Formation of clathrin-coated vesicles (CCVs) in receptor-mediated endocytosis is a mechanistically well-established process, in which clathrin, the adaptor protein complex AP-2, and the large GTPase dynamin play crucial roles. In order to obtain more mechanistic insight into this process, here we established a giant unilamellar vesicle (GUV)-based in vitro CCV reconstitution system with chemically defined components and the full-length recombinant proteins clathrin, AP-2, epsin-1, and dynamin-2. Our results support the predominant model in which hydrolysis of GTP by dynamin is a prerequisite to generate CCVs. Strikingly, in this system at near physiological concentrations of reagents, epsin-1 alone does not have the propensity for scission but is required for bud formation, whereas AP-2 and clathrin are not sufficient. Thus, our study reveals that epsin-1 is an important factor for the maturation of clathrin coated buds, a prerequisite for vesicle generation.

**Keywords:** clathrin-coated vesicle; endocytosis; membrane scission; vesicle reconstitution; vesicular transport

Clathrin-coated vesicles (CCVs) are established carriers within the endosomal system and in endocytosis. Clathrin is recruited to membranes by the tetrameric adaptor protein complexes (AP) AP-1, AP-2, and AP-3, as well as by the interaction with the monomeric adaptor of the Golgi-associated, γ-adaptin ear-containing, Arf-binding protein (GGA) family that serves various routes in intracellular transport (reviewed in Ref. [1]). Among the various locations of budding of CCVs, the formation of carriers during endocytosis at the plasma membrane is a most investigated process. During biogenesis of endocytic CCVs, AP-2 has a crucial role, acting as a central hub within the clathrin endocytic interactome [2], and several cargos require AP-2 for their endocytosis. Whereas for a long time there has been no evidence for the existence of clathrin-coated structures at the plasma membrane without AP-2 [3–5], very recently evidence in live cells was provided for clathrin-coated structures that function in the ligand-induced internalization of the epidermal growth factor receptor (EGFR) in AP-2 knockout cells, with epsin proteins as adaptors [6]. In contrast, these vesicles did not support the uptake of the constitutive internalized transferrin receptor. However, whereas the EGFR is recycled back to the cell surface when internalized in AP-2-positive CCVs, a lack of AP-2 destined this receptor for degradation and does not support EGFR-dependent AKT signaling [6].

**Abbreviations**
ANTH, AP180 N-terminal homology domain; AP, adaptor protein complex; Arf, ADP ribosylation factor; BAR, Bin/amphiphysin/Rvs; BPLE, brain polar lipid extract; CALM, clathrin assembly lymphoid myeloid leukemia; CCB, clathrin coated bud; CCP, clathrin-coated pit; CCV, clathrin-coated vesicle; CHC, clathrin heavy chain; CLC, clathrin light chain; CME, clathrin mediated endocytosis; COPI, coat protein complex I; EGFR, epidermal growth factor receptor; ENTH, epsin N-terminal homology; GGA, Golgi-localized, γ-adaptin ear-containing, Arf-binding protein; GUV, giant unilamellar vesicle; PI, phosphatidylinositol; PIP2, phosphatidylinositol-(4,5)-bisphosphate; SUV, small unilamellar vesicle; TGN38, trans-Golgi network integral membrane protein TGN38.
yeast, AP-2 does not bind to clathrin [7,8]; rather, the yeast homologues of epsin and AP180, Ent1/2 and Yap180/02, are essential for clathrin recruitment [9]. Furthermore, depletion in yeast of AP-2 does not cause a reduction of CME [7,8] as compared to mammalian cells [3–5]. A function of AP-2 in yeast seems to be in the uptake of some special cargos, such as the yeast toxin K28 [10] and the stress sensor protein Mid2 [11].

A model for the formation of AP-2 CCVs was put forward, in which AP-2 undergoes a large-scale conformational change, initiated by phosphatidylinositol-(4,5)-bisphosphate (PIP2) and stabilized by cargo binding [12,13]. In this mechanism, both large AP-2 subunits play a key role in initial membrane binding, whereas binding sites within the μ-subunit of the complex drive a rearrangement from a closed to an open conformation. Only in this conformation, all four PIP2-binding sites and the two cargo binding sites for the interaction with YxxΦ and dileucine motifs become coplanar, which allows their simultaneous interaction with PIP2 and cargo-containing membranes [12,13].

In addition to clathrin and AP-2, there is a set of proteins with specialized functions assisting vesicle biogenesis. This process can be subdivided into five steps [14]: nucleation, cargo selection, coat assembly, scission, and uncoating of the vesicle in order to fuse with the target membrane.

Besides clathrin and AP-2, the large GTPase dynamin, implicated in the separation of CCVs from the plasma membrane, plays a crucial role in receptor-mediated endocytosis, in the scission of clathrin-coated endocytic buds to release free vesicles.

Whereas most of the adaptors in CCV biogenesis (e.g., AP-1, AP-3 and GGA) are recruited to their donor membranes dependent on the small GTPase ADP ribosylation factor (Arf), AP-2 binds to the plasma membrane independent of Arf. Rather, AP-2 harbors binding sites for PIP2 within the plasma membrane. For the formation of CCVs other than those with AP-2, Arf is likely to play a distinct role in the mechanism of vesicle scission via its myristoylated amphipathic helix, as has been shown for COPII vesicle release [15]. In the predominant model of AP-2 CCV release, dynamin has to catalyze GTP hydrolysis, thus inducing a conformational change within dynamin that leads to separation of the CCV from the plasma membrane. However, other studies pointed out that dynamin may not be the direct or exclusive scission factor for CCVs. For AP-2 CCVs, epsin that like Arfs contains an amphipathic helix [epsin N-terminal homology (ENTH) domain] is an accessory protein [16]. Epsin overexpression was shown to rescue vesicle release in dynamin knockdown cells [17].

In clathrin-mediated endocytosis (CME), clathrin-coated pit (CCP) nucleation is initiated through the recruitment of adaptor proteins and clathrin to the plasma membrane. While receptors, adaptors, clathrin, and other regulatory proteins will be continuously recruited to the edge of the growing coat, the initial clathrin patch matures to an invaginated bud that is finally separated from the membrane via dynamin [1,18]. Initially, it was believed that curvature of a CCP constantly grows with clathrin polymerization [19,20]. This model is known as the constant curvature model. However, a second model, the constant area model, has come increasingly into focus over the last years. Here, clathrin assembles into flat lattices up to a certain size before the lattice starts to remodel and becomes curved [21,22]. The area of the clathrin lattice is kept nearly constant during the conversion to a curved lattice. To introduce curvature during this remodeling step, the hexagonal flat clathrin lattice needs to be partially converted to pentagons. Although the constant area model was already put forward at a time right after clathrin had been linked to vesicle transport [23,24], it was initially not accepted due to structural and energetic arguments [19,20]. However, molecular simulations suggest that local conformational changes within the flat clathrin lattice are in fact able to induce the remodeling to a curved lattice [25]. This dynamic behavior is strengthened experimentally by fluorescence recovery after photobleaching experiments [21,26]. The question remains as to how curvature is initiated in living cells. The curvature of clathrin lattices is increased by a conformational change in the clathrin light chain (CLC) [27]. Additionally, mutational analysis revealed a key role of adaptor proteins in controlling the curvature of the clathrin lattice. If AP-2 is missing its C-terminal domain is crucial for the recruitment of endocytic accessory proteins, this observation was suggested to be caused by missing curvature-inducing accessory proteins. The exact mechanisms and regulators of curvature generation remain unclear.

In order to address mechanistic questions, CCV reconstitution studies were carried out using complete or component-depleted cytosol or truncated purified proteins for generating CCVs in vitro. Therefore, to investigate which factors are required to generate a CCV after a CCP has formed, we established an in vitro system exclusively with chemically defined components and full-length proteins to study the mechanism of CCV formation. We confirmed the role of dynamin in GTP hydrolysis-dependent membrane
scission and found that CCVs can be formed with epsin-1 in the presence or absence of AP-2, but not with AP-2 alone, in agreement with recent data in living cells [6].

Materials and methods

Materials

The following antibodies were used: anti-epsin-1 (dilution 1:100, clone B-12, order number sc-365914; Santa Cruz, Dallas, TX, USA); anti-AP-2-µ1 (1:200, K-13, sc-49150; Santa Cruz); anti-dynamin-1/2 (1:100, N-19, sc-6401; Santa Cruz); and anti-clathrin heavy chain (CHC) (1:2000, #43820, BD Transduction, https://www.bdbiosciences.com). The following plasmids were purchased or kindly provided from the following companies or research groups: pFBDM and pUCDM transfer vectors kindly provided from the following companies or research groups: pFBDM and pUCDM transfer vectors from Imre Berger (Bristol); pET32c-epsin-1 and pQE32-ΔENTH-epsin-1 from Ernst Ungewickell (Hannover); pEGFP-N1-dynamin-1 (#34680; Addgene, https://www.Origene.com/plasmids); pEGFP-N1-dynamin-2 from Volker Haucke (Berlin); pET5u-AP-2γ, pFASTBac-AP-2α and pFastBac-AP-2µ from Ute Schepers (Karlsruhe); pCMVSPORT6-AP-2β (I.M.A.G.E. ID: 7929959; Source Bioscience, https://pharma.sourcebioscience.com); pET28a-CLC a1; and pFastBac-CHC from Tomas Kirchhausen (Boston). Most used sequences were from rat, and dynamin-1 was from humans.

Cloning and protein expression

For the recombinant expression of AP-2, clathrin, dynamin-1 and 2, and mutants of these proteins, the respective cDNAs were cloned into pFBDM/pUCDM baculovirus expression system to allow the expression of protein complexes within one virus [29]. AP-2α and AP-2γ were amplified by PCR. The products were digested with Smal/NcoI and NotI/XbaI, respectively, and cloned into the respective restriction sites of the pFBDM vector. AP-2β and AP-2µ were amplified by PCR. The products were digested with Xmal/Sphi and BssHII/SalI, respectively, and cloned into the respective restriction sites of the pFBDM vector. pFBDM-AP-2α/σ was digested with Pmel and AvrII. The product was cloned into the multiplication module of the pFBDM vector. pFBDM-AP-2β/µ using the restriction sites SpeI and NruI (Fig. S3). Cloning, expression, and purification of clathrin (pFBDM-CHC/CLC) will be described elsewhere (S. Finkenberger, A. Hellwig, I. Reckmann, F. Wieland, & F. Adolf, manuscript in preparation). Two further copies of the CHC cDNA were inserted into the final expression construct to increase the protein yield of the heavy chain. To this end, the cDNA of CHC was amplified by PCR and digested either with Xmal/XhoI or with BssHII/XbaI and cloned into the respective restriction sites of the pUCDM vector (Fig. S5). The cloned constructs from the pUCDM-CHC/CHC vector and pFBDM-CHC/CLC vector were consecutively transfected into the bacmid for insect cell expression as described [29]. Dynamin-1 and dynamin-2 were amplified by PCR. Both products were digested with NotI and XbaI and cloned into the respective restriction sites of the pFBDM vector (Fig. S6). For affinity purification, a tobacco etch virus cleavable Twin-Strep-tag was cloned to the S′-end of AP-2α, dynamin. If not otherwise stated, the final constructs were transferred from the pFBDM vector into the bacmid for insect cell expression as described [29]. The expression of proteins in Sf9 insect cells was essentially done as described previously [30]. In brief, 0.75 × 10⁶ cells were seeded in each well of a six-well plate. After attachment for 15 min at room temperature, the Sf9 cells were transfected with the isolated bacmid using Xtreme Gene HP (Roche, Penzberg, Germany) and incubated for approximately 60 h at 27 °C. The baculovirus-containing supernatant was collected and stored at 4 °C in the dark. This virus is referred to as the P1 virus. For a test expression, 2 mL fresh Sf9 medium [SF900 II SFM; Gibco (Thermo Fisher Scientific, Waltham, MA, USA)] was added to each well. After incubation for further 48 h at 27 °C, Sf9 cells were detached and analyzed for protein expression by SDS/PAGE. For large-scale protein expression, the P1 virus was amplified by infecting 25 mL of an uninfected Sf9 cell suspension with a density of 0.8 × 10⁶ cells per mL with 0.3, 0.6, 1, and 3 mL of the P1 virus stock in 250-mL Erlenmeyer flasks and incubated for 3 days at 27 °C under agitation. Following incubation, the infected Sf9 cell suspensions were analyzed for protein expression by SDS/PAGE. Sf9 cell suspensions with the highest protein expression level were centrifuged for 5 min at 2000 g for 4 °C. The virus-containing supernatant was collected and stored at 4 °C in the dark. This virus is referred to as the P2 virus. Large-scale expression (500 mL Sf9 cell suspension) was carried out in 2-L roller bottles at a cell density of 2 × 10⁶ cells per mL. Suitable amounts of the P2 virus stock necessary to infect the large-scale expression culture were determined by test expressions. To this end, 25 mL of an uninfected Sf9 cell suspension with a density of 2 × 10⁶ cells per mL was infected with 0.15, 0.3, 0.6, and 1 mL of the P2 virus stock in 250-mL Erlenmeyer flasks and incubated for 3 days at 27 °C under agitation. Following incubation, the infected Sf9 cell suspensions were analyzed for protein expression by SDS/PAGE. Sf9 cell suspensions with the highest expression level were centrifuged at 4 °C for 5 min at 2000 g for 4 °C. The virus-containing supernatant was collected and stored at 4 °C in the dark. This virus is referred to as the P2 virus. Large-scale expression (500 mL Sf9 cell suspension) was carried out in 2-L roller bottles at a cell density of 2 × 10⁶ cells per mL. Suitable amounts of the P2 virus stock necessary to infect the large-scale expression culture were determined by test expressions. 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KpnI and XhoI (Fig. S4). His$_{6}$-$\Delta$ENTH-epsin-1 was amplified by PCR, digested with NdeI and XhoI, and cloned into the respective restriction sites of the pET29a vector (Fig. S4). Epsin-1 and its truncated version $\Delta$ENTH-epsin-1 were expressed in Escherichia coli BL21(DE3) pLysS. The expression was started with 1 mM IPTG at 0°C for 4 h before bacteria were harvested.

**Protein purification**

Proteins fused to a Twin-Strep-tag (AP-2, clathrin, dynamin) were purified on Strep-Tactin Sepharose beads (IBA Lifesciences, Göttingen, Germany) according to the manufacturer’s procedure. GST-tagged full-length epsin-1 was purified on Glutathione Sepharose 4 Fast Flow beads (GE Healthcare, Chalfont St. Giles, UK) according to the manufacturer’s procedure and subsequently incubated with thrombin (10 U·mg$^{-1}$) overnight for tag cleavage. AP-2 and epsin-1 were further purified by size-exclusion chromatography. To this end, an appropriate column was connected to an AKTAprime plus system (GE Healthcare) and equilibrated with storage buffer (25 mM HEPES pH 8.0, 300 mM potassium acetate, 0.05% 1-thioglycerol, and 10% glycerol). Chromatography conditions were chosen according to the manufacturer’s protocols. The ENTH-truncated epsin-1 was tagged with an N-terminal His$_{6}$-tag and purified using Ni Sepharose 6 Fast Flow. Tagged protein was bound to the beads in the presence of 25 mM imidazole, and the column was washed with three CV of purification buffer supplemented with 25 mM imidazole and successively eluted with 60, 80, 100, 150, and 300 mM imidazole. The 100 mM elution was used for the experiments. All purification steps were done in 25 mM HEPES pH 8.0, 300 mM potassium acetate, and 0.05% 1-thioglycerol supplemented with a protease inhibitor cocktail (Roche) using a gravity flow column (Bio-Rad, Munich, Germany). Last, the purification buffer was exchanged with storage buffer. Aliquots were flash-frozen and stored at $-80\,^\circ C$.

**Liposome preparation**

All lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and stored at $-20\,^\circ C$ overlaid with argon to prevent oxidation. The brain polar lipid extract (BLEP)-derived lipid mixture was supplemented with P(4,5)P$_2$ and $\Delta$-Golgi network integral membrane protein TGN38 (TGN38)-lipopeptide as indicated. The lipid mixtures were dried using a vacuum concentrator (RVC 2-18, Christ, Osterode). Dried lipid films were overlaid with argon and stored at $-80\,^\circ C$ until use. Liposomes were resuspended to the desired concentration in an appropriate volume of assay buffer (25 mM HEPES pH 7.5, 150 mM potassium acetate), vortexed until homogeneity, and subjected to ten freeze-thaw cycles to make them unilamellar. To this end, the liposome suspension was immersed for 5 min into liquid nitrogen and immediately thawed at $37\,^\circ C$ for 5 min.

**GUV preparation**

Giant unilamellar vesicles (GUVs) were essentially prepared as described earlier [31]. To this end, 5 µmol of a dried, plasma membrane-like lipid mixture (phosphatidylethanolamine:phosphatidylethanolamine:phosphatidylserine:phosphatidylinositol:cholesterol=36:20:1:15:3:25) supplemented with 1 mol% P(4,5)P$_2$ and TGN38 lipopeptide was dissolved in 700 µl reconstitution buffer (25 mM HEPES/KOH pH 7.4, 150 mM potassium acetate, 1 mM DTT, and 100 µM EDTA) containing 1.3% octyl-$\beta$-D-glucopyranoside (OG). The lipid solution was rapidly diluted threefold with reconstitution buffer, allowing formation of small unilamellar vesicles (SUVs). Generated SUVs were subsequently desalted using a PD-10 desalting column (GE Healthcare) equilibrated with 25 mM desalting buffer [1 mM HEPES/KOH pH 7.4, 20 mM trehalose, 1% glycerol (v/v), 1 mM DTT, and 100 µM EDTA] according to the manufacturer’s spin protocol. Recovered SUVs were snap-frozen in liquid nitrogen in 500 µl aliquots and stored at $-80\,^\circ C$ until use. Prior GUV preparation, the SUVs need to be desalted once more to eliminate trace amounts of detergents and salts. To this end, an SUV aliquot was thawed and applied to a PD MidiTrap G-25 column (GE Healthcare) equilibrated with desalting buffer. After the sample had entered the packed bed completely, 550 µl desalting buffer was added. SUVs were eluted with 1.4 mM desalting buffer into a 1.5-ml low-binding reaction tube (Biozym Scientific, Hessisch Oldendorf, Germany). The eluate was centrifugated in a TLA-55 rotor (Beckmann Coulter, Munich, Germany) at 130 000 g for 2 h at 4 °C. Next, the pellet was resuspended in a total volume of 20 µl and spread as a uniform layer with a diameter of about 10 mm onto the surface of a 25 × 25 mm (0.05 mm thick) platinum foil (Alfa Aesar, Heysham, UK). The platinum foil was attached to a glass support with double-sided adhesive tape. Subsequently, the liposome film was dried for 1 h in a vacuum (50 mbar). An O-ring (20 × 2 mm) was placed onto the platinum foil, surrounding the dried lipid film to seal the chamber. The electroformation chamber was filled with 550 µl swelling buffer (1 mM HEPES/KOH pH 8.0, 272 mM sucrose, 1 mM DTT) and covered with a second platinum foil. GUVs were generated overnight at 10 Hz and 1 V at 4 °C. The next day, GUVs were detached from the bottom by gentle pipetting and transferred into a 1.5-mL low-binding reaction tube.

**Density gradient centrifugation**

Following incubation of liposomes with proteins, 5% of each reaction mixture were used as the input sample. A
solution of 65% sucrose (w/w) was added to the residual reaction mixtures to a final concentration of 40% (w/w) sucrose, and the samples were mixed thoroughly. Then, these solutions were transferred to 0.7-ml SW60 tubes and carefully overlaid with 200 μl of 30% sucrose (w/w) and finally 50 μl assay buffer (25 mM HEPES pH 7.5, 150 mM potassium acetate). All sucrose solutions were prepared in assay buffer. The gradients were centrifuged for 1 h at 250 000 g at 4 °C in a SW60 swinging bucket rotor (Beckman Coulter) to isolate liposomes coated with proteins from the reaction mixture. One hundred microliter from the top of the gradient was taken to collect the protein-coated liposomes (top fraction). The proteins of the inputs and the top fractions were separated on a 10% SDS/PAGE and analyzed after Coomassie blue staining or immunoblotting. The western blot results were quantified with image studio lite 5.2.5 (Li-COR, www.licor.com).

**CCV reconstitution assay**

Before each set of experiments, the GUVs were separated from aggregates, multimellar GUVs, and small liposomes with sizes corresponding to diameters typical for endocytic CCVs by differential centrifugation. To this end, the GUV suspension was diluted 1:10 in an iso-osmotic assay buffer and analyzed after Coomassie blue staining or immunoblotting. The western blot results were quantified with image studio lite 5.2.5 (Li-COR, www.licor.com).

**Results**

**Functionality of the recombinant proteins**

In receptor-mediated endocytosis, clathrin adaptors need structural motifs to bind to the inner leaflet of the plasma membrane. In this study, the full-length adaptors AP-2 and epsin-1, as well as a truncated epsin-1, ΔENTH-epsin-1, were used. For AP-2, it is known that the adaptor is initially recruited to membranes via PIP₂, which leads to a conformational change, called the open conformation. This conformation is subsequently stabilized by binding to a cargo signal motif and further PIP₂-binding sites [33–35]. Likewise, epsin-1 possesses a PIP₂-binding site to recruit this endocytic protein to the inner leaflet of the plasma membrane via its N-terminal ENTH domain. The interaction with the plasma membrane-enriched lipid PIP₂ induces the formation of an additional helix, referred to as ‘helix zero’ (H0), which inserts into the inner leaflet, thus causing an asymmetry that results in membrane curvature [36,37]. To test the mentioned dependencies for the binding of AP-2 and epsin-1 to membranes, synthetic liposomes were supplemented with PIP₂ and a lipopeptide containing the sequence YxxΦ, the cargo signal of the trans-Golgi network integral membrane protein TGN38.
The ENTH-truncated epsin-1 variant was recruited to membrane via the interaction of its N-terminal His-tag with a nickel chelating lipid [38]. For binding studies, the various adaptors were incubated with liposomes and clathrin and bound material was subsequently separated via sucrose gradient centrifugation and analyzed by immunoblotting. As expected from the literature, AP-2 and epsin-1 did not bind to membranes without any endocytic signal and clathrin did not get recruited to membranes without membrane-bound adaptor (Fig. 1A,B). Efficient epsin-1 recruitment could only be observed when PIP2 was present, and in case of AP-2, the presence of both signals, PIP2 and TGN38-lipopeptide, is needed for efficient recruitment to membranes (Fig. 1A,B). In case of ΔENTH-epsin-1, liposomes were supplemented with a nickel chelating lipid to allow specific recruitment of the truncated variant through the interaction of its N-terminal polyhistidine-tag to the liposomal membrane (Fig. 1B).

Reconstitution of endocytic clathrin-coated vesicles

After assessing functionality of the proteins used in this study (Fig. 1), the capability of the purified components (Fig. 1C) to generate CCVs was tested. in vitro studies of CCV formation were carried out to date using complete or component-depleted cytosols [39]. AP-2 CCV formation was described by some authors to occur in the presence of the nonhydrolyzable nucleotide GTPγS [40,41]. As a starting point for our experiments to generate CCVs in vitro, we referred to a published study [38]. With the use of the artificial clathrin adaptor ΔENTH-epsin-1, clathrin, dynamin-1, and GTP, these authors were able to generate CCVs.

Fig. 1. Membrane binding of AP-2 (A) and epsin-1 (B). BPLE-derived liposomes (0.5 mg·mL⁻¹) and clathrin (0.8 µM) were incubated with AP-2 (0.5 µM) or epsin-1 (1 µM) or ΔENTH-epsin-1 (1 µM). For AP-2 and epsin-1, the extract was supplemented as indicated with 5% (w/w) PIP2 and/or TGN38-lipopeptide and in case of ΔENTH-epsin-1 with 5% (w/w) of a nickel chelating lipid to allow specific recruitment through the interaction of its N-terminal polyhistidine-tag to the liposomal membrane. After incubation at 37 °C for 15 min, the bound material was subsequently separated by flotation in a sucrose gradient. The top fraction was analyzed by immunoblotting for the presence of coat proteins. IRDye® 800CW Streptavidin (Rockland) was used to visualize AP-2 via the interaction with the Twin-Strep-tag. (C) 0.5 µg of each purified protein used in the present study was resolved on a 10% SDS/PAGE and stained with colloidal Coomassie.
Whereas we reproduced the results of this study (Fig. S1), these experiments did not allow to compare full-length clathrin adaptors, or their combinations, for their efficiency to generate CCVs. To minimize the risk of analyzing CCVs that originate from coating of small liposomes, the above authors incubated the liposomes first with ΔENTH-epsin-1, and then pelleted adaptor-bound liposomes in a low-speed centrifugation step. The supernatant was discarded, and the pellet was resuspended for further incubation steps. When we used the full-length proteins AP-2 and epsin-1 individually, the adaptor-bound liposomes were not pelleted as efficiently, and when using both adaptors simultaneously, the resulting pellet could not be resuspended, most likely due to cross-bridging of the liposomes by the interaction of AP-2 with epsin-1. To overcome these problems, and to use a liposomal system of lipid bilayers with (relatively) low curvature, that is more similar to the curvature of the plasma membrane, we switched to GUVs as donor membranes. These liposomes can be concentrated, and aggregates and small liposomes with diameters similar to those of endocytic CCVs can be removed by differential centrifugation before incubation with the proteins. To reconstitute CCVs in vitro, a two-step approach was established: In the first step, GUVs were incubated with adaptors and clathrin at 25 °C for 30 min, and in the second step, dynamin-2 and GTP were added for a further 30-min incubation at 37 °C. After pelleting the donor membranes at 10 000 g for 10 min, the cleared supernatant was subjected to a freshly glow-discharged carbon-coated copper grid and analyzed by negative stain electron microscopy.

The results obtained reveal a dynamin- and GTP-dependent formation of endocytic CCVs. No CCVs could be detected when AP-2 alone was used as clathrin adaptor (Fig. 2, compare A and D). Only when epsin-1 was present, either alone (Fig. 2, compare B and E) or in addition to AP-2 (Fig. 2, compare C and F), CCVs were observable in the presence of dynamin-2 and GTP. When omitting dynamin-2, neither with epsin-1 alone (Fig. 2J) as clathrin adaptor nor together with AP-2 (Fig. 2K), any CCVs were generated. To further challenge the need for GTP hydrolysis by dynamin for vesicle biogenesis, reaction conditions were chosen to abolish the GTPase activity either by the addition of the nonhydrolyzable GTP analog GTPγS (Fig. 2L) or by the introduction of a version of dynamin-2 with lysine substituted by alanine in position 44 lacking the capability to bind GTP [42,43] (Fig. 2M). In both conditions, no CCVs were observed. As a further control, we analyzed whether dynamin-2 vesiculates liposomes by itself, without a need of bud formation as a prerequisite for CCV biogenesis. To this end, GUVs were first incubated with dynamin-2 and GTP for 30 min at 37 °C; then, GTP hydrolysis was blocked by the addition of 5 mM EDTA, and the sample was further incubated with epsin-1 and clathrin for 30 min at 37 °C (Fig. 2O). Only clathrin aggregates and cages were observed, similar to incubating the whole set of components without GUVs (Fig. 2N).

The donor membranes from conditions without GTP were pelleted for epoxy resin embedding to analyze ultrathin sections by electron microscopy for the presence of clathrin-coated buds. Surprisingly and in contrast to the literature [36], no buds are observable when using AP-2 alone (Fig. 2G). Rather, it seems that clathrin structures, most likely empty cages, are connected to the liposomal surface by the interaction of clathrin with its adaptor AP-2. Invaginated membrane structures, coated with clathrin, could only be observed when AP-2 was replaced by epsin-1 (Fig. 2H) or used together with epsin-1 as clathrin adaptor (Fig. 2I). Interestingly, the average diameter of the CCVs generated with epsin-1 alone is about 50 nm and thus 10 nm smaller as compared to those generated with epsin-1 and AP-2 together (about 60 nm) (Fig. 3). This result argues for a vesicle size regulating function of clathrin adaptors when acting cooperatively.

**Discussion**

**Binding of full-length recombinant adaptors and clathrin to liposomes**

AP-2 and epsin-1 are recruited to the plasma membrane via PIP₂ [35,44]. In case of epsin-1, the so-called N-terminal ENTH domain is necessary for its targeting to PIP₂-containing membranes [37]. Another important feature of epsin-1 is its membrane remodeling activity to induce positive curvature in membranes, which relies on the insertion of an amphipathic α-helix into the inner leaflet of the membrane, referred to as helix zero [36]. At high concentrations, epsin-1 alone is able to tubulate or even vesiculate PIP₂-containing liposomes, a property affected by the PIP₂ concentration. Binding and vesicle reconstitution experiments carried out in the present study were performed at much lower epsin-1 (up to 40 times less) and PIP₂ (up to 10 times less) concentrations as compared to other studies [17,36,45]. Hence, epsin-1-driven tubulation or vesiculation was not observed under all assay conditions tested. In case of AP-2, PIP₂ does play a fundamental role in membrane recruitment; however, AP-2 has to interact additionally with a cargo peptide to establish a stable interaction with the plasma.
Reconstitution of clathrin coated vesicle from GUVs

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In liposome-based recruitment assays, carried out in the present study, AP-2 and epsin-1 showed binding characteristics as expected from the literature (Fig. 1).

Reconstitution of clathrin-mediated endocytosis

With the minimal protein machinery at hand required to generate endocytic CCVs, we firstly conducted experiments to generate CCVs from liposomes along the lines of Dannhauser and Ungewickell [38], who generated CCVs using the artificial clathrin adaptor DENTH-epsin-1, clathrin, and dynamin-1 together with GTP. The presence of CCVs was analyzed using electron microscopy of ultrathin sections of epoxy resin-embedded samples. Whereas the results are nicely reproducible, the efficiency of generating CCVs was very low in both cases. Similar results were obtained using dynamin-2 instead of dynamin-1 (data not shown). Thus, the components used in the present study are functional in terms of vesicle generation. We then went on to further develop the reconstitution system to address functions of the main endocytic clathrin adaptor AP-2 and of full-length epsin-1, and a role of epsin-1 in generating CCVs even in the absence of dynamin and GTP, as was suggested from knock-down studies [17].

To this end, we established a GUV-based system to generate CCVs in vitro. This approach demonstrated a dynamin- and GTP hydrolysis-dependent mechanism.
of CCV formation, in line with most of the literature. An involvement of epsin-1 in scission was not verified as suggested by Boucrot et al. [17]. However, the results revealed an important role of epsin in the formation of CCPs, the precursors of vesicles. Most importantly, AP-2 alone turned out not to be able to form clathrin-coated evaginations on membranes; rather, clathrin aggregates were recruited to the membrane as shown on electron micrographs (Fig. 2G). This finding is in contrast to published data [35]. Under conditions used in the present study, only when epsin was present, buds and vesicles were observed (Fig. 2H, I and B, C). From the literature, it is known that epsin is displaced to the rim of a growing bud [20, 46] and that it can induce membrane curvature by the insertion of an α-helix into the membrane [36]. As AP-2 itself is not able to induce membrane curvature, epsin at the rim of a CCP is likely to induce the curvature necessary for bud formation, which is subsequently stabilized by clathrin. Our data support a CCP maturation model in which the coat of a CCP grows first as a flat lattice before the membrane starts to bend forming a CCP (constant area model (reviewed by Lampe et al. [47])). In vivo, it is unclear which factors initiate or induce curvature to the membrane. The data obtained in this study suggest epsin to be such a protein. The strongest evidence in support of epsin-1 as an initiator of curvature is observations by electron microscopy of liposomes. When AP-2 and clathrin were added, no evaginated clathrin-coated structures could be observed. However, when epsin-1 was added together with AP-2 and clathrin, or even without AP-2, such evaginations were clearly visible. Thus, epsin-1 as an example of A/ENTH domain-containing proteins plays an important role in the transition of flat clathrin lattices to invaginated clathrin-coated structures by introducing curvature to the membrane and therefore overcomes membrane tension, which has an antagonistic role in the generation of curved clathrin structures [22, 48, 49].

In the in vitro generation of CCVs, epsin-1 as a clathrin adaptor seems to be much more efficient than a 1 : 1 combination of epsin-1 and AP-2. This is most likely due to GUV aggregation that is observed when both adaptors are used simultaneously (Fig. S2). However, while incubating all components in solution, enough vesicles were generated to allow statistical analyses of their diameter. Vesicle size was not homogeneous; rather, a broad Gaussian distribution was observed. For CCVs generated with epsin-1 alone, the bell-shaped curve reveals that most vesicles have a diameter of about 50 nm (median: 49 nm). Reconstitutions with epsin-1 plus AP-2 gave rise to vesicles with a diameter of about 60 nm (median: 58 nm) in average (Fig. 3C). These findings are in line with AP-2 knock-down experiments using small interfering RNA, in which a reduced vesicle size was observed [50]. Note that under these conditions, the endocytic uptake of transferrin and the amount of clathrin-coated structures are remarkably reduced [3, 50]. Although clathrin adaptors obviously play a certain role in defining the final vesicle size, Bin/amphiphysin/Rv (BAR) domain-containing proteins that are present in all stages during bud formation [51] could also be a vesicle size determining factor, as these domains can sense or even induce curvature [52]. The epsin-related and AP180 N-terminal homology domain (ANTH) domain-containing proteins clathrin assembly lymphoid myeloid leukemia (CALM) and AP180 were already shown to influence CCV size [50, 53], and more recently, the endocytic protein NECAP1 was reported to be important for their efficient recruitment to CCPs [53]. The increase in vesicle size upon NECAP1 depletion is similar to observations upon CALM and AP180 depletion [50, 53]. Indeed, NECAP1 does not directly influence vesicle size; rather, it is necessary for efficient recruitment of CALM to CCPs. Hence, it is not exactly known what the main factors are that determine the size of a CCV. The above proteins and underlying mechanisms are not mutually exclusive, as membrane curvature-inducing or curvature-sensing proteins are also interactors for the scaffolding protein clathrin, for example, amphiphysins [55].

Experiments by us and by others have shown that CME can be reconstituted in vitro. Similar to the results of Dannhauser and Ungewickell [38], the minimal requirements are a clathrin adaptor, clathrin, dynamin, and the hydrolysis of GTP by dynamin to separate formed buds from the membrane. However, the main endocytic clathrin adaptor AP-2 alone turns out not to be sufficient to generate CCVs. Rather, the endocytic membrane remodeling protein epsin was necessary and was also able to generate CCVs even in the absence of AP-2. From this perspective, it is of interest how other curvature-sensing and/or membrane remodeling proteins would influence in vitro reconstitutions of CCVs. Examples comprise BAR domain-containing proteins, or the epsin-related proteins CALM and AP180, abundant in CCVs in amounts similar to AP-2 [56, 57]. The ANTH domain of the huntingtin-interacting protein 1-related protein (Hip1r) homologue in yeast (Sla2) cannot tubulate GUVs, other than the ENTH domain of yeast epsin (Ent1), but influences the membrane-bending activity of the ENTH domain when coassembled on membranes via direct interactions between the membrane-binding
domains [58,59]. Moreover, in vivo studies in yeast have shown that the coassembly of Sla2's ANTH domain with the ENTH domain of Ent1 is critical for endocytosis [58]. In contrast, extensive tubulation was observed when the mammalian CALM was incubated with liposomes [50]. A synergistic effect was shown on vesicle release from supported bilayers with excess membrane reservoir (SUPER) templates for the BAR domain-containing proteins amphiphysin and dynamin-2, whereas endophilin and sorting nexin-9 (SNX9) had an inhibitory effect on the release of vesicles [60]. Reconstitution of CCVs from GUVs provides a powerful tool for similar functional and mechanistic studies to further investigate the biogenesis of CCVs and, thus, helps refine our mechanistic view of the formation of endocytic CCVs.

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Author contributions

JB has performed all biochemical experiments and written parts of the text; AH has performed electron microscopy and proof read the manuscript, FW has supervised the work and written the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. EM pictures of epoxy resin-embedded sample of vesicle formation experiment with DENTHepsin-1.

Fig. S2. Fluorescence microscopy analysis of the interaction of AP-2 and epsin-1 that causes GUV aggregation.

Fig. S3. Cloning strategy for the expression of AP-2 in Sf9 cells.

Fig. S4. Cloning strategy for the expression of epsin-1 in E. coli.

Fig. S5. Cloning strategy for the expression of clathrin in Sf9 cells.

Fig. S6. Cloning strategy for the expression of dynamin in Sf9 cells.

Table S1. List of oligonucleotides used for cloning and mutagenesis.

Table S2. List of lipids used in this study.