 M sucrose) were prepared in HME buffer

Superdex 75 column binding bufer(runing buffer): 20 mM HEPES, pH7.5, 200 mM NaCl, 1 mM EDTA, 1 mM DTT

TAE: 0.04 M Tris-Actic acid, 0.001 M EDTA

TBS: 20 mM Tris-HCl, 137 mM NaCl, pH 7.6

TBST: TBS+0.1%Tween-20

TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH8.0

2.1.8 Equipments

- PCR thermocycler: Biometra TRIO-Thermoblock^{TM} (Germany)
- Centrifuge: Optima^{TM} LE-80K, BECKMAN (USA)
- The HPLC apparatus SPD-6AV/LC-9A from SHIMADZU (Germany) was equipped with a Superdex-75 colum HR 10/30 (Pharmacia Biotech, Freiburg, Germany)
- Inverted immunofluorescence microscope (Olympus IX70 type, Japan) was equipped with a confocal image recording system (Improvision, Coventry, England)
- Liquid scintilation counter: Trib-Carb (PACKARD, Franckfurt a. M, Germany)

2.2 Methods

2.2.1 Amplification of DNA fragments coding for the dynamin II domain PHD, PRD and PCP by polymerase chain reaction (PCR)

2.2.1.1 Primer design

The PH domain of dynamin II was cloned by using the primers

PHD1(27mer). 5' --GGA AGA TCT CAG CAG AGG AGC ACG CAG--3'

PHD2(26mer). 5' --GGG GAA TTC GCC AGC TCG GAG GAA CG----3'

PRD was cloned using the primers

PRD1(27mer). 5'-- GGA AGA TCT AGC ACT GTG TCC ACG CCT ---3'

PRD2(26mer). 5'---TTA GAA TTC GTC GAG CAG GGA TGG CT-----3'

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PCP was cloned using the primers

PHD1(27mer). 5' --GGA AGA TCT CAG CAG AGG AGC ACG CAG--3'

PRD2(26mer). 5'---TTA GAA TTC GTC GAG CAG GGA TGG CT-----3'

For DNA subcloning, a Bgl II restriction site was inserted in the upper primers and an EcoR I restriction site in the lower primers. 5´ ends of primers were extended by three bases to improve restriction enzyme cleavage. Primer design was optimized using the computer program WINSTAR-Primerselect to minimize putative self dimers, primer pair dimers and hairpin formation.

2.2.1.2 PCR reaction and product analysis

cDNA fragments of dynamin II domains were generated by PCR using three pairs of primers (Figure 10 A) based on the full length cDNA of human dynamin II (refer to Appendix A)

The PCR experiments were performed according to the protocol of the PCR kit supplied by InViTek (Berlin, Germany)

PCR reaction composition:

Reagents	Volume added	Final concentration
dNTPs(1.25mM)	16µl	200 mM
primers(4µM)	2.5-5µl/each	0.1-0.2 µM
$MgCl_2$(50mM)	4µl	2 mM
10× reaction buffer	10µl	1×
5×enhancer buffer	20µl	1×
Template(0.5ng/µl)	1.0µl	0.5 ng
CombiPol^{TM} DNA Polymerase	0.5µl	2U
Distilled water	44-60µl	
Total volume	100µl	

The PCR mixture was covered with 80 µl of sterilized light mineral oil to prevent evaporation.

Parameters of PCR reaction steps:

	Tem.(°C)	Time(sec)
Annealing:	54	60
Polymeraization	74	60
Denaturation	95	60

The reaction was continued over 20 – 25 cycles. Thereafter, product formation was completed by incubation at 74 °C for 10 minutes. The PCR products were analyzed by electrophoresis on 1.0 % EB-agarose gel in TAE buffer according to Sambrook.J et al.(1989).(Figure 10 B)

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2.2.1.3 Purification of PCR products

PCR reaction mixtures were frozen at –20°C, and the upper layer of mineral oil was removed. Amplified DNA was purified using the PCR purification Kit (InViTek).

2.2.2 Construction of plasmids

2.2.2.1 Plasmids used for expression of PHD, PRD, and PCP in *E. coli*

pTrcHis2C -based plasmids

The pTrcHis2C is a pUC-derived expression vector designed for efficient recombinant protein expression and purification in *E. coli*. High levels of expression are possible using the *trc* (*tr*p-*la*c) promoter and the *rr*nB anti-termination region. Isopropyl-β-D-thiogalactopyranoside (IPTG) is used to induce expression of inserted gene. The C-terminally fused peptide encodes for the *myc* epitope as a detection tag and for a $(His)_6$ peptide that allows us to purify the recombinant protein on Ni^{2+} chelate agarose (see Appendix B).

pTrcHis2C and purified PCR fragments of dynamin II were double digested with restriction enzymes Bgl II and EcoR I at 37°C for 1 h. DNA fragments were separated by EB-agarose gel electrophoresis and recovered from the gel using the QIAGEN® II Gel Extraction kit. Thereafter, 20-50 ng vector DNA and 100-200 ng PCR product were mixed for ligation for 12-18 h at 12-16°C by 1 unit of T4 DNA ligase in a total volume of 20µl (Figure 6 A).

E.coli Top-10 competent cells were transformed according to the standard protocols (Sambrook.J et al., 1989) with 5-10 µl ligation mixture. Positive colonies were selected from ampicillin-containing LB agar plates. The insertion of DNA fragments was confirmed by double digestion of plasmid DNA with Bgl II and EcoR I. (Figure 6 B)

The inserted dynamin II DNA fragments of the plasmid pTrc-PHD, pTrc-PRD and pTrc-PCP were PCR sequenced on an ABI-PRISM automatic DNA sequencer (Versin 2.1.1) using the corresponding primers. 200 µg of recombinant plasmids were purified using the QIAGEN® plasmid maxi kit.

pET-30a(+)-based plasmids

Expression of dynamin II domains with pET-30a(+) vector resulted in fusion proteins that contain an N-terminal $(His)_6$ tag followed by a S-peptide tag (Figure 7 and Appendix C).

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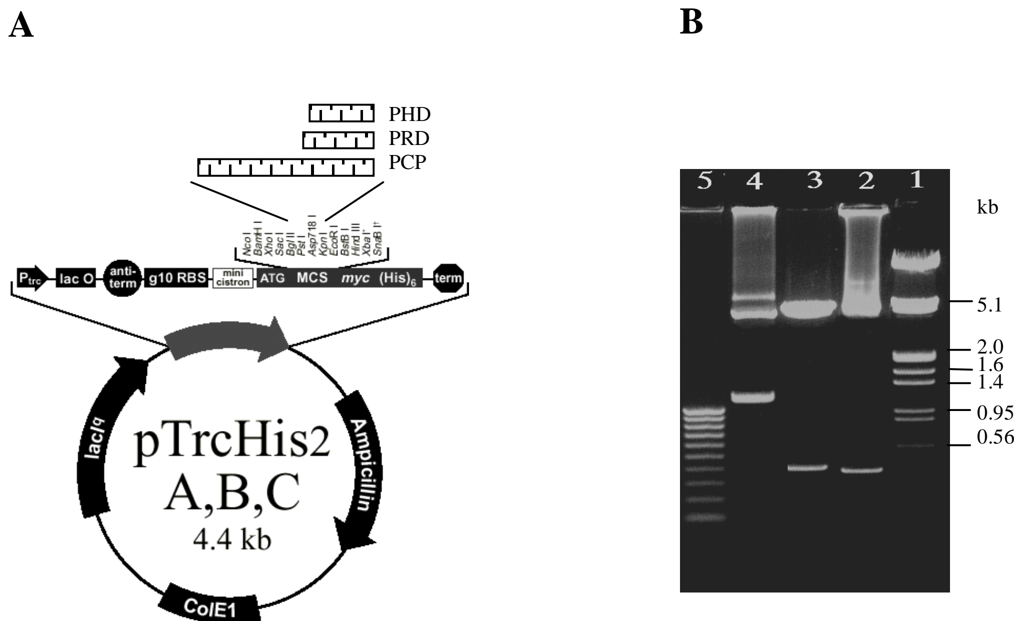
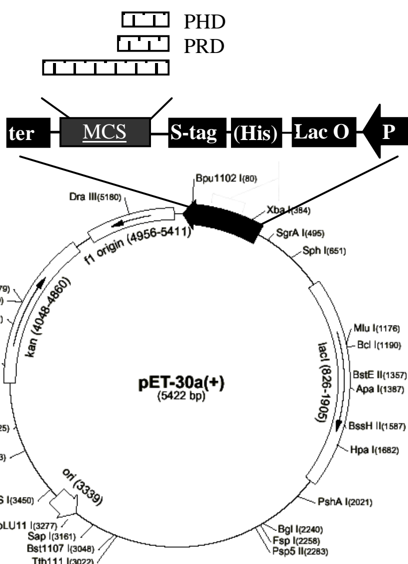


Figure 6. Construction of pTrcHis2C based plasmids. The PCR amplified dynamin II fragments, representing PHD, PRD and PCP, were cloned into pTrcHis2C vector between Bgl II and EcoR I sites (A). The recombinant plasmids were then digested with Bgl II and EcoR I and analyzed by EB-agarose-gel electrophoresis (B) [1: λDNA-Hind III-EcoR I; 2: pTrc-PHD; 3: pTrc-PRD; 4: pTrc-PCP; 5: 1 kb DNA ladder. Size of inserted fragments are 0.37 kb, 0.39 kb and 1.1 kb, respectively].



DNA fragments of dynamin II domains were subcloned into pET-30a(+) vector as follows:

Figure 7. Construction of pET-30a(+) based plasmids. The three fragments of dynamin II domains that had been cloned in pTrcHis2C vector were respectively subcloned into pET-30a(+) vector. Note that both $(His)_6$ and S-peptide were tagged at N-terminus.

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DNAs coding for the three dynamin II domains were obtained by restrictin cleavage of pTrc-PHD, pTrc-PRD and pTrc-PCP, respectively, with Bgl II and Hind III restriction enzymes, and purified using QIAGEN® II Gel Extraction kit. pET-30a(+) was digested with BamH I (compatible with Bgl II) and Hind III and purified as above. Dynamin II domain fragments were ligated with the linerized pET-30a(+) vector as described above. The resulting three recombinant plasmids, pET-30a(+)-PHD, pET-30a(+)-PRD and pET-30a(+)-PCP, were transformed into *E.coli* HMS174 for plasmid amplification or into *E.coli* BL21(DE3)pLysS for expression of dynamin II domain proteins.

2.2.2.2 Plasmids used for expression of dynamin II domains in mammalian cells

Transient expression of EGFP-tagged dynamin II domains

To study the localization of dynamin II domains in mammalian cells, EGFP tagged dynamin II domains were expressed using pEGFP-C1 constructs (Figure 8 and Appendix D). pEGFP-C vector encodes the GFPmut1 variant which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr to enhance the fluorescence light yield. The coding sequence of the EGFP gene contains more than 190 silent base changes which correspond to human codon-usage preferences. Insertion into pEGFP-C1 results in fusion proteins containing an N-terminal EGFP.

In order to preserve the reading frames and to avoid additional DNA sequencing work, the PRD and PCP fragments of plasmids pET-30a(+)-PRD and pET-30a(+)-PCP were cut by Bgl II and Hind III and ligated as above into pEGFP-C1 between Bgl II and Hind III sites. The PHD fragment was obtained by digestion of pTrcHis2C-PHD with Bgl II and EcoR I. The fragment was inserted into pEGFP-C1 vector between the Bgl II and EcoR I sites. The resulting three recombinant plasmids, named pEGFP-PHD, pEGFP-PRD and pEGFP-PCP (Figure 8), were transformed into competent *E.coli* DH5α for large scale preparation of plasmid DNA.

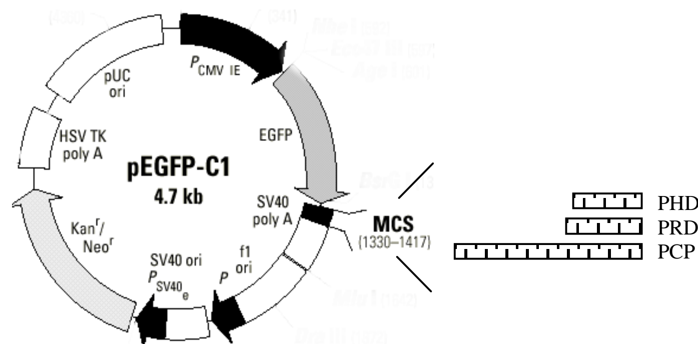

Figure 8. Construction of pEGFP based plasmids. The three fragments of dynamin II domains were respectively subcloned into pEGFP-C1 vector and fused to EGFP tag at the C-terminus.

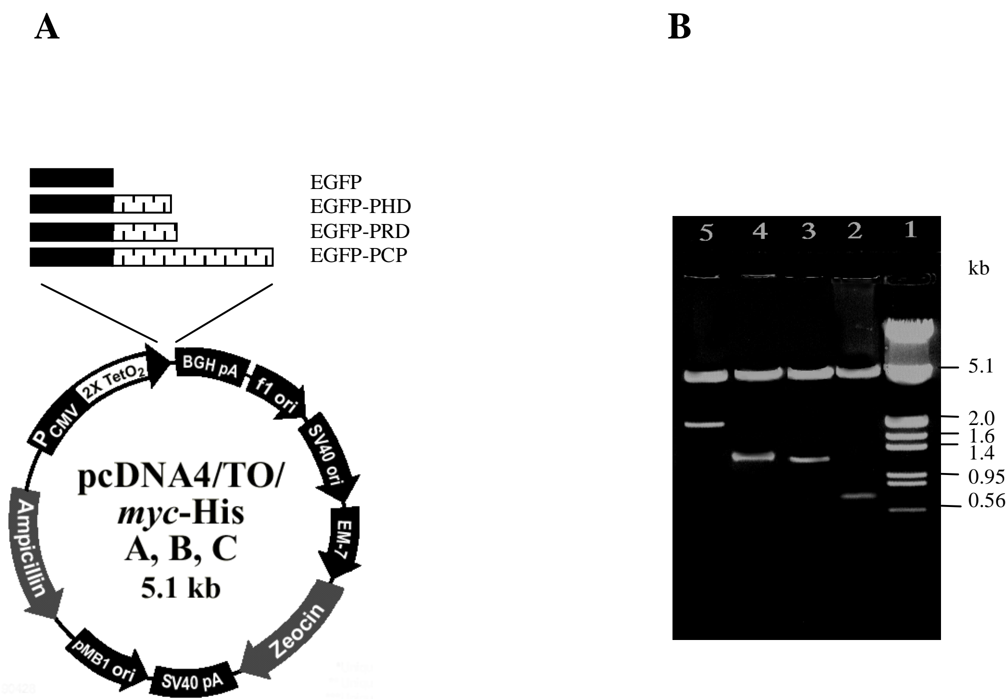

Figure 9. Construction of pcDNA4/TO/*myc*-His based plasmids. EGFP or EGFP tagged dynamin II domains were subcloned into pcDNA4/TO/*myc*-His vector (A). The inserted fragments were identified by restriction digestion and analyzed by EB-agarose gel electrophoresis (B). [1: λ DNA-Hind III-EcoR I; 2: EGFP(0.75kb); 3: EGFP-PHD(1.1kb); 4: EGFP-PRD(1.2kb); 5: EGFP-PCP(1.9kb). The DNA bands of pcDNA4/TO/*myc*-His vector in lanes 2-5 were indicated by 5.1 kb marker]

Tetracycline-regulated expression (Tet-on) of dynamin II domains

The pcDNA4/TO/*myc*-His vector containing the human CMV promoter with two tetracycline operator 2 (TetO2) sites and was used for tetracycline-induced expression of dynamin II domains. The TetO2 sequences bind 4 Tet repressor molecules in form of dimers. In the absence of tetracycline, the Tet repressor is expressed from the cotransfected pcDNA6/TR plasmid and represses the expression of the target gene. Addition of tetracycline to the cells blocks expression of Tet repressor and derepresses the hybrid CMV/TetO2 promoter. The gene of interest is then expressed at levels depending on the tetracycline concentration (see Appendix E).

DNA fragments of the above pEGFP-C1 vector constructs coding for EGFP as well as the three dynamin II domains were digested by Eco47 III (compatible with EcoR V) and Sal I (compatible with Xho I), and subcloned into pcDNA4/TO/His vector between EcoR V and Xho I sites (Figure 9). The obtained plasmids, pcDNA4-EGFP, pcDNA4-EGFP-PHD, pcDNA4-EGFP-PRD and pcDNA4-EGFP-PCP, coded for fusion proteins with a C-terminal extension by *myc* peptide and $(His)_6$ peptide.

2.2.3 Expression and purification of recombinant dynamin II domains in *E.coli*

2.2.3.1 Cultivation and sonication of transformed *E.coli*

For growing *E.coli* transformed with pTrcHis2C-based plasmids, 50 ml of LB medium containing ampicillin was inoculated with a single recombinant *E.coli* colony. For pET-30a(+) constructs transformed *E.coli*, M9E medium containing kanamycin and chloramphenicol was used. After growing at 37°C for overnight under vigorous shaking, the culture was transferred into 1000 ml of the same growth medium and incubated for 3-4 h until the OD_{600} reached a value between 0.4 and 0.6. After addition of IPTG to a final concentration of 0.4 mM (for pTrcHis2C constructs) or 1 mM (for pET-30a(+) constructs), incubation was continued for 3-4 h under vigorous shaking. Cells were harvested by centrifugation at 4,000 rpm at 4°C for 30 min (BECKMAN J6-HC, rotor Js-4.2). Pellets of about 1.2 g wet cells were washed once with 20 ml of PBS and stored at -80°C.

The *E.coli* pellet was resuspended in 8 ml of native binding buffer (NBB, 20 mM sodium phosphate, pH 7.8, 1 M NaCl, 10 mM β-mercaptoethanol) containing 100 µg/ml of egg lysozyme and incubated for 15 min on ice. After addition of protease inhibitor solution and

RNase A (10 µg/ml), the bacterial suspension was sonicated at 4°C four times with 10-second bursts at 80-90% of maximum intensity setting (sonicator). Thereafter, the suspension was flash frozen in liquid nitrogen and thawed at 37°C. After 3-4 cycles, debris was removed by centrifugation at 4,000 g for 20 min. The clear lysate supernatant was filtered through a 0.8µm syringe filter and then used for Ni-NTA-agarose chromatography.

2.2.3.2 Purification of the $(His)_6$ -tagged proteins by Ni-NTA-agarose affinity chromatography

E.coli lysate was loaded onto a column containing 0.5 ml of NBB-equilibrated Ni-NTA agarose. For batch purification, the resin was incubated with gentle shaking at 4°C for 3-5 h and then settled down by gravity or centrifugation (800 g, 5 min). After removing the supernatant (flow through) by careful aspiration, the unspecific bound proteins were removed by washing the resin with NBB of pH 6.0 and NBB containing 50 mM imidazole. Finally, the $(His)_6$-tagged protein was eluted with NBB containing 250 mM imidazole. Fractions were analyzed by SDS-PAGE using a 12 % acrylamide gel. Peak fractions were pooled for purification by Superdex 75 column chromatography.

2.2.3.3 Purification of the S-peptide-tagged proteins by S-protein agarose affinity chromatography

E.coli lysate was mixed with ¼ volume of 8 M urea and loaded onto S-protein agarose column equilibrated with binding buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl and 2 M urea. After incubation for 1-2 h at 4°C, the S-agarose was washed four times with binding buffer. The S-peptide tagged proteins were eluted with binding buffer containing 3 M $MgCl_2$ and analyzed by SDS-PAGE. Protein fractions were pooled and run on a Superdex 75 column for further purification.

2.2.3.4 Superdex-75 column chromatography

The Superdex-75 column HR 10/30 connected to a Shimadzu HPLC system was equilibrated with two volumes of running buffer (20 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.5 mM DTT). Protein samples between 1 and 5 mg per 0.5 ml were loaded and proteins were separated at a flow rate of 0.5 ml/min. Fractions of 0.25-0.5 ml were collected and analyzed by SDS-PAGE.

2.2.4 Determination of protein concentration

If not indicated, protein concentrations were determined by using Bradford Reagent according to manufacturer's protocol (Sigma, USA), in which BSA was used as a standard. For spectroscopic measurements, proteins were dialyzed against 20 mM sodium phosphate, pH 7.0, 0.2 mM DTE, 50 mM NaCl and protein concentrations were determined spectrophotometrically using absorption coefficients of $E^{1\%}_{280} = 2.73$ for the recombinant his-s-PRD and $E^{1\%}_{278} = 16.8$ for the recombinant PHD-his that were calculated from amino acid compositions (Gill and von Hippel, 1989).

To determine membrane protein concentrations, membranes were first solubilized in SDS buffer (50 mM Tris pH 6.8, 2%SDS) at RT for 20 min before spectroscopic measurements.

2.2.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Proteins were solubilized in SDS-sample buffer, heated at 95°C for 5 min and centrifuged for 2 min at 10,000 g. The clear lysates were loaded and separated by discontinuous SDS-PAGE under reducing conditions (Sambrook.J et al., 1989) using a continue gel or a gradient gel containing 12% or 6-25% of bisacrylamide/acrylamide, respectively, at a ratio of 1:29. Gels were stained with Coomassie brilliant blue R250. For Western blotting, proteins were electrophoretically transferred onto NC membranes (Protran BA85, 0.45µm, Schleicher & Schuell). The NC membranes containing transferred proteins were blocked in 5 % defatted milk solubilized in TBST, washed with TBST and incubated with primary antibodies. Immunoreactive bands were detected with an appropriate secondary antibody conjugated to HRP. After each step, the membranes were washed four times with TBST. Membranes were developed with Phototope chemiluminescent reagent (BioLabs, Germany) and were exposed to autoradiographic film (Eastman Kodak, Rochester, NY). To estimate molecular weights, phosphorylase B (97.4 kD), albumin bovine (67.0 kD), albumin egg (45.0 kD), carbonic anhydrase (29.0 kD), trypsin inhibitor (21 kD) and lysozyme (14.0 kD), were used as standard proteins.

2.2.6 Binding of proteins to lipids measured by a dot blot assay

Lipids of bovine brain acetone extract were dissolved in chloroform-methanol (1:1) to 10 mg/ml. 0.5-1 µl of the lipid solution was spotted onto nitrocellulose membrane. After drying, the nitrocellulose membranes were blocked for 2 h at 37 °C in TBS containing 3 % bovine serum albumin and were then incubated with purified proteins of interest at a final concentration of 0.5 µg/ml at 4 °C for 4 h. After washing, membranes were incubated with

primary antibodies to GST to detect GST tagged SH3 domain of amphiphysin II, with the monoclonal antibody to the *myc*-epitope to detect *myc*-tagged PHD of dynamin II or with an antibody to dynamin II to detect PRD and PCP. Bound antibodies were analyzed using HRP-conjugated secondary antibodies and the Phototope-HRP-Detection Kit.

2.2.7 Binding of proteins to biological membranes

Purified PRD or PHD (3 µg each) were incubated with 100 µg of erythrocyte inside-out vesicles (IOV) (see 2.2.8) or 30 µg of Golgi-enriched membranes (compare 2.2.13). Protein concentrations were determined according to 2.2.4. Assays were incubated in binding buffer (10 mM HEPES-KOH, pH 7.2, 30 mM NaCl, 5 mM DTE, 2 mM $MgCl_2$) for 30 min at 4°C or 37°C. In a second set of experiments, GTP, ATP, creatine phosphate and creatine phosphokinase were added in final concentrations of 0.1 mM, 1.0 mM, 0.8 mM and 200 µg/ml, respectively. A third group was supplemented in addition with 0.15 mg/ml cytosolic proteins from HepG2 cells. After incubation, membranes were pelleted for 15 min at 14,000 g through a 0.5 M sucrose cushion in HME buffer, resuspended in binding buffer and laid onto 1.2 M sucrose in binding buffer. After centrifugation, membranes were collected at the interface between binding buffer and 1.2 M sucrose and solubilized in SDS buffer. Proteins were separated by SDS-PAGE and detected by immunobloting using anti-*myc* or anti-dynamin II antibodies.

2.2.8 Preparation of erythrocyte inside-out vesicles (IOV)

Based on the method described by Sulpice et al. (1994), 5 ml packed human erythrocyte cells were diluted into 80 – 100 ml of hypotonic buffer (5 mM potassium phosphate, pH 8.0) and incubated at RT for 5 min to break the erythrocytes. Ghost membranes were pelleted by centrifugation at 35,000 g for 20 min and washed twice with the same buffer. Membranes were incubated in 40 ml Tris buffer (0.5 mM Tris-HCl, pH 8.5, 1 mM EDTA, 2 mM DTT) at 4 ^{0}C for 45 min, followed by another incubation for 45 min at 37 °C. Membranes were collected by centrifugation at 28,000 g for 30 min, washed three times with the same buffer, suspended in 5 ml of Tris buffer and filtrated through a 0.45 µm filter membrane (millipore-PVDF) for 10-15 times to form the inside-out vesicles (IOV). The diameter of IOV was about 0.1 µm. The IOV were separated from debris of ghost membrane by centrifugation at 100,000 g for 2 h through a sucrose cushion at a density of 1.03 g/ml. The vesicle fraction floated on the top of sucrose solution was collected and washed once in 40 ml 10 mM Tris-HCl, pH 7.8, 1 mM EDTA for 30 min at 40,000 g. The

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final vesicle pellet was suspended in 20 mM HEPES-KOH, pH 7.2, 0.5 mM DTT, 1 mM $MgCl_2$, 150 mM KCl.

2.2.9 Preparation of protein extracts

2.2.9.1 Preparation of rat brain proteins

According to Slepnev et al. (1998), five fresh rat brains (about 25 g) were rinsed three times with ice-cold cytosol buffer (10 mM HEPES, pH7.4, 1 mM EDTA, PIT), minced and homogenized in 30 ml of the same buffer in a glass-Teflon Dounce homogenizer at 4°C. The total lysate was centrifugated at 1000 g for 30 min. Triton X-100 was added to the postnuclear supernatant (PNS) to a final concentration of 2 %. After incubation at 4 °C for 1 h, the solubilized proteins were collected by centrifugation at 150,000 g for 1 h and desalted by dialysis against 20 mM HEPES, pH 7.4 100 mM KCl, 5 mM $MgCl_2$ and 1 % TX-100 at 4°C for 24 h with three buffer changes.

2.2.9.2 Preparation of total proteins of HepG2 cells

Confluent HepG2 cells cultivated in 20 dishes of 150 mm in diameter were collected in 20 ml of HCB150 buffer (20 mM HEPES, pH7.2, 150 mM NaCl, 1 mM DTT, 2 mM EGTA, 1 mM $MgCl_2$, PIT) and homogenized at 4 °C using a ball cracker. The PNS was obtained by centrifugation at 1000 g for 10 min and supplemented with Triton X-100 to a final concentration of 1 %. The mixture was incubated under mild shaking for 1 h at 4°C and centrifuged for 1 h at 100,000 g. Soluble proteins were stored at – 80°C.

2.2.10 Purification of amphiphysin II SH3 domain (GST-SH3A)

The expression plasmid pGEX2-amph-SH3, obtained from Dr. P. McPherson (Ramjaun et al., 1997), was transformed into *E.coli* BL21 cells. Expression of fusion protein was induced by addition of 1 mM IPTG in LB medium. The bacteria were broken by sonication. The cell lysate was centrifuged at 8,000 g for 30 min to remove the cell debris. The clear supernatant was incubated with GST-sepharose 4B in 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM $KHPO_4$ and 1% Triton X-100 for 30 min at room temperature. After washing four times, the GST-fusion protein (GST-SH3A) was eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0 at room temperature. GST-SH3A was further purified on Superdex 75 column to a final purity of more than 95 %.

2.2.11 CD and fluorescence spectra of PHD and PRD and determination of secondary structure

Circular dichroism (CD) spectra were recorded with a Jasco J-720 spectropolarimeter (Jasco, Japan) using a 0.01 cm path length quartz cell. The sample temperature was held at 25 °C. Mean residue ellipticities [Θ] ($deg \cdot cm^2 \cdot dmol^{-1}$) were calculated using mean residue masses of 118.5 Da for PHD-his and 104.7 Da for his-s-PRD. The contents of secondary structure were determined from the far-ultraviolet CD spectra according to Provencher and Glöckner (1981) and Venyaminov et al. (1993) using the program CONTIN. The tertiary structure classes of PHD-his and his-s-PRD were determined from the CD spectra by a cluster analysis according to Venyaminov and Vassilenko (1994) using a program provided by the authors.

Fluorescence spectra were measured on a RF 5001 PC fluorimeter at 25 °C (Shimadzu, Japan). To avoid the inner filter effect, the protein absorbance was held below 0.1 optical units at the excitation wavelength. Measurements were performed using microcells with 3 mm path length, and monochromator band width of 3 nm and 5 nm for excitation and emission, respectively.

2.2.12 Cell cultures

HepG2, COS-7 and HeLa cells were maintained in DMEM medium with 2 mM L-glutamine, 10 % FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C. HEK293 cells expressing tet-repressor (T-REx^{TM}293) and stable transfected T-REx^{TM}293 cell lines were cultivated in the same medium with 5 µg/ml of blasticidin or 5 µg/ml of blasticidin plus 300 µg/ml of zeocin, respectively. HepG2 and T-REx^{TM}293 cells were plated in 150 mm dishes or T-75 flasks precoated with collagen. Expression of dynamin II domains in stable transfected T-REx^{TM}293 cell lines (Tet-on) was induced by addition of 0.5-1 µg/ml tetracycline. All cells were incubated at 37 °C with 85 % humidity and 5 % CO_2.

2.2.13 Preparation of Golgi-enriched membranes and cytosolic proteins

Cells were cultivated in 150 mm dishes in DMEM with 10 % FCS at 37 °C until the cell density reaches to 85-95 %. Then dishes were cooled on ice, rinsed twice with 2 ml of ice-cold 0.25 M sucrose/HME buffer (buffer A) and collected in buffer A containing protease inhibitors (PIT). After homogenization, the cell lysate was centrifuged for 10 min at 1000 g and 4 °C to remove the cell debris. The supernatant (PNS) was laid on a sucrose step gradient of 0.8 and 1.2 M and centrifuged at 120,000 g for 30 min at 4 °C in a SW 60 rotor

(BACKMAN). The interface fraction between 0.8/1.2 M in HME sucrose, which represented the "total membrane fraction", was aspirated with a syringe connected to a long needle and mixed with an equal volume of 1.8 M sucrose/HME. The total membranes were floated through 1.18M sucrose in HME for 2 h at 120,000 g and 4 °C. The Golgi membranes that were enriched at the interface between 0.8/1.18 M sucrose were collected and mixed with two volumes of buffer A, and then centrifuged at 14,000 g for 15 min at 4°C. The supernatant was discarded and the pellet (Golgi-enriched membranes) was resuspended in proper buffer. After the first centrifugation, the top fraction of the tube containing cytosolic proteins was collected and stored at –80°C for budding assays.

2.2.14 *In vitro* assay of post-Golgi vesicle formation

2.2.14.1 Labeling of proteoglycans and preparation of Golgi-enriched membranes

Two dishes of HepG2 cells were washed twice with sulfate-free DMEM and starved for sulfate at 37 °C for 30 min. Thereafter, the cells were labeled at 37°C for 5 min with 1 mCi [^{35}S]-sulfate in 5 ml of sulfate-free DMEM. Golgi-enriched membranes were prepared as described above. The final membrane pellet was suspended in 0.25 M sucrose/HME buffer.

2.2.14.2 *In vitro* budding assay

In the first step, Golgi-enriched membranes were incubated with antibodies or purified recombinant dynamin II domain proteins for 20 min on ice. Cytosolic proteins of HepG2 cells prepared as described above were precleared by centrifugation at 100,000 g for 30 min. The protein concentration was adjusted to 0.4 mg/ml.

To form post-Golgi vesicles *in vitro*, 5 µg Golgi-enriched membranes were incubated with ATP regenerate system (1 mM ATP, 0.1 mM GTP, 0.2 mM $MgCl_2$, 0.92 mM creatine phosphate, 40 µg/ml creatine kinase) and 20 µg of cytosolic proteins in 200 µl of 0.25 M sucrose/HME buffer on ice (as a control assay for background budding) or at 37 °C for 30 min. Formed vesicles were seperated from Golgi membranes by centrifugation at 14,000 g for 15 min through 50 µl of 0.5 M sucrose cushion. The supernatant (200 µl) containing the newly formed vesicles was carefully transferred into new tubes.

2.2.14.3 Determination of [^{35}S]-sulfated HSPGs

Pelleted Golgi membranes and the floated vesicles in the supernatant fractions were solubilized in 500 µl 1 % Triton x-100/HME. After incubation for 5 min at 37 °C, 10 µl

chondroitin sulfate (10 mg/ml) and 150 µl 10 % cetylpyridinium chloride (CPC) were added. After incubation for 1 h at 37 °C, the precipitated HSPGs were collected on nitrocelloluse membrane with 0.45 µm pore size (Schleicher & Schuell, Dassel, Germany). The filter membranes were washed three times with 1 % CPC/40mM Na_2SO_4 and then dried. Radioactivity on the filters was counted in toluene-based scintillator.

Budding efficiency was calculated according to:

Budding efficiency (%)=Vesicle labeling/(Vesicle labeling+Golgi labeling)

2.2.15 Secretion of HSPGs by 293 cells

Stable transfected 293 cells expressing EGFP or EGFP-tagged dynamin II domains were depleted of sulfate by incubation in sulfate-free DMEM for 30 min at 37°C, and then suspended in 1 ml of the same medium containing 0.5 mCi of carrier-free [^{35}S]-sulfate. Cells were incubated for 3.5 min at 37°C, cooled on ice, washed three times with ice-cold DMEM and aliquoted in 0.5 ml in DMEM chase medium. Secretion of HSPGs was started by incubation of cell suspensions at 37°C for 0, 5, 10, 15, 20, 25 and 60 min, respectively. Then cells were pelleted at 500 g for 4 min at 4°C and washed once with 0.5 ml cold DMEM. Secreted HSPGs were determined in the supernatant fractions and intracellular HSPGs were determined in cell pellets as described in 2.2.14.3.

2.2.16 Calcium phosphate-mediated transfection of mammalian cells and establishment of stable cell lines

Transfection was performed according to Sambrook.J et al. (1992) with some modifications: 24 h before transfection, the exponentially growing cells were harvested by trypsinizaion and replated at a density between 10^5 and 2 x 10^5 cells/cm^2 in 60-mm tissue culture dishes in DMEM containing 10 %FCS. Before transfection, the cultured medium was replaced with 5 ml of new medium. The calcium phosphate-DNA mixtures for transfected cells were prepared as follows:

First, 220 µl 0.1 x TE (pH 8.0) containing 10 µg plasmid DNA was mixed with 250 µl 2 x HBS in a sterile 5 ml tube. Second, 31 µl 2 M $CaCl_2$ was added dropwisely with gentle mixing over a period of 30 seconds. Third, the mixture was incubated for 20-30 minutes at room temperature, during which time a fine precipitate formed. At the end of the incubation, the mixture was pippeted up and down to mildly resuspend the precipitate.

Fourth, the calcium phosphate-DNA suspension was transferred into the medium above the cell monolayer. The dish was rocked gently to mix the medium and incubated for 4 h. Thereafter, in some cases, cells were treated with 15 % glycerol for 30 sec to improve transfection efficiency.

To establish stably transfected TREx^{TM}-293 cell lines, the antibiotics blasticidin (5 µg/ml) and zeocin (100-300 µg/ml) were added into the medium 48 h after transfection. Positive clones were selected and expanded in medium containing higher concentrations of zeocin and blasticidin until the stable clones were established .

2.2.17 Detection of immunofluorescence by confocal microscopy

Cells were grown on coverslips for 24 h, rinsed with PBS at room temperature and permeablized in 20 mM PIPES, pH 6.8, 140 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5-2 mM ATP, 20 µg/ml digitonin at 37 °C for 10-20 min. Cells were either fixed for 20 min with 3.7 % paraformaldehyde in CB buffer (10 mM MES, pH 6.1, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM glucose, adjusted to pH 6.1) or in ice-cold methanol/acetone (1:1) at –20°C for 20-30 min. After rinsing with PBS, cells were incubated in blocking buffer (3 % BSA and 5 % glycerol in PBS) for 2 h at room temperature. Thereafter, cells were incubated with primary antibody for 2 h at room temperature and washed four times with PBS before incubation with Cy2- or Cy3-conjugated secondary antibody for 45 min at 25 °C. Cells were then washed extensively with PBS, rinsed briefly with distilled water, and covered with mounting reagent. Expression of EGFP fusion proteins was viewed with an Olympus microscope (IX70) equipped with a 100-W mercury lamp using a 60 x or 100 × objective lens (UPlanFI, 0.6-1.30). Wavelength of excitation/emission is 470/530 nm, 550/590 nm and 480/510 nm for EGFP, Cy3 and Cy2 filters, respectively. Images were acquired by a CCD digital camera (HAMAMATSU, C-4742-95) at 1024 × 1022 pixel resolution over a limited exposure period. For localization of dynamin II and its domains, the confocal microscope was equipped with a piezo focus drive using a 100× objective lens. Contrast and intensity of images were optimized using the Openlab program, version 2.2.2.4 (Improvision, Image Processing & Vision Company Ltd.).

2.2.18 Identification of dynamin II binding proteins

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2.2.18.1 Binding of the SH3 domain of amphiphysin II to PRD and PCP

2 µg PRD or 1 µg PCP were immobilized on to the S-protein agarose and reacted with 5 µg purified amphiphysin II SH3 domain for 2 h at 4 °C in binding buffer (20 mM HEPES-KOH, pH 7.5, 30 mM NaCl, 2 mM $MgCl_2$, 1 mM DTE and 0.1% Triton X-100). After extensive washing with binding buffer, the proteins were eluted with 2 % SDS in 50 mM Tris-HCl, pH 8.0, and analyzed by SDS-PAGE and subsequent staining with Coomassie brillant blue. As a control, the PHD attached to Ni-NTA-agarose was used, which did not bind the SH3 domain of amphiphysin II.

2.2.18.2 Binding of the SH3 domain of syndapin to PRD

10 µg SH3 domains of syndapin-1, syndapin-2 and syndapin-1 mutant (P434L) fused to GST were immobilized to GST agarose, and then incubated with 5 µg purified PHD or PRD of dynamin II. After washing, the bound proteins were eluted with 2 % SDS buffer and analyzed in SDS-PAGE.

2.2.18.3 Identification of binding proteins by affinity chromatography

Proteins extracted from rat brain or HepG2 cells (see 2.2.9) were reacted with recombinant PRD of dynamn II immobilized to S-protein agarose in 50 mM NaCl, 20 mM HEPES-KOH, pH 7.5, and 1 % Triton X-100. After washing with the same buffer containing 300 mM NaCl to remove unspecifically bound proteins, bound proteins were eluted together with PRD by using an elution buffer containing 2 % SDS. Proteins were separated by SDS-PAGE in 5-20 % gel gradient. Protein gels were either silver stained and used for peptide sequencing or blotted onto nitrocellulose membrane and immunodetected by specific antibodies.

2.2.18.4 Mass spechrometric peptide sequencing

PRD binding proteins from S-protein agarose were separated in SDS-PAGE. Individual band was excised from the Coomassie blue-stained or silver-stained gels. Proteins were in-gel digested with trypsin as described previously (Otto et al., 1996). Peptides were desalted by binding to RP18 and eluted with 5-10 µl of 60 % acetonitrile containing 0.1 % trifluoroacetic acid. For MS/MS experiment, a nanospray ionization-hybrid quadrupole-time of flight-mass spectrometer (Q-TOF; Micromass, Manchester, UK) equipped with a nanoflow Z-spray probe was used. The nanoflow source was operated at a temperature of 30°C with a nitrogen drying gas flow of about 30-50 L/h. A potential of 1.4 kV was applied to the nanoflow borosilicate glass capillary, resulting in a flow rate of 30 nL/min.

A collision energy of 28-35 V, depending on the charge state of the parent ions, was used. For protein identification, we used the sequence tag program which combined partial manual spectrum interpretation of three amino acids with the residual N-terminal and C-terminal masses of the interpreted region and the peptide mass. This information was then used for searching nonredundant translated nucleotide database .

2.2.19 Sedimentation analysis of cytosolic dynamin II

The cytosol used in sedimentation assay was prepared from transfected 293 cells using the method described above (2.2.13). Before use, cytosol preparations were precleared by centrifugation at 200,000 g for 20 min at 4 °C. 1 ml cytosol containing 3 mg proteins was dialyzed against 100 volumes of low salt buffer HCB 20 (20 mM NaCl, 20 mM HEPES, pH7.2, 1 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT) or physiological salt buffer HCB 150 (150 mM NaCl, 20 mM HEPES, pH 7.2, 1 mM $MgCl_2$, 2mM EGTA, 1mM DTT) at 4°C for overnight. The oligomerized dynamin II was then sedimented at 150,000 g for 10 min at 4°C. The pellets were washed once with the same buffer. The protein pellets containing the oligomerized dynamin II were solubilized in 2 % SDS buffer and analyzed by Western blotting using the anti-dynamin II antibody.