Identification and functional characterization of *Arabidopsis* AP180, a binding partner of plant αC-adaptin

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Summary

Clathrin-mediated endocytosis is a well-studied uptake mechanism for nutrients and signalling receptors in mammalian cells that depends on the coordinated interaction of coat proteins and endocytic network proteins to perform the internalization. In this process AP180 promotes the assembly of clathrin triskelia into coated membrane patches at the plasma membrane, while α-adaptin interacts with various network proteins that are in turn required for the budding of the coated pits. The process of clathrin-mediated endocytosis in plants has not been dissected at the molecular level, nor have the members of an analogous uptake machinery been functionally described. In this respect, we have investigated the AP180 and α-adaptin orthologs from *Arabidopsis thaliana*: At-AP180 and At-αC-Ad. Both plant proteins display the same structural features as their mammalian counterparts and fulfill the same basic functions. To identify their interacting partners, the ear region of At-αC-Ad and the C-terminal region of At-AP180 were used as fusion proteins in pull-down experiments and plasmon-resonance measurements. At-αC-Ad binds several mammalian endocytic proteins, and its interaction with At-AP180 requires the DPF motif. At-AP180 functions as a clathrin assembly protein that promotes the formation of cages with an almost uniform size distribution. Deletion of the single DLL motif abolished the assembly activity of At-AP180 almost completely, but did not affect its binding to triskelia, suggesting the existence of additional binding determinants.

Key words: α-Adaptin, AP180, Network-proteins, Endocytic clathrin-machinery, Clathrin reassembly

Introduction

Clathrin-mediated endocytosis (CME) serves to take up nutrients from the extracellular environment into a eukaryotic cell and is also required to clear the plasma membrane (PM) of signalling receptors (reviewed by Mellman, 1996; Cavalli et al., 2001). CME is initiated at specific sites of the PM where the recruitment of adaptor 2 (AP2) complexes, clathrin and endocytic network proteins takes place. This is followed by the invagination of clathrin-coated pits (CCP) and finally culminates in the release of coated endocytic vesicles into the cytoplasm. After shedding their coat, the naked endocytic vesicles are then able to fuse with an early endocytic compartment, thereby releasing their cargo into the endocytic pathway (reviewed by Gruenberg, 2001).

Dissection of each single step during the process of CME has revealed that distinct subsets of cytosolic endocytic network proteins are required for efficient clathrin and cargo recruitment (Brodsky et al., 2001; Higgins and McMahon, 2002). Thus, the various components of the clathrin machinery can be divided into two groups. The first group comprises the true coat components: clathrin, the AP2 complex, AP180, auxilin and HIP1/HIP1R (Kirchhausen, 1999; Kirchhausen, 2000a; Engqvist-Goldstein et al., 2001; Scheele et al., 2001), whereas the second group consists of cytosolic network proteins (e.g. eps15, amphiphysin, epsin and dynamin), which associate only transiently with the coat proteins, but also with each other in order to perform their functions (Kichhausen, 2000b; Slepnev and DeCamilli, 2000). A cross-section through a clathrin-coated vesicle (CCV) therefore reveals a three-layered structure: the outer coat layer of clathrin triskelia is connected via the middle layer of AP2 complexes to the inner layer of integral proteins of the vesicle membrane (Pearse et al., 2000).

Of the four mammalian AP complexes the AP2 complex, which is exclusively associated with endocytic CCV, is the best characterized (Brett et al., 2002; Collins et al., 2002). Like the others, it is a heterotetrameric complex consisting of two large (αA- or αC-, β2-adaptin, ~100 kDa), one medium (μ2-adaptin, ~50 kDa) and one small subunit (σ2-adaptin, ~20 kDa) (Kirchhausen, 1999). With the exception of the small subunit, all the others have well-described functions assigned to their specific domains. Accordingly, the C-terminal ear domain of αC-adaptin serves as a binding platform for several network proteins, which in turn contain specific αC-ear-domain binding motifs (DPF, DPW or FXDXF) in a variable number and composition (Owen et al., 1999; Brett et al., 2002). Likewise, the monomeric neuronal coat protein AP180 also contains within its C-terminal disordered region several consensus

binding sites for the αC-adaptin ear domain and also for clathrin (Morgan et al., 2000; Kalthoff et al., 2002). Since AP180 was discovered independently in several laboratories, it was formerly designated NP185 (Kohtz and Puszkin, 1988) and F1-20 (Zhou et al., 1992), as well as AP3 (Keen, 1987). The commonly used name AP180 (Ahle and Ungewickell, 1986) reflects its electrophoretic mobility in SDS gels, where it migrates as a polypeptide double its size (Morris et al., 1993). AP180 is exclusively found in neuronal cells, where it plays a crucial role in synaptic vesicle formation (Morgan et al., 1998; Yao et al., 2002), but it also promotes the assembly of uniform size clathrin cages in vitro (Ahle and Ungewickell, 1986; Ye and Lafer, 1995b). Its non-neuronal homolog CALM is less efficient as a clathrin assembly protein (Dreyling et al., 1996; Tebar et al., 1999). Like their mammalian AP180 homologs, UNC-11 from *Caenorhabditis elegans* (Nonet et al., 1999) and LAP from *Drosophila* (Zhang et al., 1998) are also implicated in CME. The N-terminal region of AP180 displays an epsin-N-terminal-homology (ENTH) domain, which enables the protein to bind specifically to PM-localized phosphatidylinositol-(4,5)-bisphosphate (Itoh et al., 2001). Thus, AP180 is supposed to play an important role in the first step of clathrin-coated pit initiation in that it tethers clathrin to the PM (Ford et al., 2001). Another group of mammalian ENTH-domain-containing proteins also involved in endocytosis are the epsins. These resemble AP180, not only in their structural features, but also in their capacity to assemble clathrin triskelia (Kalthoff et al., 2002) and to bind to α-adaptin (Wang et al., 1995), but they can not be co-purified with clathrin-coat components in a CCV fraction (Chen et al., 1998).

While mammalian CME has been dissected at the molecular level, the situation in plant cells differs remarkably. In fact, a controversial debate has gone on over decades as to whether endocytosis might be possible at all in intact plant cells. Turgor pressure has often been considered to be the main obstacle, but the turning point in this discussion was brought about by the realization that at low turgor pressures and for small vesicles (<100 nm), endocytosis is thermodynamically feasible. Because, in addition, turgor fluctuates considerably under field conditions, it is no longer considered to be an absolute factor preventing the occurrence of endocytosis in plants (for reviews see Low and Chandra, 1994; Hawes et al., 1995; Marcote et al., 2000; Holstein, 2002).

Against this background it is not surprising that only little is known about clathrin-dependent endocytosis in plant cells. CCP have been reported at the plant PM and they are especially numerous in actively growing cells with a high PM turnover rate such as root hairs and pollen tubes (Emons and Traas, 1986; Blackbourn and Jackson, 1996; Hepler et al., 2001). Nevertheless, despite the fact that some features of receptor-mediated endocytosis also seem to apply to plants, this process has not been proven unequivocally. Thus, the cargo of plant endocytic CCV still has to be identified. Signalling receptors, most of which belong to the large group of receptor protein kinases (McCarty and Chory, 2000), are probable candidates for CME, as some of them contain the conserved YXXØ internalization motif that allows for interaction with the respective adaptins (overview in Holstein, 2002; Happel et al., 2004). Although the uptake of electron-dense markers via CCP at the plant PM has been reported (overview in Low and Chandra, 1994) clathrin has not been connected to plant endocytosis events at the molecular level despite the fact that orthologs of several coat components have been described: a plant clathrin heavy chain (CHC) (Blackbourn and Jackson, 1996), a plant clathrin light chain (CLC) (Scheele and Holstein, 2002), β-adaptins (Holstein et al., 1994; Holstein and Happel, 2000), μ-adaptins (Happel et al., 1997; Happel et al., 2004) and also plant σ-adaptins (Boehm and Bonifacino, 2001). In addition, the existence of homologs to other components of the mammalian clathrin machinery might qualify them as plant network-protein candidates (Lam et al., 2001; Holstein, 2002).

To obtain a first insight into the existence of a clathrin-based endocytosis machinery in plants, we have functionally characterized the *Arabidopsis* orthologs of two key proteins involved in mammalian endocytosis: AP180 and αC-adaptin. We show that the plant αC-adaptin homolog binds to several mammalian network proteins and also to plant At-AP180, and depends thereby on the DPF-motif. Furthermore, the clathrin assembly activity of At-AP180 has been shown, which requires the presence of a single consensus clathrin binding/assembly motif.

Materials and Methods

Bio-materials

Cell suspension cultures of *Arabidopsis thaliana* (At-7) were obtained from the Max-Planck-Institute (Cologne, Germany) and grown in the dark under constant rotation (90 rpm) on a cell shaker and harvested 7 days after inoculation. Wild-type *Arabidopsis thaliana* var. Columbia was grown in the green-house with a cycle of 16 hours light and 8 hours in the dark. Brains from adult pigs were obtained from a local slaughterhouse and frozen immediately after removal of the meninges.

Sequence analysis

For the protein structure homology modeling the automated Swissmodel server with the mouse PDB 1KY6 file as a template was used. Casein kinase 2 substrate sites were detected by analysis of the At-AP180 sequence using the Prosite database. The best alignment of two sequences was performed with the LALIGN algorithm version. 2.0 (Huang and Miller, 1991).

Primer

To obtain the AP180 full-length cDNA the forward primer including a *Nco*I-site: 5′CATG*CCATGG*CATGGACGACGAGGACAATGCCGAGCAAGC3′ and the reverse primer containing a *Bam*HI-site: 5′C*GGGATCC*CGTCAACTCAAGTGCTTGGCTATGATCTTTTTCTGG3′ were used. To obtain the full-length At-αC-Ad clone the forward primer with a *Kpn*I-site: 5′GG*GGTACC*CCATGACCGGAATGAGAGGTCT CTCCG3′ and the reverse primer also containing a *Kpn*I-site: 5′GG*GGTACC*CCGAAGTAAGCCAGCAAGCATAGCTCC3′ were used. The At-αC-h-ear construct was obtained by adding the forward primer containing a *Sma*I-site: 5′TCC*CCCGGG*GACTCAAGTGCTTGGCTATGATCTTTTTCTGG3′ and the reverse primer also containing a *Sma*I-site: 5′TCC*CCCGGG*GACTCAAGTGCTTGGCTATGATCTTTTTCTGG3′. For At-αC-Ad-hΔ-ear amplification, the forward primer harboring an *Eco*RI-site: 5′G*GAATTC*CAAAGCTTCTTATGCACGCTCAACCCCC3′ and the reverse primer also harboring an *Eco*RI-site: 5′G*GAATTC*CTCAGCTTTGATTG CCAATG GATATGAGG3′ were used.

Site-directed mutagenesis

Site-directed mutagenesis was performed according to the

manufacturer's instruction using the QuikChange Site-Directed Mutagenesis Kit from Stratagene (Heidelberg, Germany).

Fusion protein constructs

All steps that require kits were performed according to the manufacturers' instructions. Total RNA was isolated from *Arabidopsis thaliana* wild-type var. Columbia leaves using the RNAeasy Plant Mini Kit (Qiagen, Hilden, Germany). The cDNA was obtained from 1 μg total RNA using the AMV reverse transcriptase (Roche, Mannheim, Germany). The cDNA was subsequently used as a template to construct the following clones in PCR reactions. The 1985 bp fragment full-length At-AP180 clone was obtained with its specific primers and cloned into the pGEMT-easy vector (Promega, Mannheim, Germany) for amplification and commercial sequencing purposes (MWG, Eberswalde, Germany). To obtain the histidine fusion protein (H_6-At-AP180), the At-AP180 full-length clone was then inserted into the *Nco*I and *Bam*HI sites of pET30a vector (Novagen, Schwalbach, Germany). Its truncated version, At-AP180ΔENTH, was obtained by releasing the first 1030 bp by digestion of a sequence-internal *Sac*I site and the vector *Not*I site. The 956 bp C-terminal fragment was subcloned into the pET30b vector (Novagen, Schwalbach, Germany) and subsequently cleaved using *Eco*RI and *Not*I. The fragment was ligated into the corresponding sites of the pGEX-4T3 vector (Amersham-Pharmacia Biotech, Freiburg, Germany) to obtain the GST-At-AP180ΔENTH clone.

The full-length At-αC-Ad clone (3039 bp) as well as the At-αC-Ad-h-ear and the At-αC-Ad-hΔear fragments were produced using the respective primers given above. All three PCR-fragments were subsequently cloned into the pGEMT-easy vector. To obtain the GST-At-αC-Ad-h-ear construct digestion with *Sma*I released the 1437 bp fragment, which was then inserted into the *Sma*I-site of pGEX4T-2 vector. To obtain the GST-fusion-protein of the truncated version, At-αC-Ad-hΔear, digestion with *Eco*RI released the 669 bp fragment, which was then inserted into pGEX4T-3 vector.

Expression and purification of fusion-proteins

H_6- and GST-fusion proteins were expressed and purified as described previously (Scheele and Holstein, 2002), with the following modifications. For induction IPTG was added to a final concentration of 1 mM or 0.5 mM for H_6- and GST-fusion proteins, respectively. Induction occured at room temperature for 3 hours.

For the optimal purification of H_6-At-AP180ΔENTH, the cytosolic supernatant obtained after the ultracentrifugation step was purified as described elsewhere (Kalthoff, 2003). The resulting supernatant was then incubated with 5 ml of 50% Ni-NTA beads. Protein solutions were changed into buffer G (see below) and then gelfiltered via a Superdex 200 column connected to a FPLC with a flow rate of 0.5 ml/minute. The fractions were analyzed on a Coomassie-stained gel and the positive fractions were pooled and used after protein concentration was determined (Bradford, 1976).

Pull-down experiments

For binding, both the GST- and the H_6-fusion proteins were changed into binding buffer G (25 mM Hepes, pH 7.1, 125 mM K^+-acetate, 5 mM Mg^{2+}-acetate) using PD-10 columns (Amersham-Pharmacia Biotech, Freiburg, Germany). For each binding experiment 10 μl packed glutathione-sepharose (GSH) beads were prepared according to the manufacturer's instructions (Amersham-Pharmacia Biotech, Freiburg, Germany) and subsequently incubated with 20 μg of the respective GST-fusion protein for 1 hour at 4°C. 7 μg of the H_6-At-AP180ΔENTH construct or 5 μg stripped clathrin from pig CCV were added to the pre-incubated GSH-beads, filled up to 200 μl final volume and incubated for 1 hour at 4°C on a rotator. The beads were washed five times with 300 μl buffer G and the final pellet was resuspended in 20 μl sample buffer (Laemmli, 1970). The samples were boiled at 95°C for 1 minute and subjected to SDS-PAGE. From each supernatant 1/40 volume and from each pellet 1/4 volume were loaded on a gel-slot. Each binding assay was independently performed three times each with three samples.

Reassembly of clathrin triskelia

Clathrin reassembly was performed in a total volume of 250 μl with 40 μg clathrin alone or in the presence of 3.6 μg GST-At-AP180ΔENTH or 2.1 μg H_6-At-AP180ΔENTH when equimolar concentrations (238 nM) were required. Dialysis was overnight against buffer S (0.1 M Mes, pH 6.5, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.02% NaN_3). The cages were obtained in the pellets after centrifugation at 120,000 ***g*** for 20 minutes in a S45A Sorvall rotor and resuspended in 100 μl buffer S. From both, the supernatant and the pellet, 80 μl-aliquots were prepared for SDS-PAGE by adding 4× concentrated sample buffer (Laemmli, 1970).

Electron microscopy

Aliquots of 20 μl from the pellets were used for negative staining that was performed as described elsewhere (Depta and Robinson, 1986). The electron micrographs were taken at 80 kV using a Philips CM10 electron microscope.

Gel electrophoresis, western blotting and protein determination

SDS-gradient gels (10-19%) were prepared as previously described (Holstein et al., 1996). Proteins were blotted onto nitrocellulose (Towbin et al., 1979) and visualized with the Supersignal West Pico ECL kit (Pierce, Rockford, USA). Protein concentrations were determined according to Bradford (Bradford, 1976).

Antibodies

Primary antibodies used in immunoblots were: H_6-antibody in a 1:2000 dilution and a GST-antibody at a 1:1000 dilution (both from Amersham-Pharmacia Biotech, Freiburg, Germany). The monoclonal antibody directed against mammalian CHC (BD, Transduction Laboratories, Franklin Lakes, USA) was used in 1:500 dilution. The antibodies, given with the respective final dilutions, against mammalian amphiphysin (1:10,000), epsin (1:1000), AP180 (1:2000), dynamin (1:1000), and eps15 (1:1000) were a gift from E. Ungewickell (Medical School, Hannover, Germany). Secondary antibodies against mouse, rabbit or goat coupled to horseradish peroxidase, were obtained from Sigma (Taufkirchen, Germany) and used in final concentrations of 1:10,000.

Gelfiltration on Superose 6/FPLC

CCV were isolated from pig brain as described elsewhere (Lindner, 1994). To remove the coat proteins from mammalian CCV, approx. 1 mg was incubated in final 3 mM EDTA and 0.5 M Tris/bufferA (0.1 M Mes, pH 6.4, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA) for 30 minutes at 4°C. Centrifugation for 1 hour at 120,000 ***g*** in a T12.50 rotor then separated the soluble coat proteins from the vesicle membranes. About 0.5 mg/0.5 ml coat proteins were applied on a Superose 6 column connected to a FPLC (Amersham-Pharmacia Biotech, Freiburg, Germany). The flow rate was 0.3 ml/minute and the fraction size was 0.5 ml. Each fraction was analyzed on immunoblots. Calibration of Superose 6 column was performed before using the molecular weight marker kit (MW-GF-1000) from Sigma (Deisenhofen, Germany).

Surface plasmon-resonance (SPR) analysis

Association of H_6-At-AP180ΔENTH with the GST-fusion proteins of

At-αC-Ad was analyzed in real-time by surface plasmon-resonance (SPR) using a BIAcore 3000 biosensor (BIAcore AB, Freiburg, Germany) and is described in detail elsewhere (Jonsson et al., 1999). In brief, an anti-GST monoclonal antibody (BIAcore AB, Freiburg, Germany) was immobilized on all four flow cells of a CM-5 sensor chip. The antibody-coated chip was subsequently used to capture the GST-fusion proteins at a flow rate of 5 μl/minute followed by the analysis of H_6-At-AP180 construct binding. All interaction experiments were performed with buffer G (see above) at a flow rate of 20 μl/minute. Association for 1 minute was followed by dissociation for 1 minute during which the buffer was perfused. A short pulse injection (15 seconds) of 20 mM NaOH/0.5% SDS was used to regenerate the sensor chip surface after each experimental cycle. H_6-AT-AP180ΔENTH was used at a final concentration ranging from 125 to 1000 nM. The association constant k_a, the dissociation konstant k_d and the equilibrium constant $k_D=k_d/k_a$ were calculated using BIAcore kinetic evaluation software, assuming pseudo first order kinetics A+B ↔ AB.

Results

Isolation and functional characterization of *Arabidopsis* αC-adaptin

A database search of the *Arabidopsis* genome has revealed two genes coding for α-(*At5g22770*, NM_122183) and αC-adaptin (*At5g22780*, NM_122184) homologs, which share a 98.5% identity at the amino acid level. Using RT-PCR, we have isolated the full-length αC-adaptin clone (At-αC-Ad) which codes for a gene product of 1013 amino acids with a predicted relative molecular mass (Mr) of 112.29×10^3. The mammalian and plant orthologs not only share the same tripartite organization, but also display a 72.6% overall similarity at the amino acid level (Fig. 1A). Compared with the mouse αC ear region (Owen et al., 1999), modeling of the complete C-terminal domain of At-αC-Ad (residues ^{718}S-^{1013}L) shows the same bilobal structure comprising the N-terminal sandwich domain (^{718}S-^{855}K), and the extreme C-terminal platform subdomain (^{856}F-^{973}L) (Fig. 1B,C).

Sequence analysis of the At-αC-Ad ear region revealed further that the critical residues within its C-terminal platform domain required for the DPF/W-motif (^{867}F-^{937}R) and for the overlapping FXDXF-motif binding (^{867}F-^{949}R), as well as the residues in the N-terminal sandwich domain required for the specific DPW-motif binding (^{749}G-^{813}Q), are almost totally conserved (Fig. 1C,D) (Brett et al., 2002). While the DPF/W and DPW binding sites each contain one homologous exchange, the FXDXF-binding site contains three exchanges, of which two are homologous (Fig. 1C,D). In particular, those residues within the DPF/W-motif, which have been shown to

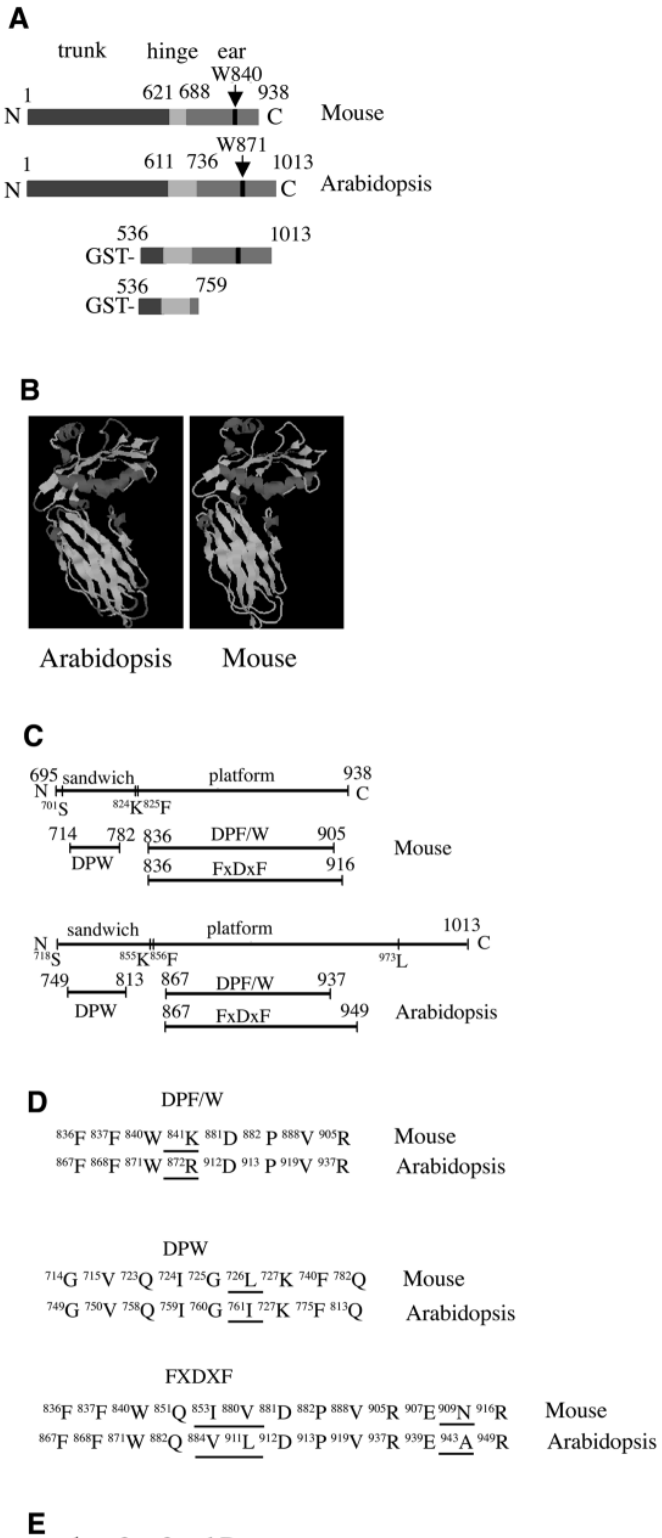

Fig. 1. Features of *Arabidopsis* αC-adaptin constructs. (A) At-αC-Ad compared with its mammalian ortholog. GST-fusion proteins of At-αC-Ad comprising either the full ear region (residues 536-1013; GST-At-αC-h-ear) or its truncated version (residues 536-759; GST-At-αC-h-Δear). (B) Three-dimensional structure modelling of the hinge ear region of At-αC-Ad was visualized using the Rasmol program, version 2.6. (C) Distribution of the specific binding sites within the two subdomains of mouse and *Arabidopsis* αC-adaptin ear regions. (D) Alignment of the three αC-ear binding-sites. Amino acid exchanges are underlined. Note the high degree of identity. (E) Coomassie-stained gels of the purified GST-fusion protein constructs of the At-αC-Ad hinge ear region. (1) GST-At-αC-h-ear, (2) GST-At-αC-h-Δear marked by a star and (3) control GST.

be absolutely crucial for network-protein interactions (mouse ^{837}F, ^{905}R, ^{840}W) (Owen et al., 1999), are totally conserved within the plant αC-adaptin sequence (^{868}F, ^{937}R, ^{871}W).

As the binding sites for the three consensus motifs are located within the two C-terminal subdomains, two GST constructs of At-αC-Ad comprising these regions were created (Fig. 1A). Both constructs lack the trunk portion but comprise the complete hinge region. The one construct containing in addition the full ear region (residues ^{536}K-^{1013}L) is named GST-At-αC-h-ear, whereas the second construct that lacks the extreme C-terminal 255 amino acids and therefore the two subdomains but comprises the residues ^{536}K-^{759}Q is thus named GST-At-αC-hΔear. This latter construct was used to prove that the same subdomains in plant αC-adaptin were also required for network-protein binding (control) and also to reveal putative binding sites that might be located within the hinge domain. The GST-fusion proteins were purified either as a 77 kDa (GST-At-αC-h-ear) or as a 50 kDa (GST-At-αC-hΔear) protein as shown in the Coomassie-stained gel (Fig. 1E). To show the conserved function of the C-terminal ear region of plant αC-adaptin, the two constructs were used in pull-down experiments together with pig brain cytosol, which served as a source of components of the mammalian endocytic clathrin machinery. After incubation, the protein composition of the pellets and corresponding supernatants were analyzed on immunoblots using the respective antibodies. The binding of the mammalian network proteins were observed exclusively in the presence of the full-length ear region from *Arabidopsis* αC-adaptin but neither when its truncated version lacking both subdomains were used nor when GST alone were used (Fig. 2). Although binding of eps15 to the ear region of plant αC-adaptin is complete, AP180 and amphiphysin bind to a lesser degree and dynamin I gave by far the faintest signal. Surprisingly, mammalian epsin did not bind at all. However, these findings indicate that the C-terminal 265 residues comprising the two subdomains of plant αC-adaptin are also required for network-protein binding, showing that plant αC-adaptin, like its mammalian counterpart, can generally function as a binding partner for cytosolic network proteins.

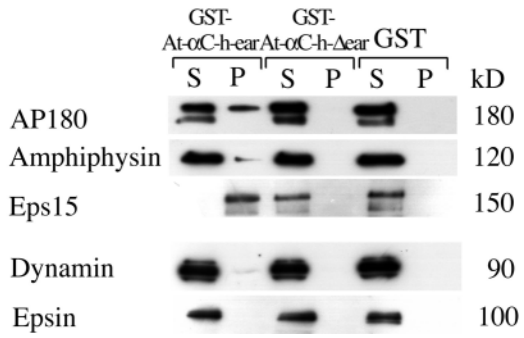

Fig. 2. Binding of mammalian network proteins to plant αC-adaptin ear region. Pull-down experiments using pig brain cytosol. Incubation with the respective antibodies shows that the proteins bind exclusively to the full-length ear construct but neither to the truncated version nor to the GST control. Note that epsin does not bind. P, pellet; S, supernatant.

Characterization of an *Arabidopsis* AP180 homolog

From the three best binding partners of At-αC-ad, we decided to isolate the plant homolog of AP180, as mammalian AP180 also functions as a clathrin assembly protein. These two features, in addition to its lipid binding capacity, qualify it to function as a tether in the early steps of CCV budding. Therefore, AP180 can be regarded as another key protein in the clathrin endocytosis machinery.

A database search in the *Arabidopsis* genome revealed several candidates of ENTH-domain-containing proteins. Subsequent multiple sequence alignment with mammalian AP180 homologs showed further that the similarities were exclusively restricted to the ENTH-domains located within the N-terminal regions of all proteins compared. Unlike the ENTH-domain of epsin 1, which is also considered to be important for coat formation (Kalthoff et al., 2002), the ENTH-domain of AP180 is not able to tubulate liposomes in vitro, and is therefore sometimes referred to as an ANTH-domain, containing a lysine-rich signature motif (K/GA(T/I)(X_6)(P/L/V) KXK(H/Y) (Kay et al., 1999; Ford et al., 2001). Thus, to verify its relationship with animal AP180s and to distinguish it from mammalian epsins we searched for a plant AP180 homolog that completely lacks the ENTH-signature motif of epsins (Ford et al., 2002) but which instead contains the ANTH-signature motif of AP180 homologs.

The ENTH domain of the *Arabidopsis* clone *At1g05020* (NM_100381) contains the first part of the ANTH-motif (^{42}KAT(X_6)P) and probably also its second part, but only at a farther distance (^{67}KKSH). Nevertheless, it reveals the highest similiarities to the ANTH domains of the AP180 homologs CALM (71%), AP180 (63%), LAP (61%) and UNC-11 (58%), but only a lower degree of similarity to mammalian epsins of around 55% (data not shown). Like other non-neuronal AP180 homologs, the plant clone investigated contains only a low number of consensus interaction sites required for the interaction with clathrin (^{442}DLL) and αC-adaptin (^{473}DPW, ^{608}DPF), as well as with other interacting network proteins (^{547}NPF) (Fig. 3A). Therefore, it was considered a true AP180 homolog and was named At-AP180, because the neuronal AP180 protein is the eponymous homolog of this group.

The At-AP180 full-length clone was also isolated using the RT-PCR approach as for αC-adaptin. At-AP180 consists of 653 amino acids with a predicted Mr of 72×10^3 and is thus of the same size as CALM (Kim and Kim, 2000) (Fig. 3A). We took advantage of the fact that all four consensus binding motifs are located within the C-terminal residues ^{336}S-^{653}S in order to create a construct lacking the hydrophobic ENTH-domain (At-AP180ΔENTH) to improve the protein yield for interaction studies. This construct was expressed either as a histidine- or as a GST-fusion protein (H_6/GST-At-AP180ΔENTH) (Fig. 3A,B). As expected, the expression levels of the truncated H_6-At-AP180 construct exceeded the yield of the purified full-length histidine-fusion protein by at least tenfold (Fig. 3B). It was possible to improve the purification of H_6-At-AP180ΔENTH even further by simply boiling the solution as described for mammalian AP180 (Kalthoff et al., 2002; Kalthoff, 2003). The successful application of this purification step already indicated that the motifs are also most probably harbored within a disordered region of At-AP180 that displays little or no conventional secondary structure. Furthermore, the full-length H_6-At-

AP180, as well as its truncated version H_6-At-AP180ΔENTH, have higher apparent molecular masses in SDS gels (100 kDa and 55 kDa, respectively) than the primary sequence predicts (72 kDa and 34.5 kDa, respectively).

At-AP180 is a binding-partner of αC-adaptin

To gain an insight into the existence of a clathrin machinery in plants at the molecular level, the interaction of the two key protein homologs, At-AP180 and At-αC-Ad, was investigated. Therefore, H_6-At-AP180ΔENTH was incubated with the two GST constructs of the plant αC-adaptin ear region (Fig. 4A). Binding was exclusively observed in the presence of the complete ear region but not with the truncated version of At-αC-Ad nor when GST alone was added, showing that the binding site for plant AP180 is located within the extreme C-terminal ear region (^{760}G-^{1013}L) of plant αC-adaptin.

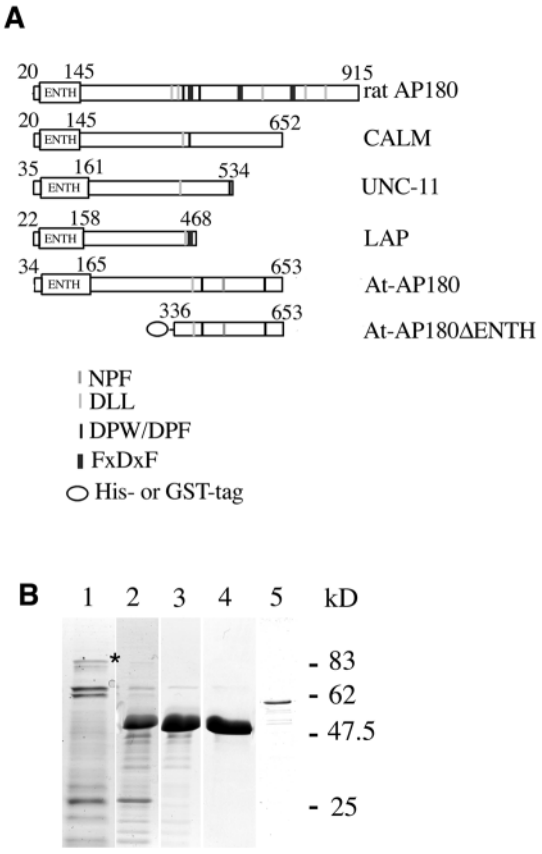

Fig. 3. Features of AP180 homologs and At-AP180 constructs. (A) Full-length At-AP180 comprises an N-terminal ENTH-domain and two putative binding sites for the α-adaptin ear region (^{473}DPW, ^{608}DPF), as well as one clathrin-binding consensus motif (^{442}DLL). The histidine or GST-fusion protein constructs of At-AP180, lacking the ENTH domain (H_6/GST-At-AP180ΔENTH), were used in pull-down and in clathrin assembly experiments. (B) Commassie-stained gel of the purification of the histidine-tagged recombinant full-length At-AP180 protein (predicted Mr is 72×10^3; lane 1, marked by a star) and also consecutive stepwise purification of the H_6-At-AP180ΔENTH fusion-protein (predicted Mr is 34.5×10^3) after incubation with Ni-NTA-beads (2) then boiled (3) and then after gelfiltration on Superose 6/FPLC (4). The purified GST-AP180ΔENTH fusion-protein has a molecular mass of 55 kDa (5).

As At-AP180 contains two α-ear consensus binding motifs, we investigated whether both motifs (^{473}DPW and ^{608}DPF) contributed equally to the binding. Therefore, we repeated the binding experiment with alanine exchange mutants of either motif (Fig. 4B). While alanine mutation of the DPF-motif reduced binding to the At-αC-Ad ear region significantly, the DPW/AAA mutation seemed not to affect the binding at all, displaying almost the same binding affinity as the wild-type At-AP180ΔENTH construct (Fig. 4A,B). Thus, we conclude that the ^{608}DPF-motif of At-AP180 is obviously crucial for binding to the ear region of plant αC-adaptin, whereas the ^{473}DPW-motif is not.

Plasmon-resonance measurements were performed next to explore the degree of binding affinity between plant αC-adaptin and At-AP180. In these experiments, the GST-fusion proteins comprising either the complete ear region or the truncated version of At-αC-Ad were coupled to CM-5 chips via GST antibodies, while the H_6-At-AP180ΔENTH construct was passed over the sensor surface to monitor the binding. With an equilibrium rate constant of 35 nM, which is lower than the controls GST (680 nM) and GST-αC-h-Δ-ear (682 nM) by a factor of 19, the binding affinity between At-AP180ΔENTH and the At-αC-Ad ear construct is rather high (Fig. 5A). Lowering the amounts of the At-AP180 construct by a factor of four and eight, respectively, reduced binding to the full ear construct, indicating that it is concentration dependent (Fig. 5B). Taken together, the C-terminal region of At-AP180 binds via its ^{608}DPF-motif to the C-terminal ear region of plant αC-adaptin.

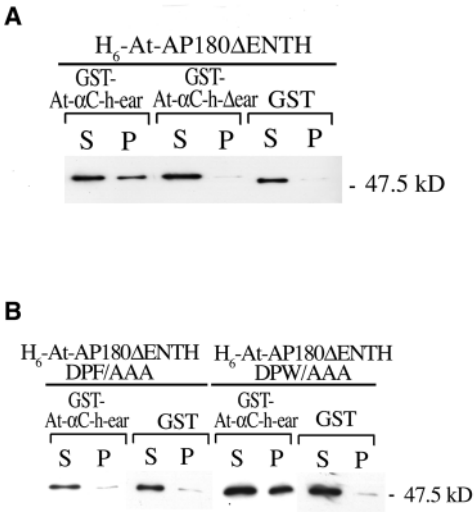

Fig. 4. Plant AP180ΔENTH binding to αC-adaptin ear constructs. (A) Pull-down experiments with the full-length ear region or the truncated version of At-αC-Ad and GST as a control together with the recombinant H_6-AtAP180ΔENTH construct show that binding requires the ear region. (B) Analysis of the consensus binding motifs of At-AP180 for binding to the αC-ear. Binding experiments with alanine substitutions mutans of either the DPF motif (At-AP180ΔENTH-DPF/AAA) or the DPW-motif (At-AP180ΔENTH-DPW/AAA) with the full ear construct of At-αC-adaptin reveal that the DPF-motif is crucial for binding. GST serves as a control. The antibody against histidine recognizes the AP180-constructs in immunoblots of (A) and (B). P, pellet; S, supernatant.

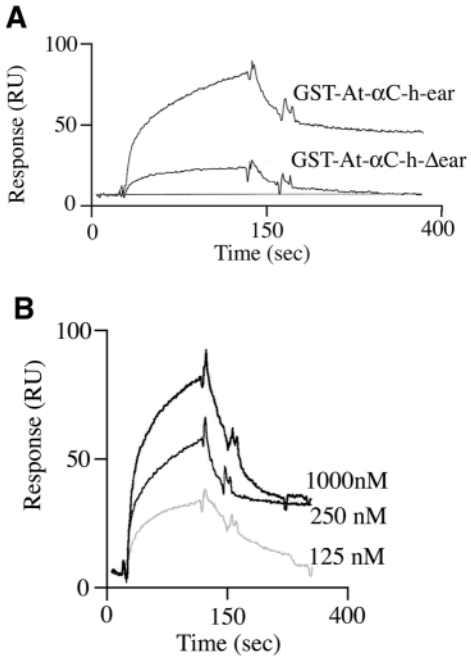

Fig. 5. Binding of At-αC-Ad and At-AP180 measured by plasmon resonance (BIAcore 3000). The full-length ear construct and the truncated version of At-αC-Ad were immobilized on CM-5 chips and H_6-At-AP180ΔENTH was passed over the sensor surface for 1 minute (association) followed by a 1 minute wash with running buffer (dissociation). (A) The full ear construct binds H_6-At-AP180ΔENTH with high affinity (k_D=35 nM) compared with the truncated version At-αC-hΔear (k_D=682 nM). (B) The binding between GST-At-αC-h-ear and H_6-At-AP180ΔENTH is concentration dependent as shown by increasing amounts of the At-AP180 construct.

At-AP180ΔENTH functions in clathrin assembly

We next investigated whether At-AP180 also functions as a clathrin assembly protein. To this end, we tested first whether its C-terminal region comprising the single conserved ^{442}DLL-clathrin assembly motif (Morgan et al., 2000) is able to bind to clathrin. Thus, the fusion-protein GST-At-AP180ΔENTH was used in a pull-down experiment with cytosolic clathrin isolated from suspension cultured *Arabidopsis* cells (Fig. 6). A complete shifting of CHC to the pellet fraction after centrifugation was observed exclusively in the presence of GST-At-AP180ΔENTH, but not in the control, attesting a specific interaction. To explore the role of the DLL-motif in regard to clathrin binding we mutated the motif into alanine residues and repeated the binding experiment. Binding of the DLL/AAA mutant occured to almost the same extent as was observed for the wild-type construct, indicating that At-AP180 clearly contains, in addition, clathrin binding motifs other than the consensus DLL-motif so far described. Nevertheless, these findings show unequivocally that the C-terminal region of At-AP180 functions in clathrin binding.

An important function of mammalian AP180 and its non-neuronal counterpart CALM is their ability to assemble clathrin triskelia into cages (McMahon, 1999). As with other mammalian clathrin assembly proteins, AP180 is also thought to promote clathrin assembly by cross-linking triskelia via multivalent interactions between its repeated DLL-motifs and

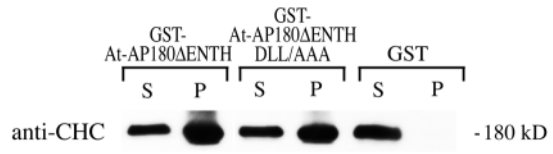

Fig. 6. At-AP180 is a clathrin binding protein. Pull-down assay with clathrin from *Arabidopsis* cytosol. The clathrin heavy-chain polypeptide (CHC, 180 kDa) is present in the pellet (P) after incubation with GST-At-AP180ΔENTH. Mutation of the clathrin binding-motif DLL for alanine (DLL/AAA) did not abolish binding; GST served as the control. Immunoblot with the anti-CHC specific antibody.

complementary binding sites in the clathrin terminal domain. As the single conserved ^{442}DLL clathrin binding motif of At-AP180 is not essential for clathrin binding we were eager to determine whether At-AP180ΔENTH might also function as an assembly protein like its mammalian ortholog. To test this, we isolated coat proteins from pig brain CCV which were subjected to Superose 6 gelfiltration to separate the triskelia completely from endogenous AP180. We made sure that the triskelia were completely devoid of endogenous AP180 by screening the gelfiltration fractions with the respective antibody (Fig. 7A). Those fractions lacking endogenous AP180 were subsequently used in all reassembly experiments. To show their assembly activity, two plant AP180 constructs were used: H_6-At-

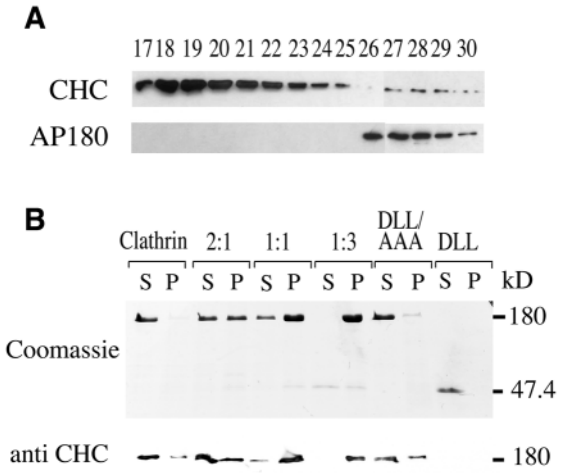

Fig. 7. At-AP180 functions as a clathrin assembly-protein. (A) Gefiltration (Superose 6/ FPLC) of coat proteins removed from pig brain CCV. The respective antibodies were used to show the separation of endogeneous AP180 in fractions 26-30 from clathrin triskelia in fractions 17-25. (B) Reassembly experiments. Successful assembly of clathrin triskelia occurs only in the presence of plant H_6-At-AP180ΔENTH. Assembly is abolished when DLL is exchanged into alanine (GST-At-AP180ΔENTH-DLL/AAA in a 1:1 stoichiometry). For reassembly, clathrin was incubated either alone or in the presence of half-molar (2:1) and equimolar (1:1) amounts of H_6-At-AP180ΔENTH, and also in its threefold excess (1:3). H_6-At-AP180ΔENTH (DLL) in the same amount as in (1:3) was also incubated alone to show that no unspecific aggregates were present in the pellets (P). Distribution of CHC and the fusion-protein constructs of At-AP180 in (1:1), (1:3), DLL/AAA and DLL are shown on a Coomassie-stained gel and on an immunoblot with the CHC antibody. S, supernatant.

AP180ΔENTH, which was added in various stoichiometric amounts, and its DLL/AAA-mutated version (GST-At-AP180ΔENTH-DLL/AAA), which still contains clathrin binding activity. Clathrin alone served as a control for spontaneous self-assembly under these conditions. The distribution of clathrin between supernatant, containing soluble triskelia, and pellet, containing assembled cages, were observed in SDS-gels and immunoblots (Fig. 7B). The clathrin triskelia alone displayed only a low tendency of self-aggregation (Fig. 7B), but the addition of H_6-At-AP180ΔENTH converted the triskelia into a fast sedimenting species. Half-molar amounts of H_6-At-AP180ΔENTH were already sufficient to induce some assembly, which could be further increased by adding equimolar amounts of H_6-At-AP180ΔENTH. With a threefold molar excess of the H_6-At-AP180ΔENTH construct all of the clathrin ended up in the pellet. The same amount of H_6-At-AP180ΔENTH when incubated alone remained in the supernatant indicating that its presence in the pellet is exclusively due to its specific CHC binding (Fig. 7B). The DLL/AAA mutant of At-AP180ΔENTH no longer contained assembly activity, which indicates that the single consensus DLL-motif of At-AP180 plays a crucial role in the assembly process. Furthermore, thorough investigation of the reassembly experiments by electron microscopy revealed that clathrin triskelia, which were assembled as a result of spontaneous self-aggregation of clathrin (Fig. 8A) or in the presence of the DLL/AAA mutant of At-AP180ΔENTH (Fig. 8C), were of negligible number compared with the high number of uniform-sized cages that assembled only in the presence of H_6-At-AP180ΔENTH (Fig. 8B). Moreover, those cages assembled without the aid of the DLL-motif seem to be more irregular in shape and somewhat larger compared with those assembled in the presence of the DLL-motif (close-ups in Fig. 8A,B,C). Analysis of the distribution of the cage size assembled with the help of the DLL-motif revealed further that from a total of 131 counted cages, 122 (93%) were between 70 nm and 90 nm in diameter, thus displaying an almost uniform size distribution (Fig. 8D). From these data we conclude that the single conserved clathrin assembly motif of At-AP180 plays a crucial role during clathrin assembly, although its mutation has no effect on clathrin binding. Therefore, plant AP180 obviously contains clathrin binding motifs that do not correspond to the consensus motifs decribed so far from mammalian clathrin binding proteins.

Discussion

Clathrin-mediated endocytosis has not been described at the molecular level in plants and neither have members of the plant clathrin-machinery been functionally studied. Therefore, we have isolated and characterized plant orthologs of two key proteins of the mammalian clathrin-mediated endocytosis machinery – namely, of α-adaptin, the specific subunit of the AP2 complex, which is exclusively associated with endocytic CCV, and AP180, which displays the strongest clathrin assembly activity but also regulates the size of cages.

At-αC-Ad is a binding partner for cytosolic network proteins

We have isolated the cDNA clone of *Arabidopsis* αC-adaptin, one of the two genes coding for plant α-adaptins that are almost identical at the amino acid level. This situation corresponds to that of mammals, which also have two α-adaptin genes sharing an overall identity of 84% – one coding for the ubiquitiously expressed αC-adaptin and the other one for the brain-specific variant αA-adaptin (Robinson, 1989; Ball et al., 1995).

Owen and co-workers (Owen et al., 1999) previously investigated the binding sites of the mouse α-ear subdomains using the αCL-construct (see Fig. 1C). We prepared an analogous construct of the *Arabidopsis* αC-adaptin ear region, GST-At-αC-h-ear, and its truncated version, GST-At-αC-hΔear, which lacks both ear subdomains. Using the pull-down approach to study protein-protein interactions, we have shown that GST-At-αC-h-ear was also capable of interacting with the same mammalian network proteins from brain cytosol as described for the mouse αCL-construct (Owen et al., 1999), but with only one exception:

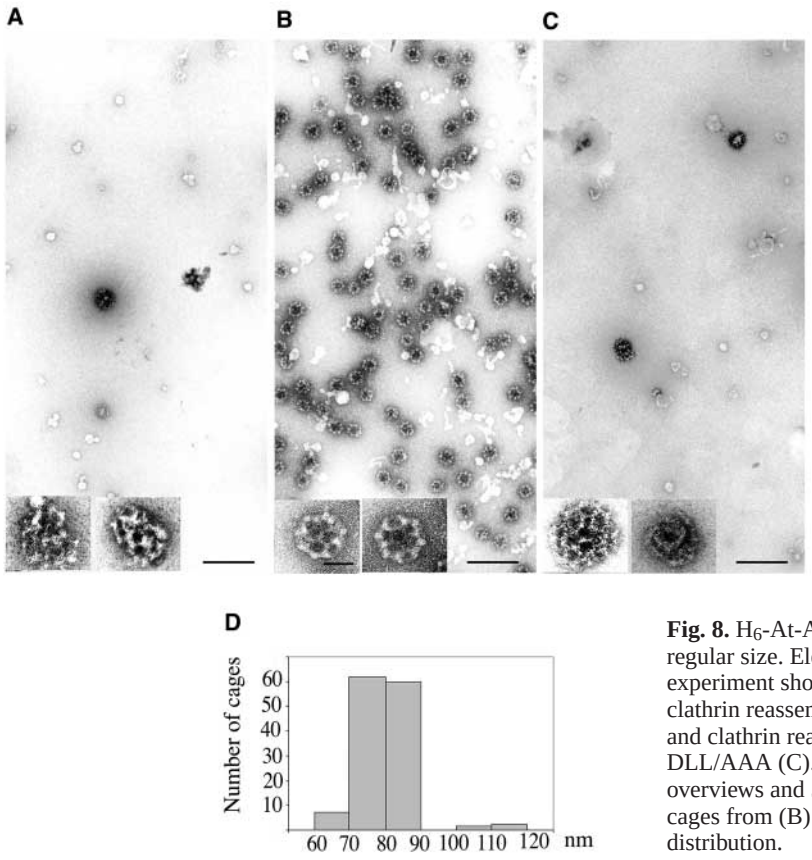

Fig. 8. H_6-At-AP180ΔENTH assembles clathrin triskelia into cages of regular size. Electron micrographs (negative staining) of the reassembly experiment shown in Fig. 7. Assembly activity of clathrin alone (A), clathrin reassembled in a threefold excess of H_6-At-AP180ΔENTH (B), and clathrin reassembled in a threefold excess of GST-At-AP180ΔENTH-DLL/AAA (C). Close-ups of the cages are inserted. Bar, 250 nm in the overviews and 50 nm in the close-ups. (D) Size distribution of assembled cages from (B) is quantified in bar charts. Note the narrow size distribution.

epsin. Interestingly, epsin contains only DPW-motifs for its interaction with α-adaptin, whereas all four mammalian binding partners of At-αC-Ad lack the DPW-motif. They contain instead two DPF- and three FXDXF-motifs in the case of AP180, one of each motif in the case of amphiphysin, 15 DPF-motifs in the case of eps15, and only a single DPF-motif in the case of dynamin I.

In our pull-down experiments the number of DPF-binding motifs per protein reflects its binding intensity, as shown for the mammalian αCL-construct (Owen et al., 1999). As the DPW-motifs of epsin have been shown to interact with both the DPF/W and the specific DPW-sites in the mouse αCL-construct (Owen et al., 1999), our result was somehow surprising, because the specific bindig site for the DPW-motif within the At-αC-Ad sequence reveals the same high degree of conservation as does the binding site for the DPF/W-motif. Therefore, it is reasonable to assume that the binding to the plant αC-ear at the DPW-specific site alone is of too low affinity and is thus not detectable under these conditions. However, binding to the DPF/W-site might probably be competed by the DPF-motif interaction of other proteins (e.g. eps15) as we used cytosol as a source for binding partners and not single purified proteins. However, it is clear that At-αC-Ad is functionally conserved and is able to serve as a binding partner for network proteins of the clathrin machinery containing an active DPW/F-binding site.

At-αC-Ad binds to plant AP180

As in mammals, recombinant At-AP180ΔENTH (see below) also binds directly to the plant α-ear region as shown in pull-down experiments and plasmon-resonance measurements. A relative high binding affinity with an equilibrium constant of 35 nM between At-αC-Ad and At-AP180ΔENTH was detected. Thus, plant AP180 obviously binds with a reasonable high affinity to plant αC-adaptin. In addition, the plasmon-resonance measurements confirmed the data already obtained by the pull-down experiments – namely, that the hinge region of plant αC-adaptin lacks extra binding sites as the k_D value for the binding to GST-αC-hΔear was the same as for the GST control.

Plant AP180 contains neither a FXDXF-motif nor a WDW-motif, the latter only recently having been described to interact with α-ear and also with the terminal domain of CHC (Scheele et al., 2003), but instead contains one DPW- and one DPF-motif. Therefore, the next question we addressed was whether the DPF-motif, which is the minimum of α-ear binding-motif resemblance between plant and mammalian AP180 proteins, is crucial for binding. While alanine mutations of the DPW-motif had almost no impact on binding, the mutation of DPF by alanine reduced the binding significantly. Thus, the DPF-motif is clearly required for the interaction with plant αC-adaptin, whereas the DPW-motif is not. This was also shown by the failure of mammalian epsin to bind to the At-αC-Ad ear-region. Taken together, the plant αC-adaptin ear region is not only able to bind to several mammalian network proteins, all of which contain DPF-motifs, but it also binds directly to At-AP180 with a rather high affinity. The failure of the DPF/AAA mutant of the At-AP180ΔENTH construct to bind to At-αC-ear underlined the importance of the DPF-motif for this interaction.

Features of At-AP180

Several putative *Arabidopsis* homologs of AP180 were previously taken into account in ENTH sequence comparison studies of mammalian proteins, but these plant homologs have not been functionally characterized (Ford et al., 2001). The plant sequences either lacked the consensus motif for α-adaptin (Gupta and Gray, 1999) or for clathrin binding (Mao et al., 2001), and therefore obviously do not meet the criteria to fulfill the functions of an AP180 homolog.

We have isolated an *Arabidopsis* AP180 clone that contains within its ENTH domain part of the ANTH-domain signature motif, which is present in all AP180 homologs. In addition, the plant clone harbors clathrin and α-ear consensus binding motifs, which are functionally active in our protein interaction studies. Furthermore, the C-terminal region of At-AP180 also seems to lack a conventional secondary structure like the mammalian AP180 (Kalthoff et al., 2002), since the H_6-At-AP180ΔENTH construct is functionally intact after boiling, a feature that has simplified its purification process (Kalthoff, 2003). Although the plant AP180 does not contain an acidic stretch in its middle portion, At-AP180 resembles the neuronal AP180 in that its isoelectric point is also very acidic (4.75 vs 5.1) (Morris et al., 1993). Thereby, the acidic part is restricted to its C-terminal half (residues 337-658 with a pI of 3.9), which is in total agreement with the high solubility of the At-AP180ΔENTH construct and its abnormal behaviour in SDS-gels. At-AP180 further resembles its non-neuronal AP180 homologs, in that it contains an ^{547}NPF-motif, which is required for interactions with EH-domain-containing network proteins. An NPF-motif is not present in the neuronal AP180 protein.

Further sequence analysis has revealed that from the four putative casein kinase 2 substrate sites within At-AP180, one is adjacent to the DLL-motif (^{442}DLL*SLD*), whereas another is close to the DPF-motif (^{608}DPF*TTFE*) and a third overlaps with the FS-motif (*T*^{622}*FSE*), which has recently been described as a clathrin interacting motif of auxilin (Scheele et al., 2003). Thus, it is possible that At-AP180 interactions are regulated via phosphorylation of these motifs, given that a casein kinase 2-like activity is associated with plant CCV (Drucker et al., 1996).

At-AP180 is a clathrin assembly protein

An important feature of AP180 (Lindner and Ungewickell, 1992; Ye and Lafer, 1995a; Ye and Lafer, 1995b), CALM (Kim and Kim, 2000; Kim et al., 2000), LAP (Zhang et al., 1998) and epsin 1 (Kalthoff et al., 2002) is the ability to assemble clathrin triskelia into cages. Hence, a homogenous population of clathrin cages is only formed in the presence of mammalian AP180 (Ahle and Ungewickell, 1986; Morris et al., 1993; Ye and Lafer, 1995a). Like their mammalian homologs, LAP and UNC-11 are also able to maintain the size of synaptic vesicles (Zhang et al., 1998; Nonet et al., 1999). The clathrin binding motif, which in the case of rat AP180 and its homologs is the DLL-motif, is also found in various other clathrin binding proteins, e.g. HIP1 (Legendre-Guillemin et al., 2002) and auxilin (Scheele et al., 2003).

An investigation of the C-terminal 317 residues of the At-AP180 sequence revealed, in addition to the DLL-motif, two further clathrin interacting motifs (^{622}FS, ^{608}DPF), which have

only recently been discovered (Scheele et al., 2003) and were therefore not considered in our investigations. Thus, At-AP180, like LAP and CALM, obviously lacks the high number of DLL motifs that are present in the neuronal AP180 homolog (Morgan et al., 2000).

As in yeast (Wendland and Emr, 1998) and mammals (Ye and Lafer, 1995a), the clathrin assembly activity of the plant AP180 also resides within its C-terminal region (At-AP180ΔENTH). So, mutating the single DLL-motif into alanine residues seems to have had no effect on the binding activity of At-AP180ΔENTH towards clathrin but abolished its assembly function almost completely.

How do our results fit with the current understanding of clathrin assembly mediated by the five DLL-motifs and the seven variations of this motif in rat AP180? These numerous motifs are thought to cross-link the triskelia in order to assemble them, as progressive deletion caused the loss of clathrin assembly activity (Morgan et al., 2000). This assumption is supported by the studies on auxilin by Scheele and co-workers (Scheele et al., 2003), who have claimed that a single DLL-motif is not sufficient for assembly functions. On the basis of our findings it is tempting to speculate that the DLL-motif is highly conserved between species and functions as an assembly regulating motif, whereas other motifs only contribute to clathrin binding. In animal AP180s the clathrin binding motifs are also DLL-motifs, whereas in plant AP180, with the exception of the FS- and DPF-motif, they obviously do not match any of the described clathrin binding consensus motifs such as consensus clathrin box motifs I or II (Lafer, 2002) or clathrin TD binding-motifs such as WDW or NWQ (Scheele et al., 2003). It would be difficult to imagine how the plant homolog fulfills its functions if motifs other than the so far described clathrin consensus binding motifs do not facilitate the clathrin binding. In this regard, the FS- and the DPF-motif of At-AP180 are promising candidates. Our systematical screening of the At-AP180 C-terminal residues might eventually reveal additional CHC-binding motifs in the near future.

However, the significance of the DLL-motif was also shown in our electron micrographs, which show cages of regular size only in the presence of the single DLL-motif, whereas in its absence the cages seem to be of a more irregular shape. Because larger quantities of clathrin were required for our experiments, we used the more readily available mammalian CHC for the clathrin assembly studies. CHC are highly conserved proteins and also contain functionally conserved domains, as shown previously by their interaction with the plant CLC (Scheele and Holstein, 2002). The use of mammalian CHC was also favored by the consideration to show unequivocally the assembly effect of the recombinant plant At-AP180. CHC completely devoid of endogeneous AP180 were therefore a prerequisite. This condition was fulfilled only when mammalian clathrin was used, as a monoclonal antibody specific for mammalian AP180 allowed us to monitor the successful separation. Unlike rat AP180, which functions in a 1:1 stoichiometry, At-AP180ΔENTH drove triskelia completely into cages only when present in excess. This might be due to the lack of the ENTH-domain in the plant construct, which in mammalian AP180 and CALM contains an additional clathrin binding site of a type I clathrin box motif (Ye and Lafer, 1995a; Tebar et al., 1999). Whether the N-terminus of At-AP180 might also bind to triskelia and probably support assembly, as does the N-terminus of CALM (Kim and Kim, 2000), has not been tested. However, At-AP180ΔENTH was also capable of maintaining a narrow size distribution of cages that matches perfectly with those described for the mammalian AP180 activity (Ahle and Ungewickell, 1986; Morris et al., 1993; Ye and Lafer, 1995a). The ability of plant AP180 to create a cage population within a narrow size range fits perfectly the requirements of plant endocytosis in intact plant cells, as thermodynamic considerations have revealed that the maximum size of endocytic vesicles may not exceed 100 nm (Gradmann and Robinson, 1989). We cannot claim that plant AP180 is alone responsible for regulating cage size as other plant assembly proteins have so far not been investigated. In our studies, clathrin alone displayed a very low tendency of aggregation, which corresponds to the situation when the DLL/AAA mutation of At-AP180ΔENTH was used, suggesting once again an assembly regulating function for the DLL-motif during cage assembly.

Taken together, our data point to the existence of an endocytosis clathrin machinery in plants. Because there are only two highly related α-adaptin genes in *Arabidopsis*, and the plant αC-adaptin homolog displays the same basic function as its mammalian counterpart, which, in turn, is the specific subunit of the endocytosis-specific AP2 complex, one can proceed from the assumption that the plant αC-adaptin might also be involved in plant CME. The αC-adaptin binding and clathrin assembly activities of the plant AP180 homolog only supports this notion. The specific interactions of both proteins put them without doubt in the context of clathrin-dependent endocytosis in plants.

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