The β -appendages of the four adaptor-protein (AP) complexes: structure and binding properties, and identification of sorting nexin 9 as an accessory protein to AP-2

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Adaptor protein (AP) complexes are essential components for the formation of coated vesicles and the recognition of cargo proteins for intracellular transport. Each AP complex exposes two appendage domains with that function to bind regulatory accessory proteins in the cytosol. Secondary structure predictions, sequence alignments and CD spectroscopy were used to relate the β -appendages of all human AP complexes to the previously published crystal structure of AP-2. The results suggested that the β -appendages of AP-1, AP-2 and AP-3 have similar structures, consisting of two subdomains, whereas that of AP-4 lacks the inner subdomain. Pull-down and overlay assays showed partial overlap in the binding specificities of the β -appendages of AP-1 and AP-2, whereas the corresponding domain of AP-3

displayed a unique binding pattern. That AP-4 may have a truncated, non-functional domain was indicated by its apparent inability to bind any proteins from cytosol. Of several novel β -appendage-binding proteins detected, one that had affinity exclusively for AP-2 was identified as sorting nexin 9 (SNX9). SNX9, which contains a phox and an Src homology 3 domain, was found in large complexes and was at least partially associated with AP-2 in the cytosol. SNX9 may function to assist AP-2 in its role at the plasma membrane.

Key words: adaptin, coated vesicle, endocytosis, intracellular transport.

INTRODUCTION

Adaptor protein (AP) complexes are tetrameric proteins in the cytosol that participate in the sorting of proteins and the formation of transport vesicles in the late secretory and endocytic pathways (reviewed in [1–4]). Four different APs are known in higher eukaryotes, designated AP-1, AP-2, AP-3 and AP-4. They all consist of two large subunits (called adaptins) of 85–130 kDa (γ and β 1 in AP-1, α and β 2 in AP-2, δ and β 3 in AP-3, and ϵ and β 4 in AP-4), one medium-sized subunit of $\approx$ 50 kDa (μ 1–4) and one small subunit of $\approx$ 20 kDa (σ 1–4). Subunits of the same type show varying degrees of similarity and are proposed to have the same general function in the different APs. The μ -subunits have a binding site for tyrosine-based sorting signals in transmembrane proteins, and by this means they function to collect cargo into the forming transport vesicle. The β -adaptins participate in the assembly of clathrin, at least in AP-1 and AP-2, a process that is thought to be the driving force for the deformation of the membrane into a coated pit at the *trans*-Golgi network and plasma membrane, respectively (reviewed in [5–7]).

The large subunits of APs are divided into functionally and structurally distinct domains: a trunk domain interacting with other subunits in the complex, an exposed appendage domain, and, between them, a flexible hinge domain. The subunits share significant homology in their N-terminal trunk domains, whereas no relationship has been detected in the hinge and appendage domains at the primary structure level. The exceptions are β 1 and β 2, which share high degrees of homology throughout their

structure. The hinge regions of β 1, β 2 and β 3 contain a sequence motif (LLN/DLD), called the clathrin box, which constitutes the main clathrin-binding site in these APs [6,8,9]. It is, however, not yet settled whether AP-3 assembles clathrin *in vivo* [10,11]. In addition to the clathrin box, a second site for clathrin binding is proposed to reside in the appendage domains of β 1 and β 2 [12].

The appendages of the large subunits of AP-2 are targets for regulatory proteins that are involved in the various phases of vesicle formation, scission from the plasma membrane and vesicle uncoating. The binding properties of the α -appendage have been studied thoroughly, and have revealed the involvement of several regulatory proteins, such as AP180, auxilin, amphiphysin, Eps15 and epsin [13–18]. Surprisingly, in spite of the fact that the appendages of AP-2 show low sequence similarity, a recent investigation showed that several of these accessory molecules also bound to the β 2-appendage domain [12]. It therefore seems that the appendage domains have overlapping binding specificities and may co-operate for the recruitment of regulatory factors. This conclusion is substantiated by the fact that the α - and β 2-appendages showed a remarkable similarity in their tertiary structures [12,17,18]. Both the α - and β 2-appendages fold into two tightly packed subdomains. The C-terminal subdomain consists of a platform-like β -sheet surrounded by three α -helices. The platform structure was in both cases shown to bind proteins with one or more copies of DPF or DPW motifs, such as the aforementioned regulatory proteins.

Since the structural and functional details are known only for the appendages of AP-2, it is of interest to compare the properties

Abbreviations used: AP, adaptor protein; CALM, clathrin assembly lymphoid myeloid leukaemia protein; GST, glutathione S-transferase; PNS, post-nuclear supernatant; SNX9, sorting nexin 9; SH3 domain, Src homology 3 domain; PX domain, phox domain; ENTH, epsin N-terminal homology domain; EH, Eps15 homology domain.

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of the appendage domains of all four AP complexes. Furthermore, it may be envisioned that several hitherto unknown cytosolic proteins play a role in the coat-assembly process through interactions with AP appendages. In the present report, we have studied the structure and function of the β -appendages of the four AP complexes. We find that the β -appendages of AP-1, AP-2 and AP-3 are functional and bind specifically to a number of proteins from haematopoietic cell cytosol, some of which were unknown previously. The corresponding domain of AP-4 is truncated and has most probably lost its binding property. As a starting point for the identification of novel accessory proteins to APs, we identified sorting nexin 9 (SNX9) as a binding partner to AP-2. SNX9 contains both an Src homology 3 domain (SH3 domain) and a phox domain (PX domain), structures that are present in other proteins with implicated functions in sorting and/or coat-formation processes at membranes. SNX9 may function to connect coat components with cargo and membrane lipids.

EXPERIMENTAL

Secondary structure prediction and sequence alignment

Amino acid sequences of the β -subunits of human AP-1, AP-2, AP-3A (the ubiquitously expressed isoform of AP-3) and AP-4 was taken from SWISS-PROT (accession nos Q10567, P21851, O00203 and Q9Y6B7). Trunk domains were aligned as defined by Pfam (<http://www.sanger.ac.uk/Pfam/>; Adaptin-N, accession no. PF01602). Sequences starting from a conserved motif in the trunk domain [W(I/L)(V/L/I)G(E/V)(Y/N/H)] [19] were analysed for regions of low complexity to define the hinge regions by programs in PredictProtein site at <http://maple.bioc.columbia.edu/pp/>, and for secondary structure prediction by Jpred2 at <http://jura.ebi.ac.uk:8888/>. Jpred2 is a consensus prediction program for protein secondary structure that uses six different prediction methods (PHD, DSC, PREDATOR, NNSSP, MULPRED and ZPRED) [20]. The input was in single-sequence mode and the Brookhaven Protein Database was bypassed to allow for validation of the result against the published X-ray structure of the β 2-appendage [12]. The results in Figure 1(A) show the consensus predictions of all methods.

Sequences C-terminal to continuous regions of low complexity of β 1–4 (appendage domains) were aligned manually. The starting point was a published alignment between appendages of β 2- and α -chains, based on similarity in tertiary structure [12]. Where necessary, gaps were introduced between predicted secondary elements to optimize the alignment.

Constructs

Plasmid constructions were carried out using standard molecular-biology techniques [21]. cDNAs encoding the C-terminal appendage domains of the human β 1 (bp 2095–2847, amino acids 699–949), β 2 (bp 2074–2811, amino acids 692–937) and α C (bp 2068–2787, amino acids 690–929) subunits were amplified by PCR from a human heart Quick-clone cDNA library (Clontech, Palo Alto, CA, U.S.A.), using 5' primers containing an *Eco*RI site and 3' primers containing an *Xho*I site (Oligo DNA-Technologies, Aarhus, Denmark). The resulting products were subcloned into the *Eco*RI–*Xho*I sites of the pGEX-5X-1 vector (Amersham Bioscience, Uppsala, Sweden), generating GST- β 1c, GST- β 2c and GST- α c constructs. All PCR products were sequenced to confirm accurate amplification. GST- β 3Ac and GST- β 4c fusion constructs encoding amino acids 810–1094 of the human β 3A subunit and 601–739 of the human β 4 subunit, respectively, cloned in-frame into the pGEX-5X-1 vector, were

kind gifts from Esteban Dell'Angelica (National Institutes of Health, Bethesda, MD, U.S.A.) [22,23]. The fusion constructs were transfected into DH5 α , BL21(DE3) and BL21(DE3) pLysS (GST- α) *Escherichia coli* strains.

Protein expression and purification

Glutathione S-transferase (GST), GST- β 3Ac and GST- β 4c were expressed in a BL21 (DE3) *E. coli* strain at 37 °C for 3 h in LB medium supplemented with appropriate antibiotics, following induction by 0.5 mM isopropyl β -D-thiogalactoside at an attenuation of 0.8 at 600 nm. For expression of GST- β 1c, GST- β 2c and GST- α c, the temperature and isopropyl β -D-thiogalactoside concentration were changed to 25 °C and 0.2 mM, respectively, due to problems with insolubility. Bacteria were harvested at 4 °C, washed once in PBS (137 mM NaCl/2.7 mM KCl/8 mM Na₂HPO₄/1.5 mM KH₂PO₄, pH 7.4), and lysed in 20 mM Hepes/KOH/5 mM EDTA, pH 7.2, containing a protease-inhibitor mixture (10 μ g/ml each of pepstatin, chymostatin and leupeptin/2 μ g/ml aprotinin/1 mM PMSF) using a Bead Beater (Biospec Products, Bartlesville, OK, U.S.A.), or repeated freezing–thawing. After centrifugation at 100 000 *g* for 60 min, the supernatant was subjected to affinity purification using glutathione–Sephacryl 4B beads (Amersham Bioscience) according to the manufacturer's instructions. Fusion proteins were purified further by gel filtration in 20 mM Hepes/KOH, pH 7.0, on a column of Sephacryl S-200 HR (Amersham Bioscience). Pooled protein fractions (1–2 mg/ml) were aliquoted, frozen in liquid nitrogen and stored at –80 °C until use.

CD spectroscopy

Purified GST fusion proteins were incubated overnight with Factor Xa (Amersham Bioscience) according to the manufacturer's instructions to cut off GST from the respective appendage domain, followed by dialysis overnight against 20 mM Hepes/KOH, pH 7.2. GST and uncleaved fusion protein were removed by absorption to glutathione–Sephacryl 4B beads and remaining protein was gel filtered in 50 mM sodium phosphate buffer, pH 7.2, on a column of Sephacryl S-200 HR. CD spectra were recorded at 23 °C on a Jobin Yvon-spex CD 6 spectrometer (Longjumeau, France) by using a quartz cuvette with a 0.5 cm optical pathlength. The spectra were measured in 50 mM sodium phosphate buffer, pH 7.2, at a protein concentration of 5 μ M. All spectra (average of three scans) were corrected by subtraction of the spectrum of a reference buffer, identical to the protein buffer. Ellipticity is reported as the mean residue molar ellipticity ($[\theta]$, in deg·cm²·dmol^{–1}), according to $[\theta] = [\theta]_{\text{obs}} \times \text{mrw} / 10 \times l \times c$, where $[\theta]_{\text{obs}}$ is the ellipticity (deg), mrw is the mean residue molecular mass, *c* is the protein concentration (in g/ml) and *l* is the optical pathlength of the cell (in cm).

Subcellular fractionation

K562 and HL-60 cells, grown in RPMI 1640 with 10% foetal calf serum and antibiotics to a density of $\approx 1 \times 10^6$ cells/ml, were harvested and washed twice in PBS and once in HES buffer (15 mM Hepes/KOH, pH 7.0/1 mM EDTA/0.25 M sucrose). All procedures were performed on ice or at 4 °C. Cells were resuspended in 5 vol. of HES buffer containing protease inhibitors and homogenized gently by passage through a ball-bearing device [24]. The homogenate was centrifuged for 10 min at 800 *g*. The nuclear pellet was washed once with 1 vol. of HES buffer and supernatants from both centrifugations were pooled to yield a post-nuclear supernatant (PNS). For the preparation of cytosol,

Figure 1 Description and analysis of investigated appendage domains

(A) Secondary structure predictions and sequence alignments of C-terminal regions of human β 1, β 2, β 3A and β 4. Sequences were analysed as described in the Experimental section and aligned manually to obtain the highest degree of similarity. Gaps (indicated by dots) were introduced only in regions where no secondary structure was predicted. For comparison, secondary structure elements of β 2 as determined by X-ray crystallography [12] are shown above the sequences. Numbers refer to amino acid positions in β 2. Predicted α - and β -structures are shown as dark and light boxes, respectively. Amino acids conserved in three of the chains are indicated by • below the sequences, and two of these symbols aligned vertically indicate residues conserved in all four chains (Cons). (B) Domain structure of full-length β -chains. The core domains (as defined by Pfam) are shown as light grey boxes, regions with low complexity as bold lines, and C-terminal subdomains 1 and 2 as white and dark grey boxes, respectively. The scale at the top indicates the amino acid number. The start of the C-terminal regions expressed and analysed in the present study are shown by arrows. (C) CD spectra of expressed and purified appendage domains of β 1, β 2, β 3A and β 4.

PNS was centrifuged at 20000 *g* for 10 min followed by centrifugation at 150000 *g* for 1 h. The clear solution between a small pellet and the lipid phase at the top was aspirated. The protein concentration was 3–4 mg/ml. For some experiments, cytosol was concentrated further to 10–20 mg/ml by centrifugation in a Macrosep 10K filtration device (Filtron Technology Corporation, Northborough, MA, U.S.A.). The cytosol was aliquoted and stored at -80°C .

For preparation of gel-filtrated cytosol, 20 ml of concentrated cytosol was adjusted to 125 mM potassium acetate and 1 mM dithiothreitol and incubated for 10 min on ice. After centrifugation at 200000 *g* for 30 min, the supernatant was gel-filtrated with a flow rate of 12 ml/h on a Sepharose CL-4B column (1.5 cm $\times$ 116 cm; Amersham Bioscience), equilibrated with 125 mM potassium acetate/25 mM Hepes/KOH, pH 7.0. Fractions of 10 ml were collected and stored at -20°C .

For the separation of total membranes and cytosol (see the experiment shown in Figure 6A, below), PNS was centrifuged for 2 h at 100000 *g*. Membranes were resuspended in HES buffer containing protease inhibitors to the same volume as PNS and cytosol.

A fraction enriched with clathrin-coated vesicles was prepared as follows. PNS (30 ml) was adjusted to 125 mM potassium acetate and incubated for 10 min on ice. The bulk of membranes were pelleted by centrifugation at 10000 *g* for 20 min in a Beckman JS13.1 rotor, and the supernatant was centrifuged for a further 1 h at 150000 *g*. The pellet was resuspended by pipetting in 600 μl of 25 mM Hepes/KOH, pH 7.0, containing 125 mM potassium acetate and 2.5 mM magnesium acetate (HKM buffer) and layered on top of a step gradient consisting of 35, 45, 55 and 60 % sucrose in HKM buffer (800 μl of each). Centrifugation was performed in a Beckman SW60 rotor at 100000 *g* for 20 h. The interphase at 45/55 % sucrose was collected, diluted 5-fold with HKM buffer, and centrifuged for 1 h at 150000 *g*. The pellet was finally resuspended in 200 μl of HKM buffer and stored at -80°C . The yield was 0.5 mg of protein from 155 mg of PNS protein.

Pull-down assay

All samples were centrifuged at 20000 *g* for 10 min before use. Glutathione-Sepharose beads were loaded with GST fusion proteins by incubation of 25 μl of a 50 % bead slurry in PBS buffer with 50 μg of fusion protein for 3 h at 4°C . Beads were recovered by centrifugation and washed twice with PBS buffer. Loaded beads were incubated by gentle agitation in a total volume of 200 μl for 2 h at 4°C in 500 μl Eppendorf tubes, together with cytosol containing additional protease inhibitors and Triton X-100 (final concentration, 0.1 %, w/v). The beads were sedimented by centrifugation and 90 % of the supernatant was removed. To allow rapid separation of beads from unbound molecules, the tubes were placed upside down in 1.5 ml Eppendorf tubes containing the following step gradient (from bottom to top): 0.4 ml of 17 % glycerol/27 % sucrose, 0.2 ml of 17 % glycerol, and 0.6 ml of 0.5 % Triton X-100. All solutions were made in 20 mM Hepes/KOH/1 mM EDTA/1 mM MgCl_2 , pH 7.0. The tubes were centrifuged for 30 s at 20000 *g*, and sedimented beads were collected and boiled in SDS sample buffer. The eluted material was analysed by SDS/PAGE and immunoblotting. All pull-downs shown were repeated at least twice with similar results.

For identification of proteins that bound to the fusion proteins by sequencing, pull-down experiments was scaled-up 10-fold (a total of 35 mg of cytosol protein was used for each reaction). After passage through a glycerol/sucrose gradient as described

above, the beads were washed additionally once with 20 mM Hepes/KOH, pH 7.0, and twice with the same buffer containing 0.1 % Triton X-100. Elution was performed by incubating the beads in 0.5 % deoxycholate in 50 mM Tris/HCl, pH 8.0, for 30 min at room temperature. This procedure retained most of the fusion protein to the beads.

Protein sequencing

Proteins obtained from pull-downs were separated by SDS/PAGE and transferred to Hybond-P membrane (Amersham Bioscience). The membrane was stained briefly with Coomassie Brilliant Blue and a piece with a protein band at 80 kDa (band no. 11 in Figure 2, see below) was excised. The protein was either N-terminally sequenced directly in an Applied Biosystems Procise 494 Sequenator, or treated with 3.3 mM CNBr in 70 % formic acid for 90 min at room temperature prior to sequencing for seven cycles. Fragmentation of the protein with CNBr yielded three to five residues/cycle (amino acids DEYSN, DGPR, LFAEG, KVAL, KSAEP, EYDI and LPI in cycles 1–7, respectively). Analysis of possible sequences starting with methionine using all permutations of obtained amino acids against the human NCBI human sequence database, using a program at <http://alpha10.bioch.virginia.edu/fasta/cgi/> (FASTF), gave four sequences with a perfect match against SNX9 (accession no. AF121859; ⁸MYDFAAEP, ²⁶³MYGLKSYI, ³⁸³MEPEAPDL and ⁴⁰⁹MDDGVKEL). No other sequence in the database showed a significant match. N-terminal sequencing gave no yield.

Protein overlay assay

Eluted material from pull-down assays was subjected to SDS/PAGE and transferred to Hybond-P membrane that was incubated in 5 % skimmed milk in PBS overnight at 4°C . The membrane was incubated for 1 h at 4°C with 1 % skimmed milk in PBS containing 0.25 mg/ml of fusion protein. After repeated washings in 0.05 % Nonidet P-40 in PBS, bound fusion protein was detected by chemiluminescence using goat antibodies against GST (Amersham Bioscience).

Immunoprecipitation

K562 cells were harvested and washed three times in PBS. The cell pellet was resuspended in PBS containing protease inhibitors to a concentration of 250 μl of packed cells/ml. Nonidet P-40 was added to a final concentration of 1 % and the cells were lysed by incubation on ice for 10 min. The sample was centrifuged at 20000 *g* for 10 min and 75 μl of the supernatant was incubated with the monoclonal antibodies 100/2 or AP.6 (for details, see the next section), or without primary antibody, for 5 h at 4°C . Protein A-Sepharose beads (10 μl ; Amersham Bioscience) were saturated with rabbit anti-mouse Ig antibodies (Dako, Glostrup, Denmark) and were added to the samples. After an additional 6 h of incubation with gentle agitation, the beads were washed once in 1 % Nonidet P-40 in PBS and finally passed through a glycerol/sucrose gradient, described above. Immunoprecipitated proteins were analysed by SDS/PAGE and immunoblotting.

Electrophoresis and immunoblotting

Proteins were resolved in 7 % polyacrylamide gels in the presence of SDS, and were either stained with colloidal Coomassie Brilliant Blue [25] or transferred to Hybond-P using a Semi-Dry transfer cell (Bio-Rad, Hercules, CA, U.S.A.). Blots were blocked over-

Figure 2 Detection of cytosolic proteins interacting with GST-appendage-domain fusion proteins

GST and GST fusion proteins were incubated with 2 mg of cytosol and complexes were pulled-down with glutathione–Sepharose. Bound proteins were detected by colloidal Coomassie Brilliant Blue staining after SDS/PAGE. Major proteins that interacted specifically with β -appendages are indicated by numbers between lanes and discussed in the text. Recombinant proteins can be seen as strong bands below the 66 kDa marker in each lane, except GST (26 kDa) and GST- β 4c (42 kDa), which were allowed to migrate out of the gel to increase the resolution in the high-molecular-mass region. Migration of molecular-mass markers is indicated to the right.

night in PBS containing 5% skimmed milk at 4 °C. Incubations with primary and secondary antibodies were performed in 2% skimmed milk in PBS for 1 h at room temperature, followed by repeated washes in 0.05% Tween-20 in PBS. Primary antibodies used were: rabbit anti-Eps15 (H-896), goat anti-epsin (R-20) and goat anti-CALM (C-18; raised against a peptide from clathrin assembly lymphoid myeloid leukaemia protein) from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), mouse monoclonal anti-clathrin (clone 23) from BD Transduction Laboratories (Lexington, KY, U.S.A.), mouse anti- α -adaptin (100/3) and mouse anti- γ -adaptin (100/2) from Sigma-Aldrich (St. Louis, MO, U.S.A.) and mouse anti- α -adaptin (MA1-064) clone AP.6 from Affinity Bioreagents (Golden, CO, U.S.A.). Rabbit anti-SNX9 antibodies against recombinant human SNX9 [26] were a kind gift from Carl P. Blobel (Memorial Sloan-Kettering Cancer Center, New York, U.S.A.). Secondary horseradish peroxidase-conjugated antibodies used were anti-rabbit and anti-mouse Igs from Amersham Bioscience, and anti-goat Igs from Dako. ECL+ Plus reagent and Hyperfilm ECL (Amersham Bioscience) were used for chemiluminescence detection. Quantification of immunoblotting results was done from digitized images of the X-ray films.

RESULTS

Previous alignment studies have revealed that the β -subunits of AP complexes are related in the N-terminal trunk domains [19,22,23,27]. β 1 and β 2 are in addition similar in their hinges and appendage domains, but the corresponding regions of β 3A (and the neuronal-specific isoform β 3B) and β 4 do not obviously share homology with the other two members. The hinge and/or the appendage of β 4 were suggested to be shorter, whereas that of β 3A/B seemed to be longer. We analysed the sequences of the four β -adaptins by a combination of several secondary structure

prediction methods. The analyses delimited the hinge regions that started at the end of the previously defined trunk domains (at amino acids 550–600), and ended at variable positions in the different β -chains where significant predicted structure appeared (see Figure 1B). The appendages of β 1, β 2 and β 3A were of the same size ($\approx$ 230 amino acids), whereas that of β 4 was about half the size. Sequences of the four β -appendages are shown in Figure 1(A). To validate the secondary structure predictions, the predicted structure of β 2 was compared with its structure determined by X-ray crystallography [12]. In almost all positions, a good correlation was found between the prediction and the crystal structure. In a few cases, the wrong signature of the element was assigned (e.g. in the middle of subdomains 1 and 2 a predicted α -structure should be a β -structure). Manual sequence alignment revealed notable homology between the appendages. This is especially significant in the outer subdomain (which contains the binding platform in β 2), where six residues were fully conserved, and a number of amino acids were shared between three of the four chains (Figure 1A, •). Most importantly, when the sequences were aligned, the predicted secondary structure elements also aligned, albeit in some cases with different signatures. Taken together, it is conceivable that not only do β 1 and β 2 have related appendage structures, but that also the C-terminal of β 3A is folded similarly.

From comparative analyses of the sequences it is clear that the short appendage of β 4 is more related to subdomain 2 than to subdomain 1. The immediate sequence upstream of the last 120 amino acids only shows regions of low complexity without any sequence relatedness to subdomain 1 of the other β -chains. In fact, there is no room for another subdomain in β 4, since the trunk domain ends less than 100 amino acids before the start of subdomain 2. Subdomain 1 in α - and β 2-chains is proposed to function as a scaffold to stabilize the outer binding subdomain [17,18]. It seems that β 4 is truncated, in the way that it is lacking

Figure 3 Identification of cytosolic proteins bound to GST fusion proteins by immunoblotting and overlay

GST and GST fusion proteins were incubated with 1.6 mg of cytosol and complexes were pulled-down with glutathione–Sepharose. Bound material was separated by SDS/PAGE, and proteins were transferred to Hybond-P filters. **(A)** The filter was cut at protein markers 82 and 118 kDa (Pre-stained molecular-mass markers; Bio-Rad) to create three pieces, which were incubated separately with antibodies against Eps15, epsin and CALM. The upper filter was reused to detect clathrin. To visualize the interaction of clathrin with GST- β 1c and GST- β 2c, the upper filter was re-exposed to X-ray film for a longer time (Over-exposed). In order to estimate the yield in binding, 40 μ g (for clathrin, epsin and CALM) or 140 μ g (for Eps15) of cytosol was analysed in parallel by SDS/PAGE and immunoblotting (Input). The estimated molecular masses of the detected proteins are shown to the right. **(B)** Filters were incubated separately in solutions containing GST fusion protein as indicated. Bound fusion proteins were detected by immunoblotting with antibodies against GST. Different exposures of the upper and lower parts of the filter are shown. Migration of Eps15 and epsin as determined by immunoblotting are indicated, and arrowheads denote unidentified proteins that specifically interacted with GST- β 2c. Numbers refer to bands visualized in Figure 2. Molecular-mass markers are indicated on the right.

the scaffold subdomain. The domain structure of β -adaptins is shown in Figure 1(B). A similar analysis of α -, γ -, δ - and ϵ -adaptins, as presented here for β -adaptins, revealed that all but the γ -chain aligned with both subdomains 1 and 2 (results not shown).

To experimentally examine the structure and function relatedness of the four β -adaptins, the appendages were expressed as GST fusion proteins. The fusion proteins were cleaved, and the appendages were purified and analysed by CD spectroscopy to explore their α/β content (Figure 1C). As expected, the spectra of β 1 and β 2 were very similar, showing a mixture of both α - and β -structure consistent with the published crystal structure of β 2. Interestingly, β 3A and β 4 showed a higher proportion of α -helices, as revealed by an absorbance maximum shift at 190–195 nm and by the broadening of the negative signal at 210–220 nm. In the case of β 4, this alteration conforms to the suggestion that it only consists of subdomain 2. All α -helices in the β 2-appendage are located in subdomain 2, and subdomain 1 only consists of β -structure. An appendage without subdomain 1 would therefore yield a higher proportion of α -structure. To further validate this interpretation, a difference spectrum between β 2 and β 4 was calculated. The result showed a pure β -spectrum (results not shown), which confirms the idea that the appendage of β 4 is made up of subdomain 2. The higher content of α -structure in β 3A is more difficult to interpret. It may be that β 3A contains extra α -helices that are not present in β 1 and β 2, although such extra elements are not revealed by the analysis shown in Figure 1(A). However, since the CD signal is stronger for α -helices than for β -structures [28], it is also possible that the observed deviation is due to one or several of the three putative α -helices in subdomain 2 of β 3A being longer than that in β 1 and β 2. In addition, we cannot rule out the possibility that part of the hinge that is present in our β 3A construct (48 amino acids) may

form α -structure when present out of context (positioned N-terminally after cleavage).

As the appendages of β 1 and β 2 are similar, whereas β 3A and β 4 are more distantly related to β 1 and β 2, it was of interest to compare their ability for protein binding. The GST fusion proteins were used to pull down proteins from haematopoietic cell cytosol through capture by glutathione–Sepharose. Figure 2 shows the result of a binding reaction analysed by SDS/PAGE and stained with colloidal Coomassie Brilliant Blue. β 1 and β 2 showed similar binding patterns (although differences were found, see below), whereas β 3A gave a completely different pattern. β 4 did not show specific binding of any protein and gave the same result as the GST negative control.

The numbers between lanes on Figure 2 refer to the major specific bands that were consistently detected in pull-down experiments with the GST fusion proteins investigated. Some of these proteins were identified and are discussed below. Band 1 is clathrin, for which β 3A has a strong binding site in its hinge region (the clathrin box, which was included in the construct). The corresponding sequences of β 1 and β 2 were omitted from the constructs, and the small amounts of clathrin detected with these proteins were therefore only through their appendage domains, a result also shown by others [12]. Clathrin was the only protein that we could detect that was pulled-down by all three appendages. Some of the proteins pulled-down by β 2 were also bound by β 1 to approximately the same extent (Figure 2, bands 2 and 12), whereas others were more strongly bound to β 2 than to β 1 (Figure 2, bands 3, 5 and 7). For certain proteins the reverse in binding preference could be seen (i.e. β 1 bound stronger than β 2; Figure 2, bands 6, 9 and 10), and finally some proteins that bound to β 2 could not be detected with β 1 (Figure 2, bands 4 and 11). From these results, it is clear that β 1 and β 2 do not have identical binding characteristics, despite the extensive sequence

homology in their appendage domains. β 3A showed several unique bands, most of which were very weak (not marked in Figure 2). Only one protein (Figure 2, band 8) gave consistently a clearly visible band in pull-downs from cytosol. The identity of this protein is under investigation.

Previous studies have revealed the binding of several cytosolic brain proteins to the appendages of β 2- and α -adaptin [12–18]. Since some are expressed ubiquitously, or have homologues in other cells, we specifically tested for the presence of these proteins in the cytosol and pull-downs from haematopoietic cells by immunoblotting. The results shown in Figure 3(A) confirm the binding of clathrin, Eps15 and epsin to the β 2-appendage, and also demonstrate interaction with β 1 to a lesser extent. By careful analysis in parallel experiments, we could identify bands 2 and 7 in Figure 2 as Eps15 and epsin, respectively. Both Eps15 and epsin showed a variable extent of binding to β 1 in different experiments (e.g. compare results for Eps15 in Figures 3 and 4, below). The reason for this is not known. Amphiphysin was not detected in the cells investigated, and auxilin and AP180 are known as brain-specific proteins [29]. However, we also tested for the presence of CALM, which is considered as the non-neuronal counterpart of AP180. CALM displayed two bands, at 77 and 66 kDa, which most probably represent alternative splice variants from a common transcript [30,31]. CALM bound specifically to β 2 and to a very low extent to β 1. Quantification of the immunoblotting signals revealed that, compared with Eps15 and epsin, of which approximately half of the amount in cytosol was pulled-down, only a few percent of CALM was bound to β 2. CALM could not be detected as Coomassie-stained bands in Figure 2. Interestingly, none of the investigated proteins (except for clathrin) had affinity for β 3A or β 4, again emphasizing the differences in specificity of the β -appendages.

We also investigated the binding of cytosolic proteins to β -appendages by a combination of pull-down and overlay experiments. The results in Figure 3(B) show that GST- β 2c pulled-down at least six proteins that could be detected with GST- β 2c in the overlay. By comparison with normal immunoblotting on the same filter (results not shown), it could be inferred that two of the bands were Eps15 and epsin, confirming direct binding to these proteins that was not dependent on their tertiary structure. Weak interactions with Eps15 and epsin were also detected in a GST- β 1c overlay, but only when GST- β 2c was used in the pull-down (results not shown). This indicates that the two appendages bind the same isoforms of the molecules, and that the affinity to β 1 is weaker. CALM was not detected by overlays. Pull-downs and overlays with GST- β 3Ac and GST- β 4c gave no signals at all. This could mean that either the appendage has very low affinity for cytosolic proteins (as may be the case for β 4), or that the binding requires intact tertiary structure of the ligand. The latter is a likely explanation for the failure to detect clathrin by this experimental set-up, since the clathrin box of β 3A is known to interact with the β -propeller structure in clathrin [9], a structure that is destroyed in SDS/PAGE. It is possible that binding of the β 3A-appendage to other cytosolic proteins is dependent on their tertiary structure.

Several unidentified proteins were detected in the GST- β 2c pull-down/overlay assay (numbers on the left of Figure 3B correspond to the marked bands in Figure 2). We decided to try to identify some of these proteins by scaling-up the pull-downs and subjecting the resulting proteins bands to amino acid sequencing after SDS/PAGE. So far, we have identified proteins 11 and 12. Protein no. 12 was identified as α -tubulin, a protein that is known to interact with many other proteins in assays *in vitro*. The significance of this interaction is uncertain and tubulin was not investigated further. Protein no. 11 was more attractive

Figure 4 Interaction of the appendage domains of AP 2 with SNX9

Cytosol from K562 cells (2 mg) was subjected to pull-down with GST, GST- β -appendages or GST- α -appendage. Immunoblotting with antibodies against SNX9 and Eps15 was used to detect specific interaction after separation by SDS/PAGE. A faint band seen in the GST- β 3A lane with anti-SNX9 detection is due to unspecific antibody binding to a bacterial protein contaminant in the GST- β 3A preparation (also seen in Figure 2 between protein no. 8 and the fusion protein). As controls, 50 μ g of cytosol from K562 and HL-60 cells were analysed for presence of SNX9 and Eps15. The immunoblotting filter was cut at 120 kDa and the pieces were incubated separately with antibodies. Estimated molecular masses of the detected proteins are shown to the right.

for further studies. This protein was identified as a member of a large family of cytosolic proteins, named sorting nexins, which are involved in various aspects of intracellular sorting. The discovered protein was SNX9, a protein that has been only sparsely studied [26]. It possesses an SH3 domain in the N-terminus and a PX domain in the middle of the molecule. Furthermore, SNX9 contains a DPW motif immediately downstream of the SH3 domain. This sequence is the probable binding site for the β 2-appendage.

SNX9 (also called SH3PX1) is a protein of ≈ 80 kDa that is widely expressed, although the levels vary in different tissues [26]. When cytosol from haematopoietic cells investigated in the present study was tested by immunoblotting, we found that the expression was much higher in K562 cells than in HL-60 cells (Figure 4). Pull-downs combined with immunoblotting revealed that only the appendages of AP-2 bound SNX9. This result is different from that found for the other specifically tested proteins, which also showed binding to the β 1-appendage (see Eps15 included as a control in Figure 4), and is consistent with the binding pattern for band no. 11 in Figure 2. When proteins in cytosol were separated by gel filtration we found that SNX9 was mainly present in large complexes ($> 1 \times 10^6$ kDa), eluting just after the void volume on Sepharose CL-4B (Figure 5A). This behaviour was shared by most of AP-2, as well as part of AP-1 and clathrin. In contrast, Eps15, epsin and CALM eluted at positions suggesting that the majority of these molecules exist as monomers or dimers in the cytosol, or as part of small complexes.

Since the appendages of AP-2 showed affinity for SNX9, and the two proteins displayed co-elution in gel filtration, it was of interest to see if a direct interaction between these proteins could be detected in a freshly prepared cell lysate. Figure 5(B) shows that when AP-2 was immunoprecipitated with antibodies against α -adaptin, SNX9 was co-precipitated specifically, as detected by immunoblotting. In contrast, antibodies against AP-1 did not co-immunoprecipitate SNX9, again demonstrating the selective binding of this protein to AP-2. Quantification of the result revealed that only a few percent of SNX9 was brought-down by anti-AP-2 antibodies. This may indicate that SNX9 is additionally present in other types of complex, or that the interaction

Figure 5 Detection of SNX9 in high-molecular-mass complexes in cytosol

(A) K562 cytosol was gel-filtrated on a column of Sepharose CL-4B. The column was calibrated with Dextran Blue (void volume, V_0), thyroglobulin (Tg; 670 kDa), IgG (150 kDa) and haemoglobin (Hb; 64.5 kDa). Eluted fractions were analysed for total protein content and for the presence of clathrin, Eps15, α -adaptin, γ -adaptin, epsin, SNX9 and CALM by SDS/PAGE and immunoblotting. (B) A cell lysate from K562 cells was immunoprecipitated with antibodies against γ -adaptin (100/2) and α -adaptin (AP.6), or without primary antibody (Control). Immunoprecipitates were resolved by SDS/PAGE and analysed by immunoblotting with antibodies against α -adaptin (100/3), γ -adaptin (100/2) and SNX9. Due to re-probing of the immunoblotting filter, a faint α -adaptin band is still visible in the α -adaptin lane after incubation with anti- γ -adaptin. Estimated molecular masses of the detected proteins are shown to the right of both panels.

with AP-2 is affected by the dilution step necessary to disrupt the cells. Nevertheless, the specific complexes positively detected in the cytosol argue for a functional relevance of the interaction between the two proteins.

The members of the sorting nexin family of proteins show a marked divergence in their membrane localization by immunofluorescence; some of the proteins are considered as peripheral membrane proteins whereas others are distributed in the cytosol [32,33]. The distribution of SNX9 was tested by immunoblotting after separation of membranes and cytosol by ultracentrifugation. Figure 6(A) shows that the majority of the total amount of SNX9 in the cells was found in the cytosol. As controls, a small amount of epsin was found associated with membranes, and for CALM about half of the cellular content was membrane-bound. This result indicates that SNX9, if participating in a coat-formation process involving AP-2, only

associates transiently with the membrane, or that it has a role exclusively in the cytosol. We also tested for the presence of SNX9 in clathrin-coated vesicles (Figure 6B). Isolation of a subcellular fraction based on known properties of clathrin-coated vesicles showed enrichment for clathrin and AP-2 but not for SNX9, indicating that SNX9 is not part of the vesicle coat. Taken together, a potential role of SNX9 may be to assist AP-2 before, or at the beginning of, the assembly of the coat at the plasma membrane. Further studies are required to establish the functional mechanism of SNX9.

DISCUSSION

The appendage or 'ear' domains of AP complexes were visualized previously by freeze-etch electron microscopy as flexible structures protruding out from a brick-shaped core [34], suggesting a

Figure 6 Cellular distribution of SNX9

Subcellular fractions isolated from K562 cells as described in the Experimental section were subjected to SDS/PAGE and analysed by immunoblotting. **(A)** Equal proportions of PNS, cytosol and total membranes were analysed for the presence of epsin, SNX9 and CALM. **(B)** Equal amounts of protein (15 μ g) from PNS and a fraction enriched for clathrin-coated vesicles (CCV) were analysed for clathrin, α -adaptin and SNX9. Under the conditions used, the amount of α -adaptin in PNS was below the detection limit.

binding function of these domains. More recently, several proteins were identified that bound specifically to α - and β 2-appendages and which may assist AP-2 function. These proteins are in turn characterized by the presence of one or several protein- or lipid-binding modules, such as epsin N-terminal homology domain (ENTH), Eps15 homology domain (EH), BIN-amphiphysin-RVS domain (BAR) and SH3 domains, or target sequences, such as DPW/F and NPF motifs, which have the potential to form additional contacts with other factors, yielding a complex network of interactions (reviewed in [29,35,36]). The accessory proteins are implicated in the process of coat assembly and related events at the membrane, but their exact roles are not yet revealed. Most of these proteins seem to interact only transiently with AP-2, and several of them are expressed only in neuronal cells that require an efficient endocytosis machinery for synaptic vesicle recycling. It is likely that other, or additional, proteins participate in the endocytic process in non-neuronal cells.

Whereas several cytosolic proteins have been identified that associate with the appendages of AP-2, few proteins are known that associate with the corresponding structures of other AP complexes. Recent reports identified γ -synergism (an EH-containing protein), microtubule-associated protein 1A, rabaptin-5, auxilin 2 and clathrin as partners to γ -adaptin [37–40], and Eps15, epsin and clathrin were mentioned in a previous report to bind to the appendage of β 1 [12]. We show in the present study that cytosolic proteins, not only belonging to the previously known accessory factors, can be detected that show differential preference for binding to the appendages of β 1, β 2 or β 3. One of

these was identified as SNX9, whereas several of the other proteins remain to be analysed. CALM is viewed as the non-neuronal counterpart to AP180. Although their sizes differ markedly, the two proteins have common structures in their N-terminal regions [41]. CALM is a ligand of EH-domain-containing proteins (through NPF motifs) and in addition contains an ENTH-like domain and the DPF/W motif. As is the case for AP180, CALM may have an important role in vesicle-size determination by regulating clathrin recruitment to the membrane [42]. We show here for the first time that CALM indeed binds to the β 2-appendage and, to a much lower extent, to the β 1-appendage. We also find that a substantial part of CALM is associated with membranes in the steady state. This is in accordance with recent reports showing phosphoinositide-binding properties of its N-terminal ENTH-like domain [42,43]. Although CALM has been shown to have a site at the C-terminus for direct binding to clathrin [30], it should be mentioned that CALM can interact with the β 2-appendage independently of clathrin, since CALM can be pulled-down by GST- β 2c from gel-filtration fractions (see Figure 5A) in which no clathrin is present (results not shown).

Common to all identified proteins that bind to the appendage domains of β 1-, β 2- and α -adaptin is the presence of at least one copy of the DPF/W motif. This motif was suggested to be the target for the platforms in the outer subdomains of the appendages [12,17,18]. However, it is clear from our studies that other structures in the subdomains, and/or in the ligands, can also influence the interaction, as revealed by the differential binding to β 1 and β 2. Interestingly, essentially all residues constituting the platform in β 2 are conserved in β 1 (18 of 19 amino acids), together with most of the neighbouring amino acids. Overall, of the few amino acids that differ between the C-terminal subdomains of β 1 and β 2 (24 differences out of 112 amino acids) most are conservative substitutions. Therefore, it is highly likely that the platform structures are similar in β 1 and β 2. A difference between the subdomains is an insertion in β 1 of three extra amino acids that would extend the loop between two of the β -strands constituting the platform (the region around amino acid 910 in Figure 1A). Furthermore, the beginning of the adjacent strand contains the only amino acid substitution of the platforms in β 1 and β 2 (a Thr $\rightarrow$ Glu exchange). It is possible that this area, which forms an edge of the platform, could positively or negatively influence the binding of certain molecules to β 1. Curiously, whereas the appendage sequence similarity is much higher between β 1 and β 2 than between β 2 and α , the latter domains are more alike in terms of binding specificity.

The appendage of β 3A did not bind any of the tested proteins and showed a unique binding pattern of proteins from cytosol. Secondary structure prediction and sequence alignment indicated, however, that the principal structure might be similar. Although several amino acids are conserved in the outer appendage domains of β 2 and β 3A (21 of 112 amino acids), only five of those reside in positions constituting the platform in β 2. If β 3A adopt the same conformation as β 2, it can be anticipated that the binding specificity of the platform must be quite different. From this reasoning, it is not surprising that proteins other than those carrying the DPF/W motif bind to this appendage.

No protein could be detected that bound to the appendage of β 4. A possible explanation is that the number of putative binding molecules is too low in the cytosol to be detected. AP-4 is present at a much lower concentration than the other three adaptor complexes in K562 cells (R. Lundmark and S. R. Carlsson, unpublished work) and in other cells [19,23]. If putative accessory molecules, regulating the activity of AP-4, were present in comparable amounts to the adaptor complex itself, the meth-

odology used in the present study would not be sensitive enough to detect them. However, as suggested in the present report, an alternative (or additional) explanation is that the β -appendage of AP-4 has lost its binding capacity through truncation of the domain. The role of AP-4 in intracellular transport is still uncertain [44], although its μ -subunit was shown recently to be functional in the recognition of tyrosine-based signals [45]. It may be that AP-4 only needs one of the appendages to fulfil its function. That ϵ -adaptin appendage may be functional was indicated by its alignment with subdomains 1 and 2 in β 2- and α -adaptin (results not shown).

A novel protein identified in the present study was SNX9, which was one of the major proteins that bound to GST- β 2c. As found for other proteins that bind the β 2c domain, such as Eps15, epsin, amphiphysin and AP180/CALM, SNX9 also bound to the α -adaptin appendage. A difference from several of these other proteins was that no binding could be observed to the β 1 appendage. In humans, the sorting nexin family consists of at least 21 members, and homologues can be found in a variety of organisms from yeast to human. The exact mechanism by which they function is not yet known, but several of the members are directly or indirectly involved in the sorting and trafficking of membrane receptors, such as P-selectin, the receptors for epidermal growth factor, platelet-derived growth factor, insulin and transforming growth factor- β in mammalian cells, as well as the sorting of vacuolar hydrolases in yeast [32,33,46–49]. The collection of this rather diverse set of proteins into a distinct family is based on the homology of their PX domains. Variants of this domain are present in many other proteins and several groups have recently identified the function of PX domains as phosphoinositide-binding modules [50–54]. SNX9 differs from other sorting nexins by the additional presence of an SH3 domain [26]. This domain is also present in other proteins with roles in endocytosis, such as amphiphysin, endophilin, intersectin and syndapin [29,35,55]. SH3 domains bind to proline/arginine-rich domains, including those in dynamin and synaptojanin. The features of SNX9 therefore fit well with a role in AP-2-mediated endocytosis. SNX9 was identified originally as a protein interacting with the cytoplasmic domains of the plasma-membrane-localized metalloprotease disintegrins MDC9 and MDC15, and was proposed to regulate the function of these proteases by influencing their intracellular processing [26]. In *Drosophila*, the orthologue of SNX9 was shown recently to be present in a complex that connects the axonal guidance receptor Dscam to the actin cytoskeleton [56]. In accordance with our results, SNX9 was in addition detected in complexes with AP-2. It is conceivable that SNX9 functions as a link between cell-surface receptors, the cytoskeleton, membrane lipids and the endocytosis machinery.

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