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Arabidopsis μA-adaptin interacts with the tyrosine motif of the vacuolar sorting receptor VSR-PS1

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Summary

In receptor-mediated transport pathways in mammalian cells, clathrin-coated vesicle (CCV) μ-adaptins are the main binding partners for the tyrosine sorting/internalization motif (YXXØ). We have analyzed the function of the μA-adaptin, one of the five μ-adaptins from *Arabidopsis thaliana,* by pull-down assays and plasmon resonance measurements using its receptor-binding domain (RBD) fused to a histidine tag. We show that this adaptin is able to bind the consensus tyrosine motif YXXØ from the pea vacuolar sorting receptor (VSR)-PS1, as well as from the mammalian *trans*-Golgi network (TGN)38 protein. Moreover, the tyrosine residue was revealed to be crucial for binding of the complete cytoplasmic tail of VSR-PS1 to the plant μA-adaptin. The *trans*-Golgi localization of the μA-adaptin strongly suggests its involvement in Golgi-to vacuole-trafficking events.

Keywords: μ-adaptin, vacuolar transport, tyrosine-based sorting motif.

Introduction

Vesicle formation and vesicle targeting/fusion are processes essential for intracellular protein transport (Kirchhausen, 2000a). Proteins carried by vesicles may be transported either as bulk cargo or selectively through the agency of a transmembrane receptor. Clathrin-coated vesicles (CCVs) are present in all eukaryotic cells and are the vehicles of receptor-mediated post-Golgi and endocytic protein transport (Marsh and McMahon, 1999; Robinson and Bonifacino, 2001). In both cases, the extracytoplasmic (lumenal) domains of the receptors possess motifs, which are responsible for binding the transport cargo molecules, while the cytoplasmic tails of the receptors interact with adaptor (AP) complexes. Recruitment of the latter from the cytosol is followed in a highly coordinated fashion by the assembly of clathrin triskelions, providing the vesicle with a coat and culminating in vesicle budding (Hirst and Robinson, 1998; Kirchhausen, 2000a; Owen *et al.*, 1999).

Early *in vitro* binding studies on extracts from mammalian cells pointed to the AP-2 complex, a component of the CCV coat, as being the likely receptor-binding partner (Pearse, 1988; Sosa *et al.*, 1993). Yeast two-hybrid studies subsequently identified the μ1- and μ2-adaptins as interacting with a tyrosine motif YXXØ in the receptor cytoplasmic tails (Ohno *et al.*, 1995), an observation later confirmed directly by plasmon resonance (Boll *et al.*, 1996). The YXXØ motif, where Ø is a bulky hydrophobic residue and X is any amino acid, has now been well characterized (Marks *et al.*, 1997), and is present in the cytoplasmic tail of many integral proteins of the plasma membrane (PM) of mammalian cells, as well as the *trans*-Golgi network (TGN), e.g. TGN38 (Stephens and Banting, 1998; Stephens *et al.*, 1997).

In total, four AP complexes have been isolated from mammals and are termed AP-1, AP-3 (both located at the TGN; Dell'Angelica *et al.*, 1997; LeBorgne *et al.*, 1996), AP-2 (located at the PM; Pearse and Robinson, 1990), and AP-4 (located at a perinuclear compartment; Hirst *et al.*, 1999). Of these, only AP-1 and AP-2 associate with clathrin triskelions (Ahle and Ungewickell, 1989; Schroeder and Ungewickell, 1991). Each of the AP complexes consists of two large subunits of around 100 kDa, one medium subunit or μ-adaptin (approximately 50 kDa) and one small subunit or σ-adaptin (approximately 20 kDa; Kirchhausen, 1999, 2000b). While one of the large subunits is always a β-adaptin (β1–β4), the other one is specific for the

respective AP complex. This is α-adaptin in AP-2, γ-adaptin in AP-1, δ-adaptin in AP-3, and ε-adaptin in AP-4. The μ- and σ-adaptins have been named μ1–μ4 and σ1–σ4, respectively (overview by Boehm and Bonifacino, 2001).

There can be no doubt that the β1- and β2-adaptins function as clathrin heavy chain (CHC)-binding partners (Owen *et al.*, 2000; Shih *et al.*, 1995). On the other hand, the μ1–μ3-adaptins have been unequivocally established as being the main binding partners for the cytoplasmic tails of various transmembrane receptors (Heilker *et al.*, 1999). Nevertheless, the large subunits, α-, β1- and β2-adaptins have also been reported to bind to epidermal growth factor (EGF)- and asiaglycoprotein (ASGP)-receptors (Beltzer and Spiess, 1991; Rapoport *et al.*, 1998; Sorkin and Carpenter, 1993). All μ-adaptins have in common a bipartite structure with their aminoterminal one-third responsible for the interaction with β-adaptins, whereas the carboxyterminal two-thirds represent the receptor-binding domain (RBD; Aguilar *et al.*, 1997). Recently, the crystal structure of the RBD of μ2-adaptin from mouse complexed with the internalization/sorting signal peptide of TGN38 has been resolved at 2.7 Å (Owen and Evans, 1998). The amino acids crucial for binding to the tyrosine sorting motif YXXØ are located within the β-sheet strands β1, β2, β15, and β16. These authors demonstrated that two hydrophobic pockets are required for binding to the YXXØ motif, one for the binding of the tyrosine residue and the other one for the binding of the hydrophobic (Ø) residue.

Little is known about the coat proteins of CCV from plants, and even less about their interactions with putative sorting receptors (Beevers, 1996; Holstein, 2002; Robinson *et al.*, 1998a). Some coat subunits of plant CCV have been identified: CHC from soybean (Blackbourn and Jackson, 1996), CLC from *Arabidopsis* (Scheele and Holstein, 2002) one γ-adaptin (Schledzewski *et al.*, 1997), three β-adaptins (Holstein and Happel, 2000; Holstein *et al.*, 1994) and one μ-adaptin from *Arabidopsis* (Happel *et al.*, 1997), a σ1-adaptin from a chinese medical tree (Maldonado-Mendoza and Nessler, 1996), and a σ2-adaptin from maize (Roca *et al.*, 1998). However, the types and composition of putative plant AP complexes remain to be defined.

So far only one type of sorting receptor for vacuolar proteins has been identified in plants (Hadlington and Denecke, 2000; Paris and Neuhaus, 2002), and this has been alternatively named binding protein (BP)-80 or vacuolar sorting receptor (VSR)-PS1 in pea (Kirsch *et al.*, 1994; Paris *et al.*, 1997), PV72/82 in pumpkin (Shimada *et al.*, 1997), or *Arabidopsis thaliana* epidermal growth factor-like protein (AtELP) in *Arabidopsis* (Ahmed *et al.*, 1997). It is now known that these proteins are members of a family of VSRs with additional representatives having been found in *Arabidopsis* (Laval *et al.*, 1999), rice, wheat, and tobacco sequence databases (Miller *et al.*, 1999). Sequence-specific *in vitro* binding to cognate ligand peptides has been demonstrated for the VSRs of pea, pumpkin, and *Arabidopsis* (Ahmed *et al.*, 2000; Kirsch *et al.*, 1994; Shimada *et al.*, 1997). Moreover, the nature of the binding between the lumenal domain of VSR-PS1 and its cargo ligands has been elucidated (Cao *et al.*, 2000), and most recently, the vacuolar protein sorting function of VSR-PS1 verified heterologously in yeast (Humair *et al.*, 2001).

All members of the VSR family contain the putative tyrosine sorting motif YXXØ, the amino acids (Y)Tyrosine, (M)Methionine, (P)Proline, (L)Leucine (YMPL) within their cytoplasmic tails (Paris *et al.*, 1997; Shimada *et al.*, 1997). Although VSRs have no homology to the mammalian mannose-6-phosphate receptors (MPRs), a number of the proteins, which participate in vacuolar protein sorting, are nevertheless homologous to those involved in lysosomal protein transport, e.g. the target soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (t-SNAREs) AtVAMP3p and AtPEP12p (Bassham and Raikhel, 1999; Sanderfoot *et al.*, 1999), as well as a dynamin-l-like protein (Jin *et al.*, 2001). This suggests that a similar pathway to the lytic vacuole exists for hydrolytic enzymes in plants (Jiang and Rogers, 2003; Robinson *et al.*, 2000). However, unequivocal proof for an interaction between plant CCV coat components and a VSR has yet to be established.

In this paper, we now provide conclusive evidence that a plant μ-adaptin is the binding partner for the YXXØ sorting motif in the cytoplasmic tail of a plant VSR. Furthermore, we show by gel filtration analysis subcellular fractionation and immunogold staining of cryosections that the *Arabidopsis* μA-adaptin is part of a putative plant AP complex, and that it is also localized to the *trans*-Golgi.

Results

Sequence comparisons of Arabidopsis *μA-adaptin*

We have previously described the cDNA sequence of *Arabidopsis thaliana* μA-adaptin (At-μA-Ad) medium chain as sharing homologies not only with the μ-adaptin sequences of *Dictyostelium discoideum* and *Caenorhabditis elegans* but also with mouse μ1- and μ2-adaptins (Happel *et al.*, 1997). In addition, *Arabidopsis* contains four further genomic plant μ-adaptin sequences as revealed by databank searching. While At-μA-Ad is located on chromosome 5, the highly related At-μB1-Ad and At-μB2-Ad (86% identity and 90% similarity) are both located on chromosome 1 (genes T16B5.13 and F8A5.29, respectively). At-μC-Ad (gene F22K18.250) is located on chromosome 4 and At-μD-Ad (gene F25P12.96) is located on chromosome 1.

All plant μ-adaptin sequences resemble their mammalian counterparts in size: μA-adaptin 438 amino acids (aa), μB1-adaptin 411 aa, μB2-adaptin 428 aa, μC-adaptin 451 aa, and μD-adaptin 445 aa, all with predicted molecular masses of

Table 1 Comparison of similarities between At-μA-Ad (GenBank Accession no. AF009631) and μ-adaptin sequences from *Arabidopsis* and mammals by pairwise BLAST alignment (Blosum62 matrix)

μ-Adaptin	GenBank Accession no.	Identity (%)	Similarity (%)
At-μB1-Ad	AC007354	40	59
At-μB2-Ad	AC002292	40	59
At-μC-Ad	AL035356	32	52
At-μD-Ad	AC009323	32	50
μ1 mouse	AF139405	43	61
μ2 mouse	AF001923	47	67
μ3A human	AF092092	26	44
μ4 human	AF155158	26	49

around 50 kDa. Comparison of μA-adaptin sequence with the other plant μ-adaptin sequences reveals almost equal similarities (Table 1), with a slightly higher degree of similarity to μB1- and μB2-adaptin than to μC- and μD-adaptin sequences. When compared with mammalian sequences, the highest degree of similarity was observed to the mouse μ1- and μ2-adaptin sequences, while the lowest degree of similarity is to mammalian μ3A- and μ4-adaptins. Although μA-adaptin shows an almost equally high similarity to mammalian μ1- and μ2-adaptins, and cannot be correlated to either one on the basis of similarity alone, it is the only plant μ-adaptin with the highest similarity to mammalian μ2-adaptin (Table 2). As μ2-adaptin is reported to have the highest affinity and broadest specificity for YXXØ signals (Ohno *et al.*, 1998), we decided to take μA-adaptin as an exemplary representative of the plant μ-adaptins to prove their general function. Comparison of the other plant μ-adaptins with the mammalian μ-adaptins reveals a significant similarity of both μB1- and μB2-adaptins to mouse μ1-adaptin and for μD-adaptin to human μ3A-adaptin, while μC-adaptin cannot be correlated unequivocally to any of the mammalian medium subunits (Table 2).

In their paper on the crystal structure analysis of the RBD of μ2-adaptin complexed with the tyrosine motifs of TGN38 and the EGF-receptor, Owen and Evans (1998) had taken a plant μ-adaptin sequence into comparison, which is identified here as the μA-adaptin sequence. As shown in Figure 1, we have demonstrated by alignment of the five plant μ-adaptin sequences that all of the amino acids crucial for the binding of the tyrosine motif are also highly conserved in the five plant μ-adaptin sequences. Almost all amino acid exchanges within the β-sheet strands between the plant μ-adaptins are conserved, thus leading to the expectation of an analogous function for all plant μ-adaptins.

Table 2 Comparison of similarities between plant and mammalian μ-adaptin sequences using pairwise BLAST alignment (Blosum62 matrix)

	μ1 Mouse	μ2 Mouse	μ3A Human	μ4 Human
At-μB1-Ad	57/78	39/59	27/49	28/49
At-μB2-Ad	60/79	38/58	27/49	29/49
At-μC-Ad	34/52	31/53	25/43	34/57
At-μD-Ad	28/47	24/43	43/63	25/44

Identities/similarities are given in percentage. The clones are identical to those in Table 1.

Expression of the RBD of μA-adaptin

Based on the significant similarity between plant μA- and mammalian μ1- and μ2-adaptin sequences we expected the plant μA-adaptin to bind tyrosine motifs, which are located within the cytoplasmic tails of membrane proteins. To demonstrate an interaction between μA-adaptin and the YXXØ motif in pull-down assays, it was first necessary to express both proteins as fusion proteins. We made use of the fact that mammalian μ-adaptins have a bipartite structure, with the aminoterminal one-third spanning the β-adaptin-binding region (residues 1–145 in mouse μ1,2-adaptin), and the carboxyterminal two-thirds comprising the receptor-binding region (residues 147–423 of μ1-adaptin; residues 164–435 of μ2-adaptin). Separate expression of either region does not result in a loss of functionality (Aguilar *et al.*, 1997). Based on this knowledge, we have fused the carboxyterminal two-thirds comprising the RBD of μA-adaptin (residues 144–438) to a histidine tag and expressed this fusion protein, which has a molecular mass of 38 kDa (Figure 2a,b).

In order to monitor the expression of the RBD, as well as to obtain an insight into the intracellular localization of μA-adaptin, two independent sets of peptide antibodies were raised (Figure 2c). To avoid a cross-reaction with the other plant μ-adaptins, epitopes were chosen in regions of the μA-adaptin sequence, which share extremely low similarities and no continuous stretch of identical residues with either of the other four adaptin sequences (Figure 1). The peptide sequence of the first epitope is located at the border between the aminoterminal one-third and the carboxyterminal two-thirds, while the peptide sequence of the second epitope is located within the RBD region of the μA-adaptin. Both antibodies recognize their epitope when coupled to BSA but do not recognize BSA alone, indicating that labeling is specific for both antibodies (Figure 2c). In addition, both antibodies cross-reacted with the histidine-tagged RBD fusion protein of μA-adaptin in immunoblots, which was additionally confirmed by labeling with the histidine antibody (Figure 2b).

μA-adaptin is able to bind to threefold repeats of tyrosine motifs (YXXØ)

A putative tyrosine motif in the carboxyterminal end of all plant VSRs, ^{606}YMP^{609}L in VSR-PS1, corresponds to the

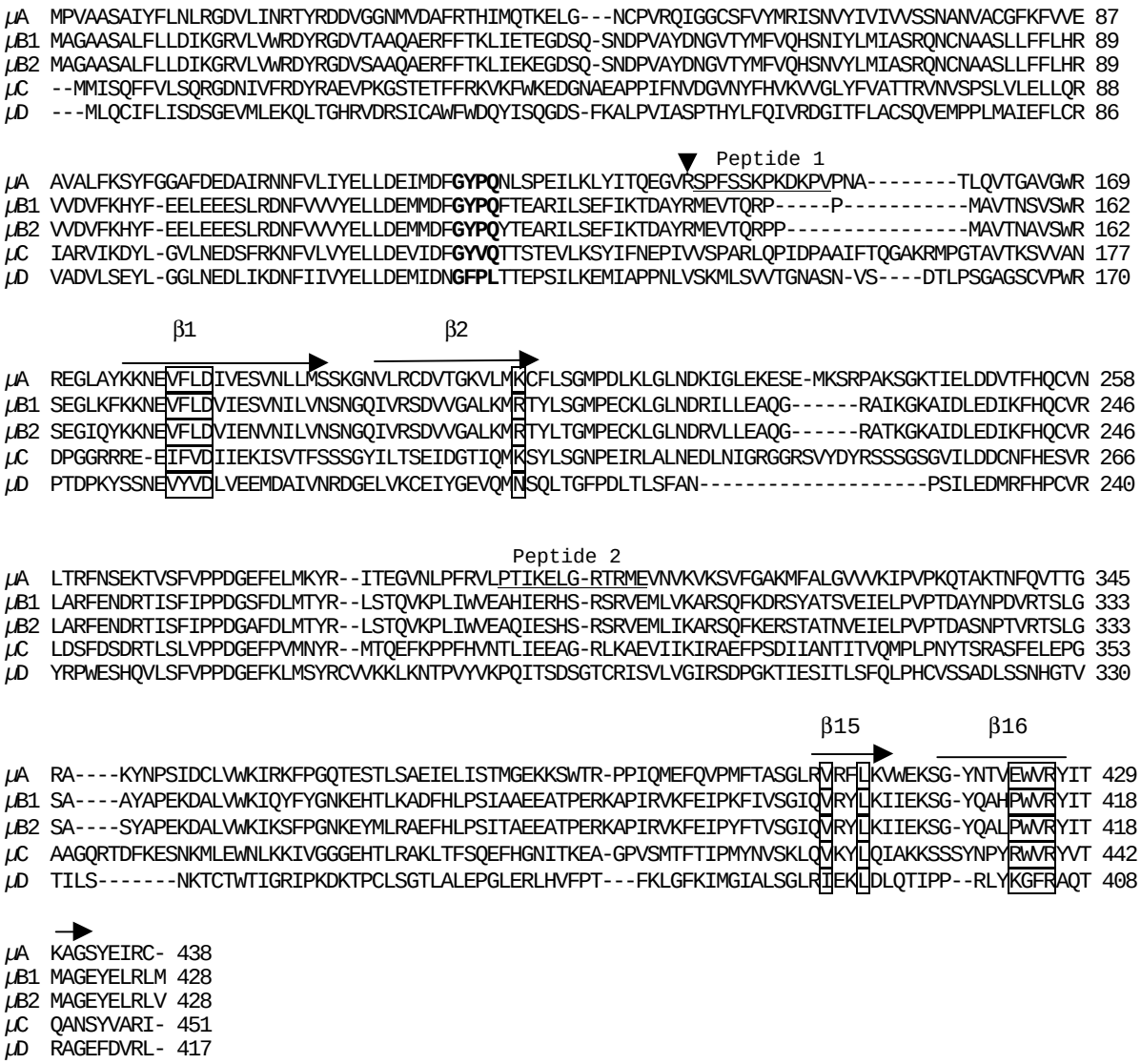

Figure 1. Alignment of *A. thaliana* μ-adaptin amino acid sequences At-μA-Ad, At-μB1-Ad, At-μB2-Ad, At-μC-Ad, and At-μD-Ad using the program CLUSTALW 1.8.

The arrowhead indicates the end of the N-terminal domain and the beginning of the RBD of μA-adaptin. The arrows correspond to the positions of beta sheet strands as given by Owen and Evans (1998). The epitope sequences of μA-adaptin for the peptide antibody #2080 (Peptide 1) and #9139 (Peptide 2) are underlined. Boxed amino acids are crucial for the binding of the YXXØ motif and the amino acids in bold letters correspond to the dileucine binding motif. All amino acid positions given in the text correspond to the μA-adaptin sequence.

consensus motif YXXØ in mammalian receptors. However, before attempting to show that the plant μA-adaptin is a binding partner for plant sorting receptors, we first investigated its binding capacity toward the tyrosine motif of TGN38, a protein that cycles between the TGN and the PM in mammalian cells (Bos *et al.*, 1993; Humphrey *et al.*, 1993; Wong and Hong, 1993). The tyrosine motif (YQRL) in the cytoplasmic tail of TGN38 is known to function in both the endocytic and the lysosomal transport pathways, and is also well known for its binding to mammalian AP complexes and isolated μ-adaptins and exhibits the highest affinity observed so far, especially when used as a triple repeat (Ohno *et al.*, 1995).

For heterologous binding experiments, the threefold repeat of the wild-type (wt) tyrosine motif of TGN38 $(SDYQRL)_{3\times}$ was expressed as a glutathione-S-transferase (GST) fusion protein, immobilized on GSH–Sepharose and incubated with the histidine-tagged RBD of μA-adaptin. A mutated sorting signal of TGN38, in which the critical tyrosine is substituted for alanine $(SDAQRL)_{3\times}$, served as a negative control. Binding of μA-adaptin to GST-$(SDYQRL)_{3\times}$ in this pull-down assay was detected by immunoblotting using a histidine antibody.

As shown in Figure 3(a), the plant μA-adaptin bound to the TGN38 motif $(SDYQRL)_{3\times}$ with high specificity while almost no binding to the control was detectable.

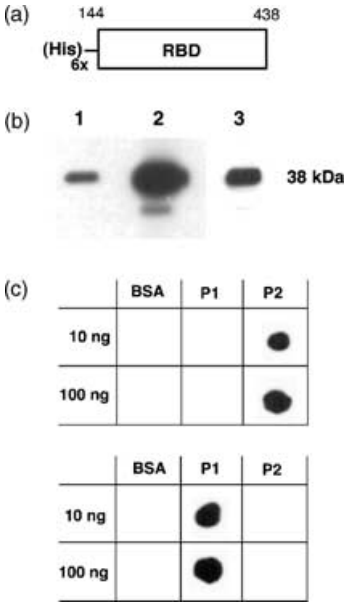

Figure 2. Receptor-binding domain of μA-adaptin.
(a) Schematic representation of the histidine-tagged RBD of μA-adaptin. The RBD comprises the carboxyterminal 294 amino acids of μA-adaptin. Numbers mark the amino acid positions.
(b) Expression of His-RBD of At-μA-Ad as 38 kDa protein on immunoblots with highly purified fusion protein. Lane 1, probed with the histidine antibody; lane 2, the μA-adaptin peptide antibody #9139; and lane 3, the μA-adaptin peptide antibody #2080.
(c) Peptides (P1, P2) that correspond to the respective epitopes (see Figure 1) were coupled to BSA and used in protein dot-blot immunoassays as described in Experimental procedures. BSA alone was used as a control. The amounts of BSA and the conjugate are given. Note that both antisera recognize specifically their respective epitopes.

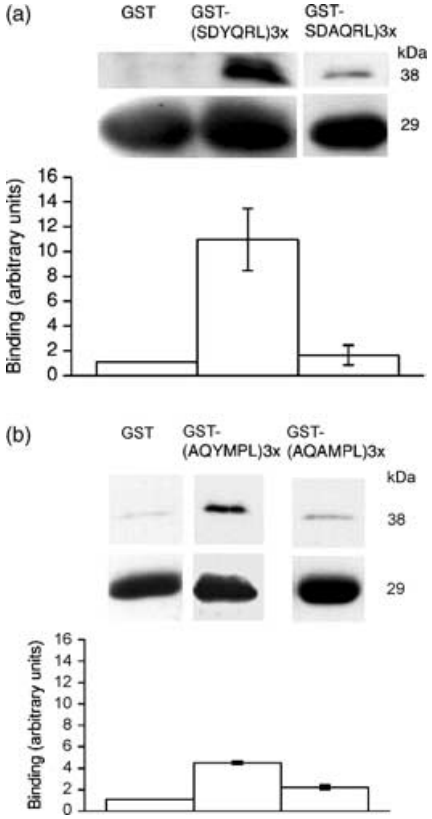

Figure 3. His-RBD-μA binds to the tyrosine motifs of the mammalian TGN38 and that of the VSR-PS1.
The threefold repeats of the tyrosine motifs of (a) TGN38 $(SDYQRL)_{3\times}$ and (b) VSR-PS1 $(AQYMPL)_{3\times}$ were tagged with GST and immobilized on glutathione–Sepharose beads in pull-down experiments. Bound RBD-μA was analyzed after centrifugation in immunoblots and quantified by densitometry. Further incubation with the GST antibody was required to confirm that equal amounts of the GST fusion proteins were added in each sample.

Furthermore, binding of μA-adaptin to the alanine-substitution mutant (Y/A) was reduced to 16% demonstrating that the critical tyrosine residue of TGN38 is also crucial for binding to plant μA-adaptin. These results provide strong evidence in support of the notion that the binding mechanism for tyrosine motifs is conserved between plant and mammalian μ-adaptins. The functional analogy between plant and mammalian μ-adaptins gains further support from our homologous binding experiments using plant μA-adaptin and the triple repeat of the plant VSR-PS1 $(AQYMPL)_{3\times}$.

As seen in Figure 3(b), the threefold repeat of the pea VSR-PS1 motif recognized the μA-adaptin, showing a weaker binding compared to the TGN38 motif, which is not surprising (see above). This interaction is nevertheless specific, as binding to the alanine substitution mutant (Y/A) was reduced by more than 60% and was not detectable in the control (GST). In additional experiments, the binding of the histidine-tagged RBD of μA-adaptin to the tyrosine motifs of TGN38 and VSR-PS1 was revealed to be saturable using 6–8 μg for TGN38 and 12 μg for VSR-PS1 (data not shown).

Interaction of VSR-PS1 cytoplasmic tail with μA-adaptin

Binding of the threefold repeats of the TGN38 and the VSR-PS1 sorting motifs has revealed that *Arabidopsis* μA-adaptin can function as a binding partner in both cases. To confirm this in a situation that more resembles the *in vivo* state, the tyrosine motif of VSR-PS1 was examined when located in the complete cytoplasmic tail sequence. In a first set of experiments, the full cytoplasmic tail sequence of VSR-PS1, as well as its alanine substitution mutant (Y/A), was fused to GST and used to assay its binding capacities to the histidine-tagged RBD of μA-adaptin. As shown in Figure 4(a), μA-adaptin binding to the complete VSR-PS1 cytoplasmic tail was clearly detectable well above over a low background binding to the control GST. However, we noticed significant binding of μA-adaptin to the mutated cytoplasmic tail, estimated as being 50% compared to binding of the wt VSR-PS1 cytoplasmic tail. To corroborate our findings through a complementary approach, we also measured μA-adaptin binding to the cytoplasmic tail of

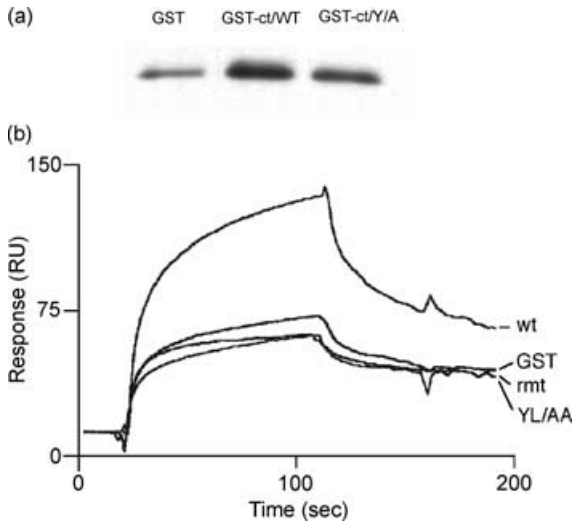

Figure 4. Binding of His-RBD-μA to the complete cytoplasmic tail (ct) of VSR-PS1.

(a) Pull-down experiments were used to investigate the binding between the ct fused to GST and His-RBD-μA. Lane 1, GST as a control; lane 2, ct/WT; lane 3, Y/A-substitution mutant.

(b) Plasmon resonance measurements. The full-length ct of VSR-PS1 (wt) was expressed as a GST fusion protein, purified and immobilized on a C1 sensor surface. A mutant fusion protein in which the tyrosine and the leucine residues of the putative sorting motif YMPL were substituted for alanine, as well as a fusion protein with a randomized VSR-PS1 ct and GST alone served as controls. Subsequent to capture of GST and the GST fusion protein (see Experimental procedures for details), μA-adaptin was passed over the sensor surface (association) for 1 min, followed by switching back to the running buffer (dissociation) and regeneration of the sensor surface. Note that strong binding of μA-adaptin was only observed for the wt GST-VSR-PS1 ct fusion protein. Mutation of the ct (YL/AA) abolished μA-adaptin binding to values, which were also observed for the non-specific binding of μA-adaptin to GST and the randomized VSR-PS1 ct fusion protein.

VSR-PS1 by surface plasmon resonance (SPR) measurements (BIAcore 3000).

The GST-tagged wt cytoplasmic tail of VSR-PS1 was captured with GST antibodies, which were immobilized on the sensor surface. Subsequently, the histidine-tagged RBD of μA-adaptin was passed over the sensor surface in order to monitor binding to the VSR-PS1 fusion protein. As shown in Figure 4(b), binding of the VSR-PS1 cytoplasmic tail to μA-adaptin was detectable and exhibited an equilibrium rate constant of 144 nM (see Table 3 for details). Simultaneously, μA-adaptin binding to two further controls in addition to GST was recorded, one comprising the alanine substitution (YL/AA) mutant of VSR-PS1, in which the putative sorting motif YMPL was altered and a control fusion protein in which the VSR-PS1 cytoplasmic tail amino acid residues were randomly mixed (GST-GSGYEHPIASER-IKDVPYMQERISQDGRRQLM-YNQAHNM). The binding of the μA-adaptin RBD to these controls was reduced to 20% (690 nM for the alanine substitution mutant and 633 nM for the random mutant), showing that binding of μA-adaptin to the VSR-PS1 cytoplasmic tail is dependent upon the sorting

Table 3 Kinetic values for the μA–adaptin interaction with the VSR-PS1 cytoplasmic tail

	μA interaction		
	k_a (M^{-1} sec^{-1})	k_d (sec^{-1})	K_D (nM)
GST	9.6×10^3	5.6×10^{-3}	584
VSR-PS1			
wt	2.8×10^4	4.1×10^{-3}	144
Randomized	6.0×10^3	3.8×10^{-3}	633
YL/AA	5.7×10^3	3.9×10^{-3}	690

Sensorgrams such as those shown in Figure 4 were used to calculate the rate constants for association, dissociation, and the equilibrium rate constant for the binding of μA-adaptin to the VSR-PS1 cytoplasmic tail. Note that the affinity of μA-adaptin for the wt cytoplasmic tail was around four times higher as compared to all other controls, showing the specificity of the μA-adaptin binding and the requirement of a functional tyrosine sorting motif.

motif. Furthermore, comparison of μA-adaptin binding to the wt VSR-PS1 cytoplasmic tail and the alanine substitution (YL/AA) mutant confirms the results obtained in the pull-down assay, namely demonstrating that the YMPL motif in the cytoplasmic tail of VSR-PS1 is necessary for binding of μA-adaptin and thus reconstitutes a classical tyrosine sorting motif.

The assembly status of μA-adaptin

A primary question is whether μA-adaptin is a subunit of a putative AP complex or exists as a single protein associated with clathrin as for example AP180/CALM (Ahle and Ungewickell, 1986; Dreyling *et al.*, 1996). In order to detect the presence of μA-adaptin in a putative plant AP complex, we chose an approach that has been used extensively for the purification of mammalian AP complexes. Therefore, we isolated CCV from *Arabidopsis* cell suspension cultures and also from pig brain for comparison. After their removal from the vesicles, the coat proteins were subjected to gel filtration using a Superose 6 column. As shown in Figure 5, clathrin from both organisms is found in fractions corresponding to the molecular mass of triskelia (670 kDa). The commercially available monoclonal antibody 100-1 recognizes mammalian β1- and β2-adaptins (Figure 5a), but also at least one β-adaptin from *Arabidopsis* (Figure 5b). This antibody has already been used to detect plant β-adaptins (Holstein *et al.*, 1994). Both the mammalian β- and μ-adaptins are clearly separated from the clathrin peak, thus confirming the successful separation of clathrin coat proteins into clathrin and AP complex peaks, the latter with an average molecular mass of about 280 kDa (Schröder and Ungewickell, 1991). The plant β-adaptin(s) elutes in fractions corresponding to the mammalian AP-complex peak, but more importantly, the plant μA-adaptin shows almost

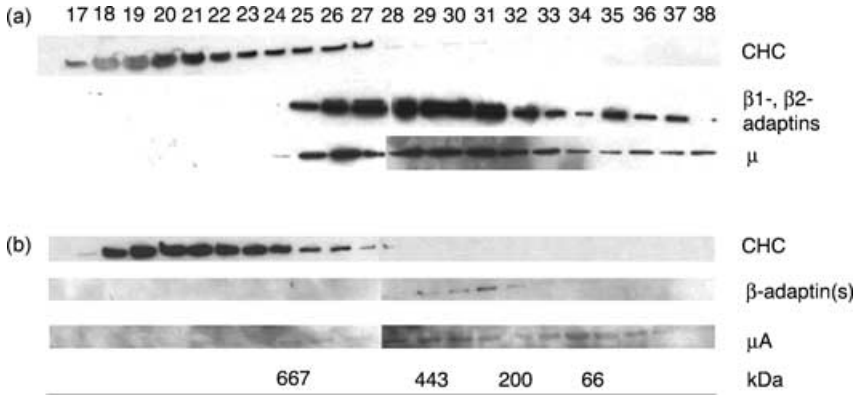

Figure 5. Immunoblots of Superose 6 gel filtration fractions from pig brain and *Arabidopsis* CCVs.
(a) Pig brain coat proteins. Clathrin triskelia are prominent in fractions 17–27, while β1/β2-adaptins as well as the μ-adaptin are present in fractions 25–37.
(b) *Arabidopsis* coat proteins. Clathrin triskelia are most prominent in fractions 18–24, while β-adaptin(s) are in fractions 28–31 and μA-adaptin in fractions 24–36. Note the same behavior of mammalian and plant AP complex subunits. Calibration of Superose 6 column resulted in the following distribution of the marker proteins: 66 kDa (fraction 32–36), 200 kDa (fraction 29–33), 443 kDa (fraction 27–31), and 667 kDa (fraction 23–28).
The fractions were screened with the respective antibody given.

the same distribution as its mammalian counterpart. The mammalian μ-adaptin has a peak in fraction 30, which is shared by the μA-adaptin, which in addition has a second peak at around 66 kDa (fraction 34), corresponding to monomers. Thus, the gel filtration characteristics of the μA-adaptin are consistent with its presence in a multimeric AP complex. As all our antibody preparations proved to be unsuitable for immunoprecipitation, we were unfortunately not able to identify the other μA-adaptin-interacting subunits.

Intracellular localization of the μA-adaptin

Subcellular fractionation of *Arabidopsis* cell cultures was performed in order to gain an insight into the intracellular localization of μA-adaptin. Therefore, microsomal membranes were separated on a linear sucrose gradient (20–55% w/w) under isopycnic conditions (Figure 6). A clear separation of PM (39.5–46.5% w/w) from the bulk of Golgi membranes (27–32% w/w) was obtained. This is shown in immunoblots using standard marker antibodies, namely against the 100 kDa subunit of PM-ATPase (Villalba *et al.*, 1991), and against reversibly glycosylated protein (RGP), a 40-kDa Golgi marker (Dhugga *et al.*, 1997).

The distribution of the TGN, of a pre-vacuolar compartment (PVC) and also of an *Arabidopsis* VSR were also scrutinized in this gradient using a commercially available antibody against the TGN localized syntaxin TLG2a (Bassham *et al.*, 2000), a polyclonal antibody directed against the PVC-localized syntaxin Syp21 (Sanderfoot *et al.*, 1998) as well as a polyclonal antibody against VSR-At1 from *Arabidopsis*, respectively (Sohn *et al.*, 2003). The distribution of the bulk of VSR membranes (28.5–37% w/w) is very similar to that of the PVC marker Syp21 (28.5–36% w/w) and also to that of the TGN marker TLG2a (30.5–38% w/w). The VSR antibody revealed a double band, indicating the possibility of two VSR isomers labeled by this antibody.

μA-adaptin (30.5–41.5% w/w) co-fractionates with the TGN, the VSR(s) and Syp21, and also partially overlaps with the Golgi. It does not correlate with the distribution of the PM marker.

Clathrin is distributed throughout the gradient. It is detected in PM-enriched fractions as well as in those fractions, which contain the PVC, the TGN and the Golgi apparatus. Such a distribution is in accordance with the sites of CCV formation in the cell (the PM, the TGN, and the recycling endosomes). It is also in agreement with the notion that clathrin functions together with VSR(s) and μA-adaptin at the TGN.

In order to confirm that the Golgi/TGN, and not the PM, is the location of the μA-adaptin, we have performed immunofluorescence studies on paraffin sections as well as immunogold electron microscopy on ultra-thin cryosections prepared from *Arabidopsis* root tips (Figure 7). As seen in Figure 7(a), a punctate labeling pattern was obtained suggestive of either the PVC, endosomes or the Golgi apparatus. In order to identify the structure labeled by the μA-adaptin antibody, we used the same peptide antibody on ultra-thin cryosections (Figure 7c,d). The gold label revealed the μA-adaptin to be restricted to Golgi stacks, especially at the periphery of their trans face. Label was absent from the PM (Figure 7c).

Discussion

Features of Arabidopsis *μ-adaptins*

In mammalian cells, the μ1- and μ2-adaptins are the most prominent binding partners at the Golgi and PM for the

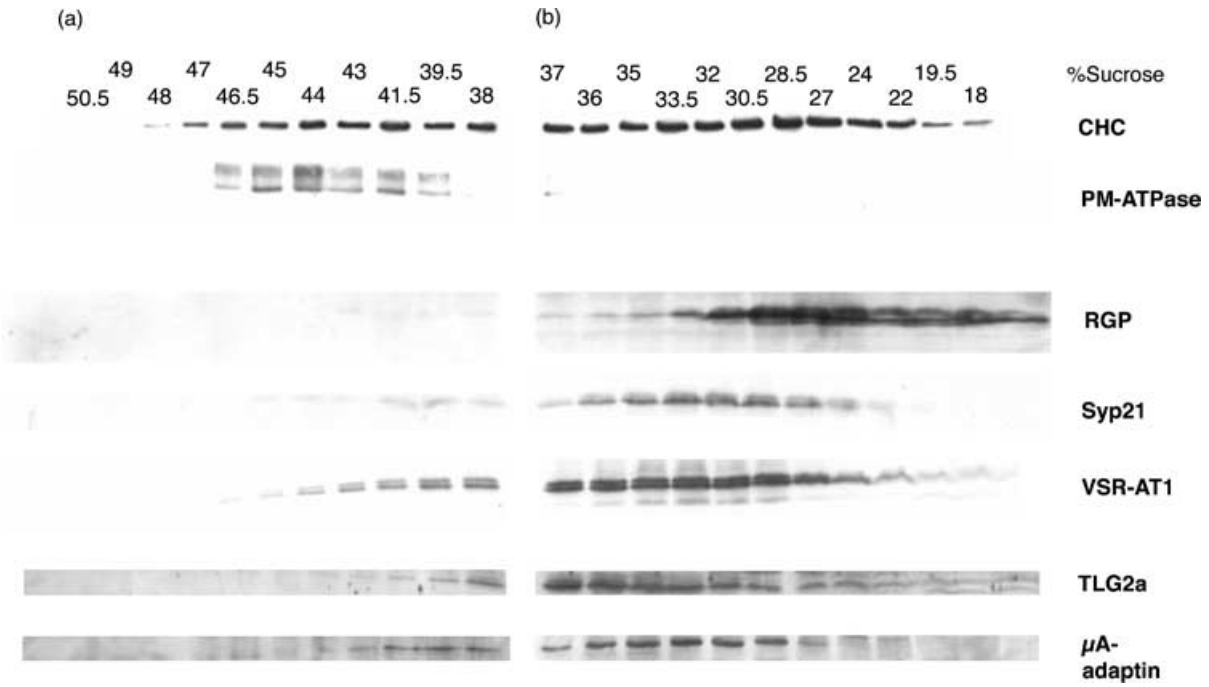

Figure 6. Equilibrium density gradient analysis of *Arabidopsis* cell culture cells.
Total cell membranes were loaded onto a linear 20–55% (w/w) sucrose gradient and spun to equilibrium. Twenty-three fractions were collected and analyzed on two separate blots. (a) Fractions of 38–50.5% (w/w), and (b) fractions of 37–18% (w/w). Both blots were screened under identical conditions with antibodies against clathrin (CHC; 180 kDa), PM-ATPase (PM; 100 kDa), RGP (Golgi; 40 kDa), TGN (TLGa2; 39 kDa), *Arabidopsis* VSR (VSR-At1; 80 kDa), and μA-adaptin (At-μA-Ad, 50 kDa).

YXXØ sorting/internalization motifs located in the cytoplasmic tails of a number of receptors and transmembrane proteins (Marks *et al*., 1997). While mammals have six μ-adaptins present in four AP complexes (Kirchhausen, 1999), yeast has three AP complexes with four μ-adaptin homologs (Yeung *et al*., 1999). *A. thaliana* has in total five μ-adaptin sequences, which in size resemble their mammalian and yeast counterparts.

All amino acids crucial for binding to the tyrosine and the bulky hydrophobic amino acid of the tyrosine motif are

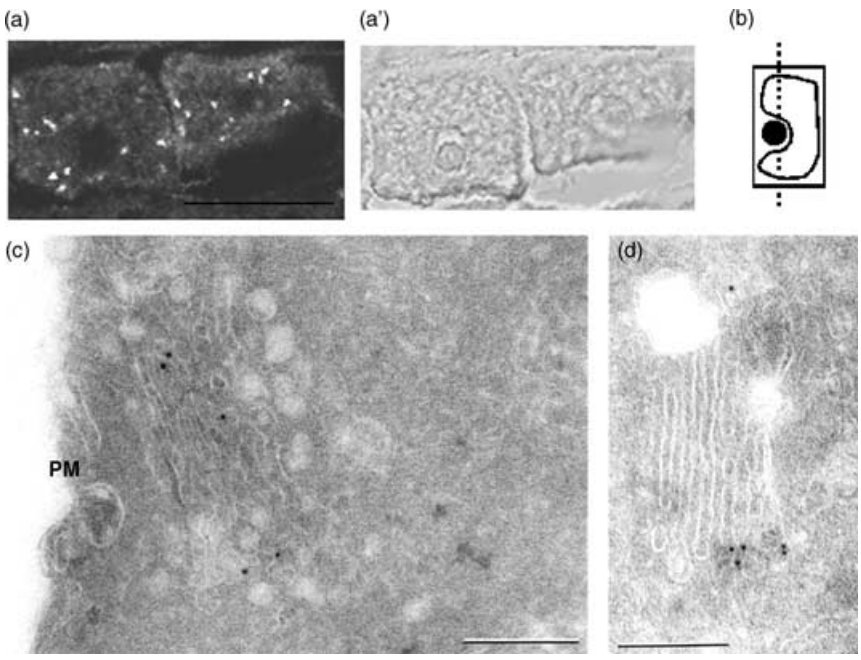

Figure 7. Immunolocalization of μA-adaptin in *Arabidopsis* cells.
(a, b) Single immunofluorescence labeling of a longitudinal paraffin section of *Arabidopsis* root tips with the #2080 peptide antibody to detect μA-adaptin (a). The corresponding differential interference contrast (DIC) image (a′) and (b) a cartoon indicating the median section of a plant cell are also given. Bar in (a) is 21 μm. Immunogold labeling of cryosections of *Arabidopsis* root tip cells with the μA-antibody #2080.
(c, d) Labeling of Golgi stacks, particularly on their peripheries at their *trans* sides. Note that the PM in (c) is not labeled. Bar in (c,d) = 200 nm.

strictly conserved in plant μ-adaptins with the exception of the exchange of ^{423}E in the μA-adaptin sequence and for that of the ^{438}R residue in the μC-adaptin sequence. Interestingly, changes at these positions are also found in μ1–μ3-adaptins from various non-plant species. The variability at this position may reflect the preference for different amino acids in the Y + 3 position in various tyrosine motifs and may point to the existence of other tyrosine motifs in plant proteins.

Sequence comparison between μA-adaptin and mammalian μ-adaptins, reveals an almost equal similarity to both, mammalian μ1- and μ2-adaptins. This is in agreement with the fact that in the tyrosine motif of VSR-PS1, the Y + 2 position (proline) and the Y + 3 position (leucine) match the preferences of both μ1- and μ2-adaptins (Ohno *et al.*, 1998). Moreover, μA-adaptin contains the consensus motif GYPQ within its aminoterminal domain, which is identical to that from mouse μ1- and μ2-adaptin. The mammalian motif is also able to interact with the dileucine signal of the invariant chain (Bremnes *et al.*, 1998), implicating similar kinds of sorting motifs in plant transmembrane proteins as well.

Compared to μA-adaptin, μB1- and μB2-adaptins possess the most appropriate amino acid sequences that are required for the structure of the third pocket, which is responsible for binding to a hydrophobic amino acid at the Y − 3 position (Owen *et al.*, 2001). A scrutiny of the cytoplasmic tail sequence of VSR-PS1 reveals that a hydrophobic amino acid is also at the Y − 3 position, as is also the case for P-selectin (Owen *et al.*, 2001). It is therefore possible that a third hydrophobic pocket in plant μ-adaptins may play an additional role in binding to tyrosine motifs (see below).

Binding of μA-adaptin to YXXØ motifs

The tyrosine motif sequences of VSR-PS1 and six *Arabidopsis* VSR homologs are 100% identical (YMPL), and in one other *Arabidopsis* VSR homolog, there is only one amino acid exchange (YIPL). Furthermore, a comparison of the full-length sequences from VSR-PS1 (NP471; Paris *et al.*, 1997) with AtELP (Ahmed *et al.*, 1997) has revealed an overall identity at the amino acid level of 72% (Paris *et al.*, 1997). Based on a BLAST search (data not shown), even higher overall similarities (82%) to other members of the *Arabidopsis* VSR family were observed, some of them described earlier as being localized at the PM (Laval *et al.*, 1999). Therefore the tyrosine motifs and the cytoplasmic tails from both plants may be considered to be equal in terms of adaptin binding.

Earlier attempts to elucidate an interaction between a receptor and an AP complex from our and other laboratories have been based solely on heterologous binding experiments. While in one set of experiments, AtELP YXXØ-peptides were used for competition (Sanderfoot *et al.*, 1998), in the other two cases, the receptor interacted with coat protein fractions, but in neither case could it be ruled out that proteins other than the AP complex subunits were responsible for the binding (Butler *et al.*, 1997; Robinson *et al.*, 1998b).

In our initial bead-binding experiments with the receptor binding domain of the μA-adaptin from *Arabidopsis*, we used the threefold repeat of the tyrosine motif of TGN38 and of the VSR-PS1 (AQYMPL) from pea, as the same strategy is an established procedure to verify AP complex binding to sorting signals in mammalian systems (e.g. Boge *et al.*, 1998; Boll *et al.*, 1996; Höning *et al.*, 1996; Heilker *et al.*, 1996; Ohno *et al.*, 1995; Rodionov and Bakke, 1998). Furthermore, the μ-adaptin subunit alone or a μ-adaptin fragment comprising the RBD as a binding partner instead of complete AP complexes in binding experiments have been successfully used previously (Aguilar *et al.*, 1997; Hofmann *et al.*, 1999; Ohno *et al.*, 1995; Rodionov and Bakke, 1998; Stephens *et al.*, 1997). Our heterologous binding experiment using the threefold repeat of the TGN38 motif proves that the RBD of μA-adaptin is functionally conserved between plants and mammals, as μA-adaptin was capable of binding to the tyrosine motif and especially because the critical tyrosine residue was also crucial for binding. Thus, binding of the heterologous TGN38 motif by μA-adaptin demonstrates the conservation of plant μ-adaptin functions.

We were able to confirm these results in homologous binding studies by using the tyrosine motif of the VSR-PS1 receptor, not only as a threefold repeat in pull-down assays, but also as a single motif located within the cytoplasmic tail in plasmon resonance measurements. The equilibrium dissociation constant we measured for the VSR-PS1 cytoplasmic tail ($K_D = 144$ nM) is within the range of values obtained in numerous studies for other AP-complex-binding studies (Heilker *et al.*, 1999). The binding of the cytoplasmic tail of VSR-PS1 to *Arabidopsis* μA-adaptin was of high specificity for the tyrosine motif because all three controls show equilibrium constants, which are more than four times lower (584–690 nM). Here, the alanine mutant (YL/AA) exhibited the lowest affinity binding, suggesting that the interaction between μA-adaptin and the VSR-PS1 motif is based on a 'two-pinned plug' rather than on a 'three-pinned plug' (Owen *et al.*, 2001). The cytoplasmic tails of VSR-PS1 and *Arabidopsis* VSR homologs contain another tyrosine motif (YMDS/A) upstream of the YMPL motif, which does not fit the consensus sequence. Furthermore, there was no other mammalian consensus sorting motif observed within the VSR-PS1 cytoplasmic tail, so that the YMPL sequence can be regarded as the only likely functional sorting motif.

In conclusion, the binding studies reported above represent a direct interaction between an AP complex subunit

and a sorting receptor motif in plants. The strong binding of μA-adaptin to TGN38 is in perfect agreement with previous studies, which show that the tyrosine motif of TGN38 exhibits by far the highest affinity towards all mammalian μ-adaptins, especially when used as a triple repeat (Boll *et al.*, 1996; Ohno *et al.*, 1995). As both the position and the residues surrounding the critical tyrosine are important determinants of interaction (Ohno *et al.*, 1996), the binding of multiple repeats of a single motif does not reflect the *in vivo* situation, and the binding values obtained cannot be compared to repeats from other motifs or even to single motifs located in their natural environment. Thus, it is not surprising that the TGN38 motif displays a stronger binding affinity towards μA-adaptin than does the plant motif itself. In our studies, we have used the pea VSR-PS1 tyrosine motif together with the *Arabidopsis* μA-adaptin. Although, they do not represent the correct *in vivo* combination, they served well to demonstrate the conserved features of receptor motif μ-adaptin interactions. Elucidation of the correct *in vivo* binding partners can only be achieved by comparative binding studies with the five plant μ-adaptins and seven *Arabidopsis* VSR homologs.

μA-adaptin is a subunit of the putative AP complex

Gel filtration chromatography is a common procedure to reveal that AP complex subunits are constituents of heterotetrameric complexes with a molecular mass of about 280 kDa (Dell'Angelica *et al.*, 1997, 1999; Hirst *et al.*, 1999; Schröder and Ungewickell, 1991; Simpson *et al.*, 1997). As a subunit of a putative plant AP complex μA-adaptin was expected to be eluted in fractions corresponding to the Stoke's radius of AP complexes. Here, we were able to demonstrate that the gel filtration characteristics of clathrin coat proteins from mammals and plants are identical, namely their separation into a clathrin and an AP complex peak. Both, the mammalian μ-adaptin and the plant μA-adaptin were found in fractions that correspond to their presence in AP complexes, as well as in fractions that correspond to their monomeric or partially assembled AP complex status (Jarousse and Kelly, 2000). While the mammalian β1- and β2-adaptins showed the same broad distribution as mammalian μ-adaptin, plant β-adaptin(s) was located in the fractions that corresponded to the position of mammalian AP complexes. As μA-adaptin and also plant β-adaptin(s) behave like their mammalian counterparts in gel filtration, we conclude that they are also subunits of a putative plant AP complex.

μA-adaptin localizes to the Golgi/TGN and not the PM

As previously mentioned, as μA-adaptin showed almost the same degree of similarity towards mammalian μ1- and μ2-adaptins, indeed with a slight preference for the latter, it might be expected to be localized to the PM rather than the Golgi/TGN.

Our immunolabeling data, however, convincingly demonstrate that the μA-adaptin is not present at the PM. They are also supported by subcellular fractionation experiments, which clearly show that μA-adaptin-containing fractions do not co-equilibrate with the PM.

In isopycnic sucrose density gradients, the distributions of μA-adaptin and VSR-At1 are almost identical, suggesting that they may be located on the same membrane. Indeed, based on the binding studies described above, this could be expected. According to Li *et al.* (2002), the bulk of VSR(s) is found at the PVC under steady state conditions. Moreover, the multi-vesiculate nature of the PVC has recently been established through the immunogold labeling of high-pressure-frozen specimens, and by the selective response of the PVC to the phosphatidyl-inositol-3 (PI3)-kinase inhibitor wortmannin (Tse *et al.*, 2004). Our gradient results showing almost identical profiles for VSR-At1 and the PVC marker Syp21, also support this co-localization. On the other hand, the TGN marker TLG2a also shows the same distribution, and as indicated by immunogold labeling, the μA-adaptin is restricted to the *trans*-/TGN region of the Golgi apparatus. However, this apparent contradiction can be explained in the following way: VSRs cycle between the TGN, where they selectively bind cargo ligands, and the PVC, where the ligands dissociate. VSRs rapidly enter and leave the TGN, where they are collected into CCV with the help of μA-adaptin-containing AP complexes. The coat of the CCV dissociates before fusion of the VSR-containing vesicle with the PVC. If ligand dissociation is slow, VSRs will accumulate in the PVC. Thus, VSRs may be present in both TGN (small amounts) and PVC (the majority) membranes, but the μA-adaptin only becomes associated with TGN membranes. This scenario is based on the assumption that retrograde transport of VSRs occurs without the mediation of μA-adaptin containing AP complexes. Whereas in yeast, retrograde transport of the VSRs occurs via retromer-coated vesicles (Seaman and Williams, 2002; Seaman *et al.*, 1998), this does not appear to be the case for mannosyl 6-phosphate receptors, which are recycled from the pre-lysosomal compartment in animal cells via AP1-bearing vesicles (Huang *et al.*, 2001; Waguri *et al.*, 2003). It is possible that in plants other medium adaptin subunits, such as the μB1/B2, which are closely related to the μ1-adaptin, serve as substitutes for this transport step.

Experimental procedures

Strains, media

Escherichia coli strains used in this study were DH5α and BL-21DE3 (Hanahan, 1985) and were grown in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989). Cell cultures of *A. thaliana* (At-7) were

obtained from the Max Planck Institute in Cologne, Germany, and grown in the dark under constant rotation (90 r.p.m) and harvested 7 days after inoculation. *A. thaliana* var. Columbia (wt) was grown in liquid culture (Gamborgs medium; #G-5893, Sigma, Deisenhofen, Germany) for 10 days under constant rotation (90 r.p.m.) with a cycle of 16 h light and 8 h dark.

Fusion protein constructs

The cDNA sequences of the threefold repeats of the TGN38 tyrosine sorting motif $(SDYQRL)_{3\times}$, of the VSR-PS1 motif $(AQYMPL)_{3\times}$, as well as of their alanine substitutions $(SDAQRL)_{3\times}$ or $(AQAMPL)_{3\times}$ were synthesized as oligonucleotides corresponding to the plus and the complementary minus strands, respectively (Nucleic Acids Product Supply, Göttingen, Germany). In the following, the cDNA sequences of the respective plus strands are given. The cDNA sequence of $(SDYQRL)_{3\times}$ is 5′-GATCCAGTGACTACCAACGTTTGAGTGACTACCAACGTTTGAGTGACTACCAACGTTTGTGA-3′. The $(SDAQRL)_{3\times}$ cDNA sequence is 5′-GATCCAGTGACGCTCAACGTTTGAGTGACGCTCAACGTTTGAGTGACGCTCAACGTTTGTGA-3′. The $(AQYMPL)_{3\times}$ cDNA sequence is 5′-GATCCGCACAATATATGCCCTTGGCACAATATATGCCCTTGGCACAATATATGCCCTTGTGA-3′. The $(AQAMPL)_{3\times}$ cDNA sequence is 5′-GATCCGCACAAGCTATGCCCTTGGCACAAGCTATGCCCTTGGCACAAGCTATGCCCTTGTGA-3′. The cDNA sequence of the GST-VSR-PS1 wt cytoplasmic tail fusion protein is 5′-GGATCCGTGTATAAATATAGAATTAGGCAATACATGGATTCTGAAATCAGAGCAATCATGGCACAATATATGCCCTTGGACAGCCAAGAAGAAGGTCCAAATCACGTCAATCATCAAAGAGGTTGAATTC-3′ with a *Bam*HI site in 5′- and an *Eco*R1 in 3′ added for insertion in frame with the GST-coding sequence into pGEX 2T (Amersham Pharmacia Biotech, Freiburg, Germany). The sequence of the randomly mixed complete cytoplasmic tail sequence of VSR-PS1 is 5′-GGATCCGGATATGAACACCCCATCGCATCTGAAAGAATTAAAGATGTCCCATATATGCAAGAAAGAATCTCTCAAGACGGTAGAAGGCAATTGATGTACAATCAAGCACATAATATGTGA-3′.

To anneal the complementary strands, 4 μg of each of the plus and minus strands were mixed, respectively, incubated for 10 min at 95°C in a water bath and cooled slowly over night (Sambrook *et al.*, 1989). Fusion of each double-stranded oligonucleotide to the GST-tag occurred via its *Bam*HI site at the 5′ end and its blunted 3′ end into the expression vector pGEX-4T-3 (Amersham Pharmacia Biotech). The vector was opened with *Bam*HI and *Sma*I prior to fusion. The construct was transformed into the DH5α strain, and the plasmid DNA was isolated using a Midiprep kit (Qiagen, Hilden, Germany) and then subjected to commercially sequencing (MWG AG Biotech, Ebersberg, Germany).

The cDNA comprising the sequence of the RBD of μA-adaptin was cut out from λ ZipLox vector using the restriction enzymes *Bst*EII (position 602 bp) and *Hin*dIII (position 1724 bp). The resulting 1122-bp fragment was then cloned into the expression vector pQE30 (Qiagen), which had been opened using *Sma*I and *Hin*dIII. Ligation finally resulted in the construct of histidine-tagged RBD of μA-adaptin ($(His)_{6x}$-RBD-μA-adaptin).

Expression and purification of fusion proteins

Luria-Bertani (0.5 l) were inoculated in a 1 : 10 dilution from an overnight culture. Cells were grown until an OD_{600} of 0.7 was obtained. For induction isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 or 2 mM for $(His)_{6x}$- and GST fusion proteins, respectively. Induction occurred at room temperature 3 h for GST fusion proteins and 2 h for $(His)_{6x}$ fusion proteins. Bacteria were obtained by centrifugation at 4000 ***g*** at 4°C for 15 min and frozen at −80°C after washing once with PBS.

Purification of GST fusion proteins was performed as described by Smith and Johnson (1988). Bacteria obtained from 0.5 l cultures were re-suspended in 20 ml of buffer A (50 mM Tris, pH 7.5, 100 mM KCl, 5 mM EDTA) plus protease inhibitors (2 mM leupeptin, 0.7 μM pepstatin, 2 g ml^{-1} aprotinin, 0.15 mM phenylmethylsulfonyl fluoride) and disrupted by sonication (six bursts of 30 sec with 90% power (Bandelin Sonoplus GM70). Centrifugation at 100 000 ***g*** for 20 min removed the cell debris. The supernatants were then incubated with 500 μl of GSH–Sepharose beads on a rotator at 4°C, sequentially washed with 30 ml of buffer A and with 10 ml of buffer B (50 mM Tris–HCl (pH 7.5), 1 mM KCl, 5 mM EDTA). Elution of GST fusion proteins was achieved at a final concentration of 5 mM glutathione.

For the purification of $(His)_{6x}$-RBD of μA-adaptin bacteria obtained from 1-l culture were re-suspended in 20 ml of lysis buffer (10 mM Tris–HCl (pH 8.0), 50 mM NaH_2PO_4, 100 mM NaCl). The cells were disrupted by sonication as described above and then centrifuged for 20 min at 100 000 ***g***. The supernatant was incubated with 750 μl Talon™ metal affinity resin (Clontech, Palo Alto, USA) for 1 h at 4°C on a rotator. The Talon™ was separated from the solution and washed sequentially with 20 ml of lysis buffer, 20 ml of washing buffer (20 mM Tris–HCl (pH 8.8), 100 mM NaCl), and 1 ml of washing buffer, including 50 mM Imidazol. An Imidazole concentration of 500 mM was used to obtain optimal elution.

Pull-down assay

For binding, both the GST fusion proteins and the $(His)_{6x}$ fusion proteins were changed into binding buffer (100 mM Tris–HCl (pH 7.5), 5 mM EDTA, 0.1% (v/v) Triton X-100; Rodionov and Bakke, 1998) using NAP-5 columns (Amersham Pharmacia Biotech). 100 μg packed glutathione–Sepharose beads were prepared according to the manufacturer's instructions (Amersham Pharmacia Biotech) and subsequently incubated with 50 μg of the respective GST fusion protein for 30 min at 4°C. The beads were washed three times with 500 μl of binding buffer and centrifuged at 510 ***g***. For each binding experiment, 30 μl of the pre-incubated GSH beads were incubated with 1–16 μg of the $(His)_{6x}$-μA-RBD construct and BSA in a final concentration of 1% (w/v), filled up to 100 μl final volume and incubated for 1.5 h at 4°C on a rotator. The beads were washed two times with 200 μl of binding buffer, and the final pellet was re-suspended in twofold sample buffer (Laemmli, 1970). The samples were boiled at 95°C for 1 min and subjected to SDS–PAGE. Each binding assay was independently performed three times with each of the three samples.

SPR analysis

Association of the VSR-PS1 cytoplasmic tail with the RBD of μA-adaptin was analyzed in real-time by SPR using a BIAcore 3000 biosensor (BIAcore AB, Freiburg, Germany). In brief, an anti-GST monoclonal antibody (BIAcore AB) was immobilized on all four flow cells of a C1 sensor chip, which has a flat carboxymethylated surface at equal densities according to the manufacturers' instructions. Subsequently, the chip was used to capture the GST fusion proteins harboring the sorting motif sequences at a flow rate of 5 μl min^{-1} followed by the analysis of μA-adaptin binding. All interaction experiments were performed with binding buffer (100 mM Tris–HCl (pH 7.5), 5 mM EDTA, 0.1% (v/v) Triton X-100) at a flow rate of 20 μl min^{-1}. Association for 1 min was followed by

dissociation for 1 min during which the binding buffer was perfused. A short-pulse injection (15 sec) of 20 mM NaOH/0.5% SDS was used to regenerate the sensor chip surface after each experimental cycle. The GST-EnvCD-derived sensor chip remained stable and retained its specific binding capacity for all experimental cycles of association/dissociation and regeneration. The $(His)_{6x}$-RBD of μA-adaptin was used at final concentrations ranging from 600 to 1000 μM. To exclude distortions because of injection and mixing, segments of the sensorgrams recorded 15–20 sec after switching from buffer flow to AP solution or 5–10 sec after switching back to running buffer were used for the calculations of association and dissociation rate, respectively.

Kinetic parameters and equilibrium dissociation constants were determined from sensorgrams recorded at different AP concentrations. The association constant k_a, the dissociation constant 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. The model calculates the association rate constant 'k_a' and the steady-state response level 'R_{eq}' by fitting data to the equation $R = R_{eq}(1 - e^{-(k_a C_n + k_d)(t - t_0)})$, where t is the time in seconds, R_{eq} is the steady-state response level, and C the molar concentration of APs in the injection solution. The steric interference factor n, which describes the valency of the interaction between μA and VSR-PS1 was set to 1. The dissociation rate constant, k_d, was determined by fitting the data to the equation $R = R_0 e^{-k_d(t - t_0)}$, where R_0 is the response level at the beginning of the dissociation phase. This model has recently been applied to describe the interactions of APs with cytosolic domains (Heilker *et al.*, 1996; Höning *et al.*, 1997) and is described in more detail elsewhere by Jonsson *et al.* (1991).

Gel electrophoresis, immunoblotting, and protein determination

SDS-gradient gels (10–19%) were prepared as previously described by 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). Bands on the ECL film were quantified by using the BASYS gel-analyzing system (BioTec Fisher, Reiskirchen, Germany). Protein concentrations were determined according to Bradford (1976).

Antibodies

For immunoblot studies, the following antibodies were used: the affinity-purified polyclonal μA-adaptin peptide antibodies #9139 (20 ng μl^{-1}) and #2080 (184 ng μl^{-1}), a polyclonal antiserum against RGP (1 : 10.000 dilution; supplied by Dr K. Dhugga, Pioneer Hi-Bred International Johnston, IA, USA), a monoclonal PM-ATPase antibody (1 : 500 dilution; supplied by Dr W. Michalke, University of Freiburg, Germany), a polyclonal syntaxin of plants (Syp)21 antibody (1 : 1000, raised according to Sanderfoot *et al.*, 1998), a monoclonal CHC antibody (1 : 500 dilution) and a monoclonal AP-50 antibody (1 : 250 dilution) both from BD, Tranduction Laboratories, Franklin Lakes (USA), the monoclonal 100-1 β1/β2-adaptin antibody (1 : 100 dilution; Sigma, Taufkirchen, Germany), the TLG2a antibody in a 1 : 1000 dilution from Rose Biotechnology Inc. (http://www.rosebiotech.com) and an $(His)_{6x}$ antibody (BioScience, Göttingen, Germany) in a 1 : 2000 dilution and a GST antibody (Amersham Pharmacia Biotech) at a 1 : 1000 dilution. Secondary antibodies against mouse or rabbit coupled to horseradish-peroxidase, respectively, were obtained from Sigma (Taufkirchen, Germany).

For immunofluorescence studies, the affinity-purified polyclonal μA-adaptin peptide antibody #2080 (184 ng μl^{-1}) was used in a 1 : 10 dilution and the secondary antirabbit IgG coupled to Cy-5 in a final concentration of 1 : 200 (Jackson ImmunoResearch Laboratory Inc., West Grove, PA, USA).

For immunolabeling on cryosections, the primary antibody was the affinity-purified μA-adaptin #2080 antibody in a 1 : 50 dilution. The secondary goat antirabbit IgG coupled to 10-nm-diameter particles of gold was obtained from BioCell (Cardiff, UK) and diluted in 1 : 30 in PBS containing 1% (w/v) BSA.

Peptide coupling to BSA

Each peptide (4 μg μl^{-1}) was coupled to H2 fraction of BSA (20 μg μl^{-1}; Applichem, Germany) via 1% (v/v) glutaraldehyde (Sigma, Deisenhofen, Germany) in a final volume of 1 ml on a rotator at 4°C for 2 h. Subsequently samples were dialyzed for 16 h at 4°C against a buffer containing 0.1 M $NaHCO_3$, 0.5 M NaCl (pH 8.3). A protein dot-blot immunoassay was made with 10 and 25 ng of either BSA alone or the conjugate. The sera of #2080 and #9139 were used in 1 : 500 dilutions each in PBS/5% (w/v) skim milk. The proteins were visualized with the Supersignal West Pico ECL kit (Pierce, Rockford, USA).

Subcellular fractionation

Twenty grams of *Arabidopsis* cell culture were homogenized in 10 ml TDKE-buffer (25 mM Tris–HCl (pH 7.0), 1 mM dithiothreitol, 10 mM KCl, 3 mM EDTA), including the final concentrations of the following antiproteases: 2 mM leupeptin, 0.7 μM pepstatin, 2 g ml^{-1} aprotinin, and 0.15 mM phenylmethylsulfonyl fluoride using a 'Zauberstab'-blendor. After filtration through three layers of miracloth, the homogenate was pre-centrifuged for 15 min at 8000 ***g*** and the resulting supernatant for another 15 min at 18 000 ***g*** in a Heraeus swing-out rotor #2704.

The supernatant was then applied on a 5-ml 60% (w/w) sucrose cushion in TDKE buffer and centrifuged for 45 min at 100 000 ***g*** in a swing-out TST28.38 rotor. The interphase was removed and put on a linear 20–55% (w/w) sucrose gradient in TDKE buffer and spun for 20 h at 100 000 ***g*** in a swing-out TST28.38 rotor. Fractions of 1.5 ml were taken, and aliquots of each fraction were subjected to SDS–PAGE after TCA precipitation.

Gel filtration on superose 6/FPLC

Clathrin-coated vesicles were isolated from pig brain as described elsewhere by Lindner (1994) and from *Arabidopsis* cell culture cells. Fifty grams of frozen cell culture were homogenized using a mortar and a pestle and subsequently re-suspended in 50 ml of buffer A (0.1 M 2-morpholinoethansulfonic acid (MES) (pH 6.4), 0.5 mM $MgCl_2$, and 1 mM EGTA, 1 mM EDTA + inhibitors, see above). Pre-centrifugation was performed as described above, and the supernatant was subsequently centrifuged (T12.50 rotor, 4°C, 120 000 ***g***, 1.5 h) to obtain the microsomal pellet. The obtained pellet was re-suspended in 4 ml of buffer A + 300 mM sucrose. A ficoll/sucrose (12.5% Ficoll 400, 12.5% sucrose) solution was added in a 1 : 1 ratio, and the mixture was centrifuged in an AT-5 rotor (40 min, 43 000 ***g***). The obtained supernatant was then fivefold diluted and centrifuged for 1 h at 110 000 ***g*** in T12.50 rotor to obtain a pellet enriched in CCV. This pellet was re-suspended in 1 ml of buffer A and 1 g Hydroxyapatite gel equilibrated before in buffer A (DNA-grade Biogel, Bio-Rad, Munich, Germany) was incubated in a batch procedure for 1 h at 4°C with slow motion

and then washed two times with 5 mM potassium phosphate (PP) in buffer A (2 min, 4°C, 600 ***g***). CCVs were removed from Hydroxyapatite gel by stepwise elution using 300, 400, and 500 mM PP in buffer A. CCV of each elution step were then subjected to centrifugation (1 h, T12.50 rotor at 110 000 ***g***), and the CCV pellets were combined in a total volume of 2 ml buffer A.

To remove the coat proteins from mammalian or plant CCV, approximately 1 mg CCV were incubated in final 0.5 M Tris/buffer A for 30 min at 4°C. Centrifugation for 1 h 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). The flow rate was 0.3 ml min^{-1}, and the fraction size was 0.5 ml. Each fraction was analyzed on SDS–PAGE. Calibration of Superose 6 column was performed using the Molecular weight marker kit (MW-GF-1000) from Sigma (Deisenhofen, Germany).

Immunofluorescence studies on paraffin sections of Arabidopsis *root tip cells*

Paraffin embedding of 5-mm-long tips of *Arabidopsis* roots from 10-day-old seedlings grown in liquid culture was performed as described elsewhere by Jauh *et al*. (1998). Immunofluorescence studies were performed as outlined by Paris *et al*. (1996) using the antibody as given above. Images were taken with the Bio-Rad Confocal laser scanning microscope MRC-10242PMT system and the laser sharp program versus 3.02, with a laser power of 1%. The recorded images were processed by ADOBE PHOTOSHOP 7.0 software.

Cryosectioning and immunogold labeling

Root tips 5 mm long were excised and transferred into a primary fixative (1.5% (w/v) paraformaldehyde, 0.2% glutaraldehyde (v/v) in 0.1 M PP buffer (pH 7.4) for 12–16 h at 4°C, then infiltrated with 2.3 M sucrose in PP buffer over night at 4°C, and finally embedded in 10% (w/v) (37°C) gelatine solubilized in PP buffer. Gelatine cubes (0.8 mm^3) were infiltrated in 2.3 M sucrose for 10 min at 4°C and then mounted onto specimen stubs, frozen in liquid nitrogen, and cut into ultra-thin sections at −80°C with a Leica UCT microtome (Leica, Bensheim, Germany). Frozen sections were picked up as described by Liou *et al*. (1996) and transferred to formvar-carbon-coated nickel grids. Immunogold labeling was performed as described by Pimpl *et al*. (2000), except that the staining procedure with uranyl acetate/oxalate was omitted.

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