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Cloning and Subcellular Location of an Arabidopsis Receptor-Like Protein That Shares Common Features with Protein-Sorting Receptors of Eukaryotic Cells[1]

Sharif U. Ahmed[2], Maor Bar-Peled[2], and Natasha V. Raikhel*

Michigan State University-Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824–1312

Many receptors involved in clathrin-mediated protein transport through the endocytic and secretory pathways of yeast and animal cells share common features. They are all type I integral membrane proteins containing cysteine-rich lumenal domains and cytoplasmic tails with tyrosine-containing sorting signals. The cysteine-rich domains are thought to be involved in ligand binding, whereas the cytoplasmic tyrosine motifs interact with clathrin-associated adaptor proteins during protein sorting along these pathways. In addition, tyrosine-containing signals are required for the retention and recycling of some of these membrane proteins to the *trans*-Golgi network. Here we report the characterization of an approximately 80-kD epidermal growth factor receptor-like type I integral membrane protein containing all of these functional motifs from *Arabidopsis thaliana* (called AtELP for *A. thaliana* Epidermal growth factor receptor-Like Protein). Biochemical analysis indicates that AtELP is a membrane protein found at high levels in the roots of both monocots and dicots. Subcellular fractionation studies indicate that the AtELP protein is present in two membrane fractions corresponding to a novel, undefined compartment and a fraction enriched in vesicles containing clathrin and its associated adaptor proteins. AtELP may therefore serve as a marker for compartments involved in intracellular protein trafficking in the plant cell.

Proteins that are transported through the secretory pathway in eukaryotic cells are imported into the ER and travel through the Golgi apparatus. Upon arrival at the TGN, vacuolar and secreted proteins are sorted and transported to their respective destinations (Bar-Peled et al., 1996; Rothman, 1996). The transport of many of these cargo proteins through the secretory pathway is mediated by small vesicles, a process requiring specific soluble and membrane proteins (Rothman and Wieland, 1996). During the transport process, vesicles carrying cargo first bud from the donor membrane, then travel to and dock at the target membrane, and finally fuse to deliver the cargo (for review, see Bassham and Raikhel, 1996; Rothman and Wieland, 1996).

Among the different types of vesicles associated with the transport of proteins along the secretory pathway in eukaryotic cells, CCVs have been best characterized. CCVs play an important role in receptor-mediated endocytosis (for review, see Pearse and Robinson, 1990; Schekman and Orci, 1996) and in targeting of proteins to the lysosome or vacuole (Dahms et al., 1989; Pryer et al., 1992; Hohl et al., 1996). During receptor-mediated endocytosis in animal cells, Tyr-based sorting signals in the cytoplasmic tails of cell-surface receptors associate with a cytosolic adaptor protein complex (AP2) at the PM. Subsequent interaction of the receptor-adaptin complex with clathrin initiates the formation of CCVs (Pearse and Robinson, 1990). These vesicles then bud from the PM and fuse with the endosomal membrane, where the receptor is delivered for further transport to the lysosome for degradation or recycling back to the PM.

Transport of some proteins from the TGN to the vacuole or PM has been shown by biochemical (Dahms et al., 1989; Glickman et al., 1989) and genetic (Seeger and Payne, 1992) analyses to require CCVs. At the TGN a different set of cytosolic adaptor complex proteins (AP1) are thought to interact with cytoplasmic Tyr motifs of transmembrane proteins, such as the M-6-PR, in forming CCVs for transport to the mammalian lysosome (Glickman et al., 1989). In yeast, homologs of the mammalian clathrin heavy chain (Lemmon and Jones, 1987) and several of the adaptin genes have been identified, and their involvement in vacuolar targeting has been demonstrated genetically (Rad et al., 1995; Stepp et al., 1995).

In plants the involvement of CCVs during endocytosis at the PM of rapidly growing cells has been described in a number of studies (for review, see Low and Chandra [1994]), and their involvement in membrane recycling from the PM has been suggested during cell-plate formation and growth of pollen tube tips (Samuels et al., 1995; Blackbourn and Jackson, 1996). In addition, CCVs are associated with the plant TGN (Staehelin et al., 1990; Driouich et al., 1993), and CCV-enriched fractions were found to contain precursors of some vacuolar seed-storage proteins (Harley and

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[2] These two authors contributed equally to the work.

* Corresponding author; e-mail nraikhel@msu.edu; fax 1–517–353–9168.

Abbreviations: CV, coated vesicle; CCV, clathrin-coated vesicle; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EST, expressed sequence tag; GST, glutathione S-transferase; LDLR, low-density lipoprotein receptor; M-6-PR, Man-6-P receptor; PM, plasma membrane; TGN, *trans*-Golgi network.

Beevers, 1989; Hoh et al., 1991). However, a more recent study reported that it is complex glycoproteins, not storage proteins, that are carried by CCVs (Hohl et al., 1996). Nevertheless, a number of studies have provided definitive evidence for the participation of CCVs in intracellular protein transport in plants. These include the recent cloning of the clathrin heavy-chain gene from soybean and the localization of the protein to the PM (Blackbourn and Jackson, 1996), the identification of a β-type adaptin from zucchini (Holstein et al., 1994), the cloning of a σ-adaptin from *Camptotheca acuminata* (Maldonado-Mendoza and Nessler, 1996), and the isolation of a putative vacuolar-targeting receptor (BP-80; Kirsch et al., 1994a) having a broad-binding specificity for plant vacuolar-targeting signals from pea CCVs (Kirsch et al., 1996).

Our laboratory is currently pursuing biochemical and genetic approaches to identify components of the protein-sorting machinery of the plant secretory pathway; however, we have adopted an additional method for the isolation of gene(s) that may encode some components of the machinery. This approach is based on the use of known functional motifs present in many of the receptor proteins involved in clathrin-dependent intracellular protein sorting in mammalian and yeast cells. We report the characterization of AtELP (*Arabidopsis thaliana* EGF receptor-Like Protein), an approximately 80-kD type I integral membrane protein that has a predicted structural organization very similar to that of mammalian and yeast protein-sorting receptors. AtELP is found in a CV population enriched for CCVs. Furthermore, it appears to be localized to a novel, intermediate compartment that may correspond to the plant counterpart of the mammalian or yeast endosome.

MATERIALS AND METHODS

Plant Growth

Ten milliliters of *Arabidopsis thaliana* ecotype Columbia cell-suspension line T87-C33, a generous gift from Dr. Michael Axelos (Institut National de la Recherche Agronomique, France), was subcultured in 50 mL of media (0.32 g L^{-1} Gamborg's B-5 with minimal organics, with the addition of 20 g L^{-1} Suc, and 1 mg L^{-1} 2,4-D at pH 5.7) and was grown on a rotary shaker at 50 rpm and 22°C under light conditions as described by Bar-Peled and Raikhel (1997). Seeds of *A. thaliana* ecotype Columbia (approximately 15 seeds) were sterilized and placed in a 125-mL flask containing 50 mL of germination medium liquid medium (4.3 g L^{-1} Murashige and Skoog salts mixture [GIBCO-BRL], 0.5 g L^{-1} Mes, 10 g Suc, 0.1 g L^{-1} *myo*-inositol, 100 μL L^{-1} of 10 mg mL^{-1} thiamine-HCl, 50 μL L^{-1} of 10 mg mL^{-1} pyridoxine, and 50 μL L^{-1} of 10 mg mL^{-1} nicotinic acid, adjusted to pH 5.7 with KOH). The sterile plants were germinated and grown in flasks placed on a rotary shaker (50 rpm) in a 22°C incubator under a 12-h fluorescent light/dark cycle. To study tissue-specific protein expression patterns, seeds were germinated either in germination medium-agar plates and grown as described above, or germinated in pots and grown in a controlled environment chamber at 22°C under a 12-h light/dark cycle: 120 μmol m^{-2} s^{-1} light intensity, 70% RH. Plant materials (roots, leaves, flowers, pollen, stems, and siliques) were collected at different times, as indicated, and analyzed.

Identification of *AtELP*

A search of the plant EST databases using the Motif Explorer program (provided by the Arabidopsis cDNA Sequence Analysis Project, Michigan State University, East Lansing) identified the products of the AtELP gene from *A. thaliana*. The Motif Explorer tool was developed by the Data Acquisition, Analysis, and Distribution Project at the University of Minnesota (Bieganski, 1995). The full-length 2314-bp *AtELP* cDNA (pSA23) was constructed in pGEX-5X-1 (Pharmacia) by an inframe fusion of two contiguous (130-bp overlap) *A. thaliana* cDNAs (EST clone nos. R90202 and R30384). DNA sequencing was performed at the W.M. Keck Foundation (Yale University, New Haven, CT). All *AtELP* DNA fragments were inserted into pGEX vectors (Pharmacia).

Generation and Purification of GST-Fusion Protein and Preparation of Antibodies

The plasmid pSA6 was constructed to produce an in-frame fusion between GST and the C-terminal half of the open reading frame of AtELP (amino acids 323–623). *Escherichia coli* (strain DH5α) cells containing pSA6 were grown in 100 mL of LB media at 37°C to A_{600} = 0.8 to 1.0. Protein expression of the GST-fusion construct was induced by adding 0.2 mM isopropylthio-β-galactoside and shifting the cell culture to 28°C. The soluble GST-fusion protein was purified by affinity chromatography as described previously (Bar-Peled and Raikhel, 1996). The fusion protein (approximately 100 μg) was emulsified with Titer-Max (CytRx, Norcross, GA) in a total of 1 mL and injected into rabbits. The rabbits were boosted three more times each with 50 μg of GST-fusion proteins emulsified with Titer-Max. Other antibodies used in this study were anti-AtSEC12 sera (Bar-Peled and Raikhel, 1997), anti-RD28, and anti-γ-TIP sera (kindly provided by Maarten Chrispeels, University of California, San Diego), anti-clathrin heavy-chain sera (kindly provided by Tony Jackson, University of Cambridge, UK), and anti-β-adaptin sera (kindly provided by Margaret Robinson, University of Cambridge, UK).

Northern Analysis

RNA was isolated from various tissues (Fig. 2C) of *A. thaliana* ecotype Columbia as described previously (Bar-Peled et al., 1995). Equal amounts of RNA were fractionated on a 6% formaldehyde/1% agarose gel, blotted, and probed with [α-^{32}P]dATP (NEN)-labeled DNA fragments from various regions of the *AtELP* cDNA that were generated with the Klenow fragment of DNA polymerase (Boehringer Mannheim) and random hexanucleotide primers.

Protoplast Preparation and Homogenization

Protoplasts were isolated from 4-d-old *A. thaliana* cell-suspension cultures essentially as described before (Bar-Peled and Raikhel, 1997) with minor modifications. Five milliliters of drained cells from two flasks of 4-d-old *A. thaliana* cell-suspension cultures was collected on a 94-μm filter. The cells were incubated with 15 mL of freshly made protoplasting solution (15.4% [w/v] Suc, 0.32% [w/v] Gamborg's B-5 minimal organics, with the addition of 100 mg caylase 345 L, and 15 mg of pectolyase Y-23) at room temperature for about 3 h on a rotary shaker (60 rpm). The treated suspension was filtered through a 94-μm metal screen, and the volume of the filtrate containing protoplasts was adjusted to about 40 mL with 15.4% (w/v) Suc and 0.32% (w/v) Gamborg's B-5 minimal organic solution. The filtrate was poured into Babcock centrifuge bottles, and protoplasts were separated from broken cells after centrifugation (1100 rpm in a HNSII clinical centrifuge swinging bucket rotor [IEC, Needham Heights, MA] for 10 min at 25°C). The floated protoplast band was collected and transferred to a centrifugation tube containing 20 mL of 0.4 M betaine, 3 mM Mes, and 10 mM $CaCl_2$, pH 5.7. Protoplasts were pelleted (for 10 min at 50*g* at room temperature), resuspended in 4 mL of cold lysis buffer containing 20 mM Hepes-KOH, pH 7.1, 13.5% (w/v) Suc, 10 mM potassium acetate, 1 mM DTT, and 0.5 mM PMSF. EDTA (1 mM) or $MgCl_2$ (3 mM) were added when indicated. Protoplasts were lysed gently by at least eight passages through a 25⅝-gauge needle and lysis was confirmed by microscopy.

Total Protein Isolation

One milliliter of packed protoplasts was passed eight times via a syringe equipped with a 25⅝-gauge needle. Five milliliters of drained cell-suspension cultures or plant tissues (up to 1 g) was ground on ice in a mortar with a pestle in the presence of 100 mg of acid-washed glass beads. Total protein was then extracted by further grinding with 4 mL of cold lysis buffer (see above) containing 1 mM DTT and 0.5 mM PMSF. Where indicated, 3 mM $MgCl_2$ was included in the lysis buffer and EDTA was omitted. Some samples were placed at 4°C on a shaker for up to 15 min, while others were extracted. Samples were further lysed by passing the homogenate 8 times through a 25⅝-gauge needle and filtered through three layers of Miracloth (25 μm; Calbiochem) to remove unbroken cells. The homogenate was termed total crude homogenate.

Differential and Suc Density Gradient Centrifugation

The total crude homogenate was further centrifuged for 10 min at 1,000*g* (4°C), and the top 3.5 mL of supernatant (termed S1) was saved. Total microsomes were prepared by centrifuging this postnuclear S1 fraction (for 60 min at 125,000*g*, r-max), generating a total membrane pellet (pellet at 125,000*g* = P125) and a soluble protein fraction (supernatant at 125,000*g* = S125). For differential centrifugation experiments, the S1 fraction was further centrifuged at 3,000*g*, generating a supernatant (S3) and pellet (P3) fraction. The S3 fraction was centrifuged again at 8,000*g* for 20 min, generating a supernatant (S8) and pellet (P8) fraction. The S8 fraction was centrifuged again for 20 min at 14,000*g* to generate S14 and P14 fractions. The S14 fraction was then centrifuged for 30 min at 55,000*g* to generate the S55 and P55 fractions. The S55 fraction was finally centrifuged (for 1 h at 125,000*g*) to generate the S125 and P125 fractions. All pellets (P1, P3, P8, P14, P55, and P125) were rinsed briefly with 1 mL of lysis buffer and further resuspended in lysis buffer containing 150 mM NaCl, 1% Triton X-100, and 1% sarkosyl. After 15 to 30 min, the solubilized pellets (P1, P3, P8, P14, P55, and P125) were spun for 15 min at 125,000*g* (4°C) to remove undissolved matter, and the supernatant was saved for analysis.

For separation of endomembrane organelles based on their densities, protoplasts were lysed in a buffer containing 1 mM EDTA (S1E) or 3 mM $MgCl_2$ (S1M). After the samples were centrifuged (at 1,000*g* and 4°C for 10 min), the supernatants in each homogenization buffer (S1E or S1M) were collected and applied either to linear 16 to 55% (w/v) Suc gradient systems or to step Suc gradients. The linear Suc gradients were buffered in 10 mM Hepes-KOH (pH 6.9) and 10 mM potassium acetate, containing either 2 mM EDTA or 5 mM $MgCl_2$, and were prepared in 12-mL Beckman ultraclear thin tubes that fit the SW40Ti rotor. The step 16 to 55% Suc gradients were made from stock solutions containing 55, 40, 33.5, 26.5, and 16% (w/v) Suc in the above buffer containing EDTA or $MgCl_2$ and were prepared by sequential layering of the following stocks into 12-mL Beckman ultraclear thin tubes: 0.75 mL of 55% Suc solution, three 0.97-mL aliquots of 40% Suc, three 0.77-mL aliquots of 33.5% Suc, two 1-mL aliquots of 26.5% Suc, and two 0.75-mL aliquots of 16% Suc solution. Three milliliters of either S1E or S1M was layered on top of the 9-mL linear or step Suc gradient, which was made in the presence of 1 mM EDTA or 3 mM $MgCl_2$, respectively. Gradients were centrifuged for 2 h at 150,000*g* in a Beckman SW40Ti rotor at 4°C. Fractions were collected from the top and the Suc concentration was determined by measurement of the refractive index. Aliquots (60 μL) of the fractions were separated by SDS-PAGE and the distribution of AtELP and other marker proteins was analyzed by immunoblotting.

Golgi Marker Enzyme Assay

The assay for the activity of the Golgi marker enzyme xyloglucan 1,2-α-L-fucosyltransferase was carried out essentially as described previously (Hanna et al., 1991). The standard assay system consisted of 200 μL of a buffer containing 25 mM Pipes-KOH, pH 6.5, 2 mM $MgCl_2$, 0.5 mg mL^{-1} purified soluble tamarind xyloglucan, 0.5% Triton X-114, and 0.7 mM GDP-[^{3}H]Fuc (740 Bq/fraction), and 100 μL of a protein sample solubilized with Triton X-114 (final 1%) from fractions of the Suc density gradients that were prepared in the presence of EDTA. The mixed enzyme reactions were allowed to proceed at room temperature for 30 to 120 min and were then terminated by the addition of 0.5 mL of 95% (v/v) ethanol to a final concentration of 70%. After chilling for 1 to 2 h on ice, the reaction mixtures were centrifuged at 14,000*g* and room temperature to pellet the

radiolabeled products. The resulting insoluble pellet was washed twice with 1.2 mL of 70% (v/v) ethanol for 2 to 3 min to remove unreacted substrate and its breakdown products. The amount of ^{3}H-fucosylated xyloglucan product was assayed by liquid scintillation spectroscopy. The activity (in disintegrations per minute) of the enzyme in each Suc fraction was measured after subtraction of blanks lacking enzyme performed with each assay.

Isolation and Purification of CVs from Developing Pea Cotyledons

Pea CCVs were isolated and purified as described previously (Harley and Beevers, 1989; Lin et al., 1992). Pea pods from field- or greenhouse-grown plants were harvested and the developing seeds (150 g) were collected and either used fresh or stored at −80°C until use. The final CV preparation consisted of a green pellet (CVI) and a yellow-green region (CVII) above the pellet. CVs recovered from each of the regions were diluted and pelleted after further centrifugation at 120,000*g* for 1 h in a Beckman 70Ti rotor. The suspended CVs of each fraction were aliquoted and frozen at −70°C for further analysis by immunoblotting.

SDS-PAGE and Immunoblotting

Proteins were quantified according to the method of Bradford (1976) using BSA as a standard. Protein molecular weight standards used as markers were purchased from Bio-Rad (broad-range marker, catalog no. 72807A). Proteins (30–50 μg/lane) were separated on either 10 or 12% modified Laemmli-reduced SDS-PAGE (Bar-Peled et al., 1991), and transferred to a nitrocellulose membrane (0.45 μm), in 10 mM Tris, 100 mM Gly, 0.05% SDS, and 10% methanol. Blots were stained with Ponceau S, incubated with blocking solutions (5% [w/v] milk powder in 1× TBS and 0.1% Tween 20) for 2 to 12 h, reacted with primary antibody at 1:100 to 1:500 dilution, washed, and then reacted with secondary antibody (1:3000) conjugated to alkaline phosphatase. Immune complexes were detected by color assay using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrates.

RESULTS

Identification of an Arabidopsis cDNA Encoding an EGFR-Like Protein

In animal cells Cys-rich regions have been suggested to be involved in receptor-ligand interactions at the cell surface during CCV-mediated endocytosis (Yamamoto et al., 1984; Lalazar et al., 1988). The mammalian EGFR is a large integral membrane protein containing several Cys-rich repeats (called EGF repeats) in the extracellular domain that are involved in interactions with its ligand (for review, see Carpenter, 1987). A specific pattern of Cys residues, termed class A or B Cys-rich repeats, has been identified within these regions of the EGFR, LDLR, and related proteins (Stanley et al., 1986; Herz et al., 1988). Class B repeats fall into two subclasses (B.1 and B.2) based on the position of the Cys residues. We therefore decided to search for proteins containing the highly conserved small peptide sequence of NNGGC present in the class B.2 Cys-rich EGF repeats of the mammalian LDLRs and related proteins (Herz et al., 1988).

A search of the Arabidopsis EST database using the Motif Explorer program resulted in the isolation of two contiguous cDNAs that were fused to construct a 2314-bp clone (*AtELP*) containing an open reading frame of 623 amino acids with a predicted molecular mass of approximately 69 kD (Fig. 1A). Southern analysis and PCR amplification of genomic DNA with primers specific to different regions of the two contiguous cDNAs and northern analyses of RNA prepared from *A. thaliana* that were probed with various regions of the two cDNAs indicated the presence of a single band of the expected size corresponding to the cDNAs (data not shown). Hydropathy analysis (Kyte and Doolittle, 1982) of the predicted amino acid sequence (Fig. 1B) indicated the presence of two hydrophobic regions (underlined in Fig. 1A). The first region at the N

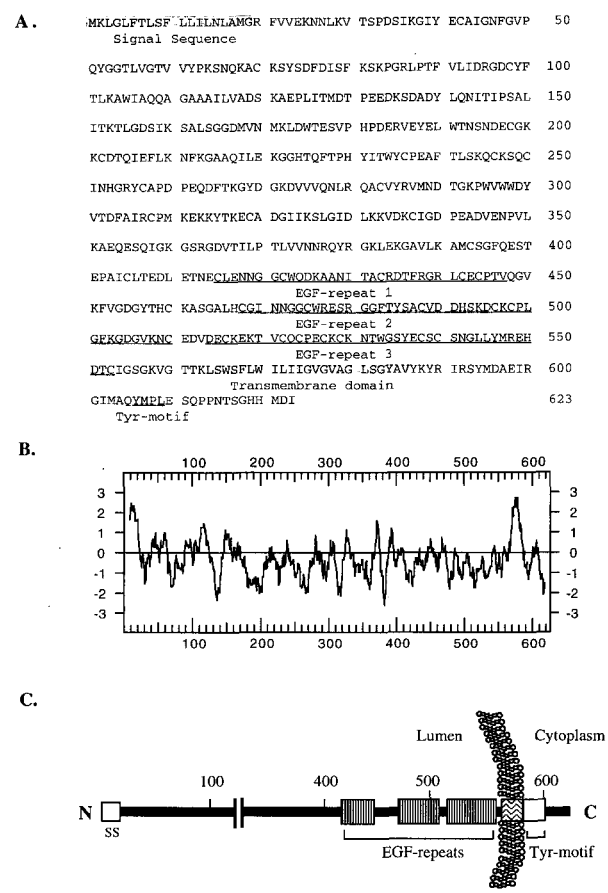

Figure 1. Predicted sequence and structure of the AtELP gene product. A, The predicted amino acid sequence of the 623-residue polypeptide encoded by *AtELP* (GenBank accession no. U86700). The predicted N-terminal signal sequence and the transmembrane domain are shaded, whereas EGF repeats and the cytoplasmic Tyr motif are underlined. B, Hydropathy analysis of the predicted amino acid sequence of AtELP using the method of Kyte and Doolittle (1982). C, Predicted structural organization of the different domains of AtELP. SS, Signal sequence.

terminus, amino acids 1 to 19, encodes a potentially cleavable signal sequence for ER import as analyzed by the Signal P program (Von Heijne, 1986; Nielson et al., 1997). The second region starts approximately 60 residues upstream of the C terminus (amino acids 564–587) and contains residues characteristic of membrane-spanning regions (Rao and Argos, 1986). Based on these observations, the cDNA was predicted to encode a type I integral membrane protein.

The rest of the AtELP protein is organized into domains (Fig. 1C) that resemble those of the EGFR and LDLRs and their related proteins (Russell et al., 1984; Herz et al., 1988). A Cys-rich (15%) region with identity to the class B Cys-rich motifs of these mammalian receptors is found between amino acids 415 and 553 of AtELP. This presumed lumenal portion of the protein consists of three tandemly repeated EGF-like motifs (underlined in Fig. 1A) that share identity (40%) with the class B (or EGF-like) motifs containing six Cys residues in a span of 40 amino acids (Herz et al., 1988). A potentially *O*-glycosylated region containing Ser and Thr residues (amino acids 556–563) is present between the third EGF repeat and the transmembrane domain (Fig. 1A). This corresponding domain of the LDLR has many clustered *O*-linked carbohydrate chains (Russell et al., 1984). In addition, there are three *N*-glycosylation sites present in the putative lumenal portion of the protein.

The sequence of the predicted cytoplasmic domain of AtELP has similarity to the yeast Vps10 protein (Marcusson et al., 1994). Within this region of the protein are two Tyr motifs (Fig. 1, A and C). The first Tyr-containing region (amino acids 589–600) shares homology with a Tyr-rich region that serves as a sorting signal in several membrane receptors involved in protein trafficking, such as the yeast CPY vacuolar receptor (Vps 10p; Lin Cereghino et al., 1995; Cooper and Stevens, 1996) and the mammalian M-6-PR (Rohrer et al., 1995). The second Tyr-containing region found between 606 and 609 resembles the Yxxϕ motif found on receptors that interact with the PM or TGN adaptor protein complexes during the formation of CCVs (Glickman et al., 1989; Ohno et al., 1995). The structural organization of these motifs in the deduced amino acid sequence encoded by the *AtELP* cDNA indicates that it encodes an EGFR-like protein that may be involved in clathrin-dependent intracellular protein trafficking in plants.

AtELP Is Membrane Associated and Conserved between Monocots and Dicots

To characterize the protein encoded by the *AtELP* cDNA, the C-terminal half of the protein (amino acids 363–623) was expressed as a GST-fusion protein in *E. coli*. The GST-fusion protein was purified by affinity chromatography over a GSH-Sepharose column and was used to raise a rabbit antiserum. To investigate the tissue specificity of the encoded protein, the serum was used to probe extracts from various Arabidopsis tissues (roots, leaves, stems, flowers, and siliques) and cell-suspension cultures. Three proteins of approximately 80 kD were detected by the serum in each tissue examined on immunoblots (Fig. 2A).

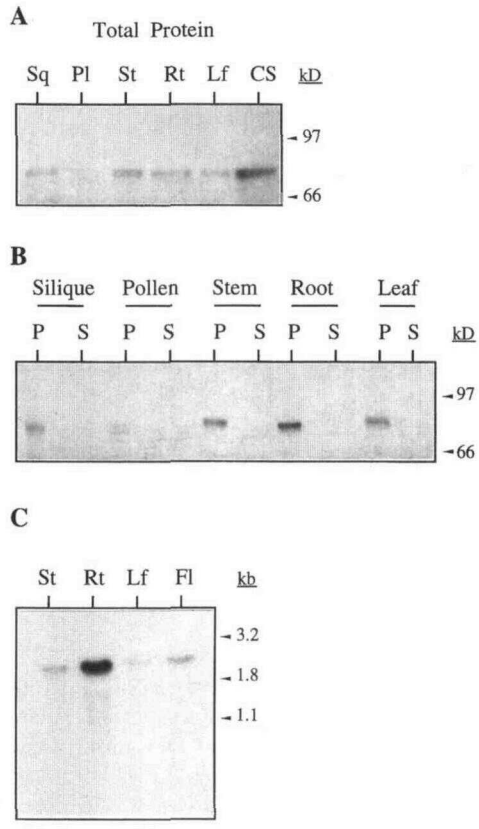

Figure 2. Distribution of AtELP in *A. thaliana* tissues. A, Total protein extracted from green *A. thaliana* siliques (Sq), pollen (Pl), or stems (St) of mature plants, roots (Rt), or leaves (Lf) from 2-week-old plants, or *A. thaliana* cell-suspension culture (CS). Equal amounts (50 μg) of proteins were separated on SDS-PAGE and immunoblotted with anti-AtELP antibodies. B, Total protein extracted from the tissues in A was further fractionated by centrifugation at 125,000*g* to generate total membrane (P) and soluble (S) fractions. Western blots were probed with anti-AtELP antibodies. The size of AtELP is about 80 kD by comparison with molecular mass standards, indicated on the right. C, Northern-blot analysis of the AtELP transcript. Total RNA (30 μg) from stems (St), roots (Rt), leaves (Lf), and flowers (Fl) was separated in an agarose/formaldehyde gel and blotted onto a nylon membrane. The membrane was hybridized with a ^{32}P-labeled fragment of the cDNA containing the AtELP open reading frame. Under high-stringency conditions using RNA from the tissues described, a single band of about 2.3 kb was found to hybridize with the probes made from different regions of the *AtELP* cDNA. Note that the AtELP antisera detected three polypeptides in the range of 80 kD. For a better resolution, please refer to Figures 5 and 6.

We were not able to reduce the relative intensity of any of the three proteins detected using protease inhibitors (data not shown), suggesting that they were unlikely to be degradation products of the same protein. Although the predicted molecular mass of the mature protein (amino acids 20–623) was approximately 67 kD, its apparent mass of 80 kD on immunoblots may be the result of posttranslational modifications of the same protein. Alternatively, the three polypeptides could represent homologs of the AtELP protein. Consistent with the later possibility, during the *A. thaliana* database search that identified *AtELP*, we identified three additional partial EST clones sharing homology

with *AtELP* between amino acids 300 and 460 (EST clone nos. T42090, T22799, and R29853).

Hydropathy analysis of the deduced amino acid sequence of *AtELP* (Fig. 1B) predicted that it is a membrane protein. To confirm this prediction, the intracellular distribution of AtELP was examined by isolating microsomal fractions at 125,000*g* and by immunoblot assays of proteins from total soluble (S) and total membrane pellet (P) fractions from various Arabidopsis tissues. The AtELP antibody detected proteins only in membrane-containing fractions, suggesting that AtELP is a membrane-associated protein. The amount of AtELP in microsomal fractions prepared from roots and rapidly dividing cell-suspension cultures was higher than in leaves, stems, siliques, or pollen, reflecting the distribution of its RNA as found by northern-blot analysis (Fig. 2C). This expression pattern (i.e. highest level in roots compared with leaves, stems, or flowers) appears to be similar to that of a number of other proteins believed to be involved in the secretory pathway in plants, such as AtERD2, AtSAR1 (Bar-Peled et al., 1995; Bar-Peled and Raikhel, 1997), and AtPEP12 (Bassham et al., 1995; Conceição et al., 1997) .

Using the *AtELP* sequence, we found homologs of AtELP in the rice (*Oryza sativa*) and maize (*Zea mays*) EST databases. The partial rice EST clone (no. D40971) shared homology with AtELP in the second EGF motif, whereas the partial maize EST clone (no. T18301) had homology in the EGF motif, the transmembrane domain, and the cytoplasmic tail containing the Tyr motif (Figs. 1A and 3A). To examine the expression of AtELP homologs in monocots and dicots, microsomal fractions were prepared from roots of Arabidopsis, tobacco, pea, maize, and rice (Fig. 3B). In all of the species tested, three cross-reactive proteins of approximately 80 kD were detected with the AtELP antisera similar to the results in *A. thaliana*. However, a weak cross-reacting band of the correct size (approximately 80 kD) was detected in rice with the AtELP antisera, even though the same amount of protein was loaded on the gel. In addition, some other small cross-reacting proteins were detected by the serum in tobacco and pea. This indicates that AtELP may be conserved between monocots and dicots, suggesting an important role for the protein.

A

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AtELP       CSGFQESTEP AICLTEDLET NECLENNGGC WQDKAANITA CRDTFRGRLC 443
Rice  EST   -A--R-T--- -V--S--IQ- ---------- --------S- -K----
AtELP       ECPTVQGVKF VGDGYTHCKA SGALHCGINN GGCWRESRGG FTYSACVDDH 493
Maize EST                                             T-P- K-I---SNEI
AtELP       SKDCKCPLGF KGDGVKNCED VDECKEKTVC QCPECKCKNT WGSYECSCSN 543
Maize EST   -EG----V-- ----E-S--- I---Q--LY- --KG-S---- --------GD
AtELP       *GLLYMREHD TCIGSGKVGT TKLSWSFLWI LIIGVGVAGL SGYAVYKYRI 592
Maize EST   DNM------- ---SKEGTA- -VG*-----V IFF-LVF--V GHTL------
AtELP       RSYMDAEIRG IMAQYMPLES QPPNTSGHHM DI                    623
Maize EST   -----S---A --------DN -
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B

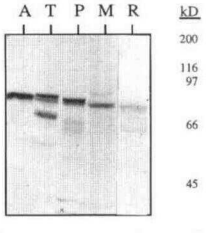

Figure 3. Homologs of AtELP are present in dicots and monocots. A, Amino acid sequence comparison between AtELP and EST clones from the rice and maize databases. Horizontal lines represent residues identical with AtELP. Asterisks mark gaps introduced into the sequence to allow maximum homology. B, Immunoblot analysis of AtELP homologs in dicots and monocots. Equal amounts of microsomal fractions prepared from roots of *A. thaliana* (A), tobacco (T), pea (P), maize (M), and rice (R) were analyzed by immunoblotting with anti-AtELP antibodies.

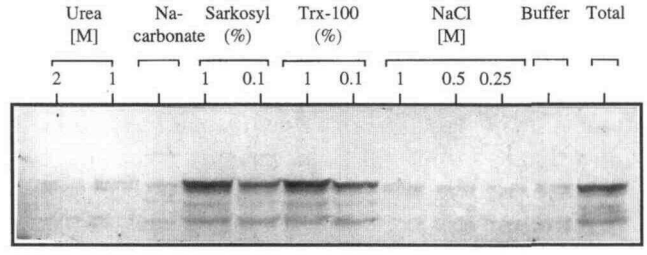

Figure 4. AtELP is an integral membrane protein. Microsomal fractions were prepared from a postnuclear (S1) fraction by centrifugation at 125,000*g* and resuspended in various buffers for 30 min on ice. The supernatants containing the resuspended pellets were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-AtELP antibodies. Microsomes were resuspended in lysis buffer containing 1 or 2 M urea (Urea); only 0.1 M sodium carbonate (Na-carbonate), pH 11.0; lysis buffer containing 1 and 0.1% Sarkosyl; 1 and 0.1% Triton X-100 (Trx-100); 1, 0.5, and 0.25 M NaCl; lysis buffer alone (Buffer), or lysis buffer containing 150 mM NaCl, 1% Sarkosyl, and 1% Triton X-100 (Total). Protein samples were analyzed on a 12% SDS-PAGE gel instead of a 10% gel as used for all other experiments. The additional bands toward the bottom of the gel are likely to be degradation products of AtELP.

AtELP Is an Integral Membrane Protein Found in a Novel Compartment

To confirm our prediction that AtELP is an integral membrane protein, various conditions and treatments to extract the protein from the microsomal pellet were examined. Equivalent amounts of microsomal protein pellet (P125) were resuspended in lysis buffer alone (Fig. 4), in lysis buffer containing up to 2 M urea, in 0.1 M Na_2CO_3, in lysis buffer containing the ionic or nonionic detergents Sarkosyl or Triton X-100 at 0.1 and 1%, respectively, in lysis buffer supplemented with NaCl at 1, 0.5, and 0.25 M (Fig. 4), and in lysis buffer containing Sarkosyl, Triton X-100, and NaCl. After incubation on ice for 30 min, the mixtures were centrifuged at 125,000*g*. The content of AtELP in the resulting supernatants was determined by immunoblotting assays after SDS-PAGE. Treatments known to be effective in extracting proteins peripherally associated with membranes (urea, salt, and alkaline conditions; Fujiki et al., 1982) were not effective in extracting AtELP from the membranes. Only nonionic (Triton X-100) or ionic (Sarkosyl) detergents extracted AtELP from the pelleted membranes (Fig. 4), indicating that AtELP was an integral membrane protein.

To determine the association of AtELP with specific cell membranes, subcellular fractionation studies were first car-

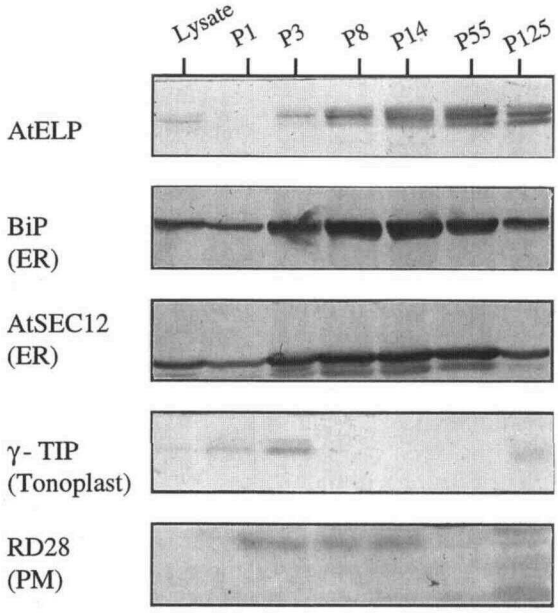

Figure 5. Subcellular fractionation of AtELP. Protoplasts isolated from *A. thaliana* cell-suspension culture were lysed and fractionated by differential centrifugation as described in "Materials and Methods." The lysate (total protein) was first subjected to a low-speed centrifugation at 1000*g*, yielding a pellet (P1) and a supernatant fraction that was subjected to further centrifugation at 3,000*g*, 8,000*g*, 14,000*g*, 55,000*g*, and 125,000*g*, with the supernatant from each spin taken for use in the next centrifugation step. These differential centrifugation steps resulted in a P1, P3, P8, P14, P55, and P125 pellet, respectively. Equal amounts of protein (50 μg) from each of the pellet fractions were separated by SDS-PAGE, transferred to nitrocellulose, and cut into strips, which were probed with antibodies against the organelle-specific proteins BiP and AtSEC12 (ER), γ-TIP (tonoplast), and RD28 (PM).

ried out using differential centrifugation, which allowed the separation of membrane-bound organelles based on their mass or density. Protoplasts were generated from actively dividing *A. thaliana* cell-suspension cultures and gently lysed to minimize the disruption of the endomembrane system during the homogenization process. Lysates were fractionated by differential centrifugation into supernatant (S) and pellet (P) fractions. The relative amounts of AtELP as well as marker proteins for various organelles were determined by immunoblot analysis. As shown in Figure 5, most of the AtELP protein was recovered in the P55 fraction (pellet fractionated at 55,000*g*), with lesser amounts in both low-speed (P8 and P14) and high-speed (P125) pellet fractions. On the other hand, the marker proteins for ER (BiP, Denecke et al., 1991; AtSEC12, Bar-Peled and Raikhel, 1997), tonoplast (γ-TIP, Gomez and Chrispeels, 1993), and PM (RD28, Daniels et al., 1994) were found mostly in the lower-speed pellet fractions (P1, P3, P8, P14). Thus, AtELP had a different fractionation pattern from these endomembrane markers, suggesting that it was not localized in these organelles.

To gain additional information about the intracellular location of AtELP, subcellular fractionation studies were next carried out by equilibrium buoyant density centrifugation using the postnuclear fraction (supernatant from a 1000*g* centrifugation) of protoplasts. This technique separates organelles on the basis of their inherent buoyant density differences and has been used to resolve the ER, mitochondria, PM, vacuole, and Golgi complex in animal, yeast, and plant cells (Walworth and Novick, 1987; Beaumelle and Hopkins, 1989; Dewitt et al., 1996; Bar-Peled and Raikhel, 1997).

Each fraction of the Suc gradient was examined for the presence of AtELP and the endomembrane marker proteins γ-TIP, AtSEC12, and RD28 by immunoblot analysis. Enzyme activity for xyloglucan 1,2-α-L-fucosyltransferase was also used as a marker for the Golgi (Hanna et al., 1991). In step Suc density gradients (Fig. 6A–C), the vacuolar membrane (tonoplast)-specific marker γ-TIP fractionated at the top of the Suc gradient and peaked at approximately 16% Suc (fraction 3), as expected. The activity of the Golgi enzyme marker fucosyltransferase and the majority of the ER-membrane marker AtSEC12 both peaked at approximately 30% Suc, corresponding to fractions 8 and 9, respectively. The pattern of distribution of AtELP in the presence or absence of Mg^{2+} was different from the tonoplast, ER, and Golgi markers. Significant amounts of AtELP were found in less-dense fractions of the gradients (nos. 4 and 5) corresponding to approximately 26.5% Suc. In addition, AtELP was found in denser fractions, which contained the ER (nos. 8, 11, and 15) and Golgi (no. 8) markers. Characteristic of ER proteins (Lord, 1987; Bar-Peled and Raikhel, 1997), the AtSEC12 peak shifted completely from fractions 7 and 8 to a higher Suc density corresponding to fractions 11 and 15 in the presence of Mg^{2+} (Fig. 6B). However, in the presence of Mg^{2+}, the pattern of distribution of AtELP did not shift in the same manner as AtSEC12 (Fig. 6, A and B), although an increase in the amount of AtELP was observed in fraction 15. Much of AtELP appeared to remain in fractions 4 to 8, as found in the presence of EDTA. These results suggest that a major population of AtELP in Arabidopsis does not co-fractionate with the ER, Golgi, or vacuolar membrane markers.

Because we were unable to resolve the PM from other endomembranes in the step Suc gradients described in Figure 6, A and B, using the RD28 marker protein, a separate linear Suc density gradient was used to investigate the localization of AtELP to the PM. On linear Suc density gradients (Fig. 6D), the majority of the PM marker RD28 peaked at approximately 40% Suc (fractions 10 and 11), whereas the peak for AtELP was found in a less-dense fraction (nos. 7, 8, and 9). Since AtELP had a different fractionation pattern than the available endomembrane markers for the tonoplast, ER, Golgi, or PM, we therefore propose that it is associated with an as yet undefined intermediate compartment that is heavier than the vacuole and lighter than the ER, Golgi, or PM.

AtELP also Appears to Be Enriched in CCVs

The structural organization of the motifs present in the deduced amino acid sequence of the *AtELP* cDNA suggested that it encoded an EGFR-like protein that may be involved in clathrin-mediated intracellular protein sorting.

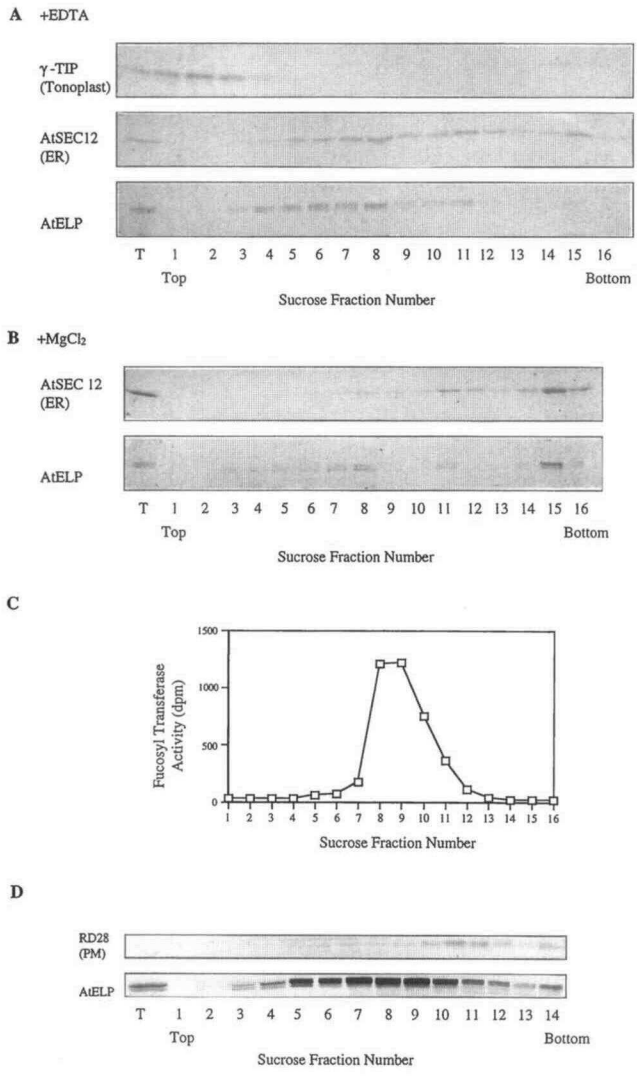

Figure 6. Suc density fractionation of AtELP. Protoplasts were made from *A. thaliana* suspension culture cells, resuspended in lysis buffer containing either 1 mM EDTA (A, C, and D) or 3 mM $MgCl_2$ (B), and then lysed. The homogenates were centrifuged at 1000*g* to remove nuclei, and the supernatant was loaded on a 16 to 55% step (A, B, and C) or a linear 16 to 55% (D) Suc gradient. After centrifugation, fractions were collected from the top to the bottom of the gradients, and 60-μL aliquots from each fraction were separated on SDS-PAGE. Proteins were transferred to nitrocellulose membrane and immunoblotted (A, B, and D) with antibodies against organelle-specific proteins as in Figure 5. The activity of the Golgi marker enzyme xyloglucan fucosyltransferase in each fraction was measured separately and expressed as disintegrations per minute per fraction and plotted (C).

Whereas *A. thaliana* was found to be a poor source of CCVs (M. Bar-Peled, S. Ahmed, and N.V. Raikhel, unpublished), developing pea cotyledons are a recognized source of these CVs (Lin et al., 1992; Demmer et al., 1993). Furthermore, the AtELP antiserum was found to cross-react with a protein of approximately 80 kD in microsomal fractions prepared from pea roots (Fig. 3B). To examine the association of AtELP with CCVs we prepared CVs that were enriched for CCVs from developing pea cotyledons. The protein profile of the purified CVs on SDS-PAGE was very similar to that reported by others in plants (Lin et al., 1992; Demmer et al., 1993; Blackbourn and Jackson, 1996), with a dominant protein of 190 kD and others of 130 to 140, 100 to 120, and 50 to 55 kD. The 190-kD band corresponding to the clathrin heavy chain (see below) was particularly enriched in the CVII fraction when compared with the CVI fraction or total protein (Fig. 7A). Immunoblotting with antibodies to the soybean clathrin heavy chain (Blackbourn and Jackson, 1996) confirmed that the 190-kD band in the pea CV preparation was the clathrin heavy chain (Fig. 7B). In addition, a 116-kD band corresponding to the plant clathrin-associated β-adaptin-type protein was also enriched in these CVs (CVII) and detected by antibodies to the mammalian β-adaptin (Robinson, 1987). In a similar manner, the AtELP homolog in pea was enriched in the CV preparations that were rich in clathrin and its associated adaptor protein. Surprisingly, the CVI fraction did not contain β-adaptin, although it contained clathrin and AtELP. However, other plant homologs of the mammalian clathrin-associated adaptor proteins of the AP1 or AP2 complex

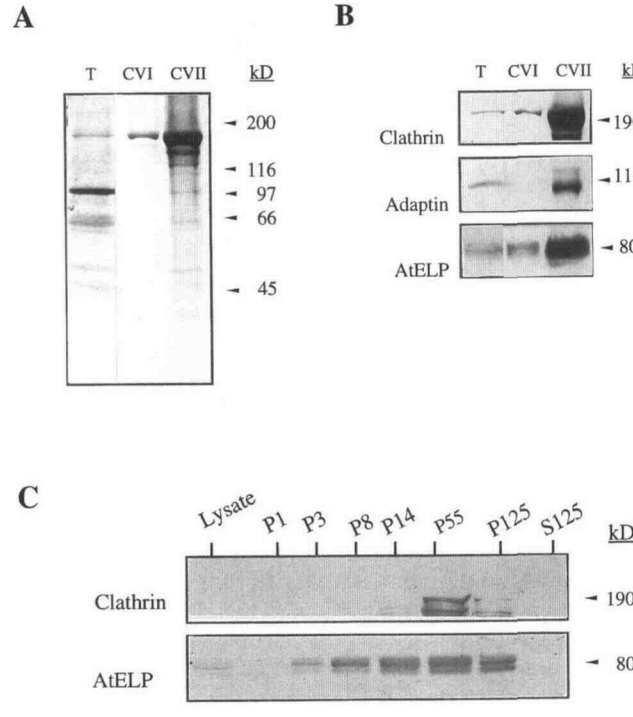

Figure 7. Enrichment of AtELP in CCVs. CV preparations from developing pea cotyledons enriched for CCVs were analyzed by SDS-PAGE. A, Coomassie brilliant blue stain of equal volumes of total homogenate (T) and fractions CVI and CVII following electrophoresis. The molecular mass marker is on the right. B, Western blot from A was probed with anti-AtELP, clathrin, and β-adaptin antibodies. The sizes of the organelle marker proteins were determined by comparison to molecular mass standards. C, Pellet fractions from differential centrifugation experiments in *A. thaliana* as described in Figure 5 were separated on SDS-PAGE and analyzed by immunoblotting with anti-AtELP and anti-CHC antibodies. Each lane (lysate, P1, P3, P8, P14, P55, and P125) contains samples identical to those in Figure 5, in addition to a supernatant from the 125,000*g* centrifugation step (S125).

may be present in this fraction that could not be detected with the β-adaptin antisera.

Differential centrifugation experiments used to determine the association of AtELP with specific cellular membranes (Fig. 5) in *A. thaliana* cell-suspension cultures indicated that the protein was associated with both low-speed (8,000*g* and 14,000*g*) and high-speed (55,000*g* and 125,000*g*) pellet fractions. To determine the association of clathrin with any of these fractions, subcellular fractions described in Figure 5 were probed with anti-AtELP and anti-CHC antibodies (Fig. 7C). Although clathrin was found mostly in the high-speed (55,000*g* and some in the 125,000*g*) pellet fractions together with AtELP, it was not detectable in the low-speed (8,000*g* or 14,000*g*) pellet fractions, where significant amounts of AtELP were also present. These results, taken together with the membrane association and subcellular fractionation data, suggest that AtELP may be present in the membranes of CCVs, in addition to the intermediate compartment described above.

DISCUSSION

In this study we have used a new method for the isolation of a gene(s) that may be involved in the plant secretory pathway by searching the EST databases with known functional motifs present in receptor proteins involved in intracellular protein trafficking in other systems. A computer search carried out with the entire protein sequences of many of these receptors (e.g. M-6-PR, Vps10p, LDLR, EGFR) was unable to identify any homology in the plant databases. However, the use of a short, highly conserved sequence of NNGGC identified a group of cDNAs from both the *A. thaliana* and *O. sativa* databases. Here we have described a unique, approximately 80-kD EGFR-like type I integral membrane protein from *A. thaliana*, AtELP. The amino acid sequence derived from the *AtELP* cDNAs shows the presence of several sequence motifs found primarily in mammalian and yeast integral membrane receptor proteins that are involved in intracellular protein sorting along the secretory and endocytic pathways. The AtELP protein appears to have four functional regions: (a) a cleavable N-terminal signal peptide; (b) a lumenal region containing three Cys-rich domains that shows homology to the EGF repeats; (c) a transmembrane segment; and (d) a cytoplasmic tail that contains two potential Tyr motifs that could be required for clustering into CCVs or for Golgi retention/retrieval.

Cys-rich domains and/or EGF-like repeats have been found in the yeast vacuolar sorting receptor Vps10p (Marcusson et al., 1994), the human EGFR and LDLRs and their related proteins (Herz et al., 1988), and the thrombomodulin receptor (Wen et al., 1987). The Cys-rich domains in the Vps10 protein may be involved in ligand binding (Horazdovsky et al., 1995), whereas in the LDLR they are believed to bind its ligands and apolipoproteins B and E (Yamamoto et al., 1984). In addition to the well-characterized EGF-EGFR interaction, in at least three cases (thrombomodulin, urokinase, and Notch), the EGF repeats participate directly in protein-protein interactions (Apella et al., 1987, 1988; Kurosawa et al., 1988; Rebay et al., 1991). The only transmembrane protein with EGF repeats described in plants, Wak1, was identified recently in *A. thaliana* as a cell wall-associated receptor-like protein kinase (He et al., 1996). The extracellular domain of the Wak1 protein contains several EGF repeats and may serve as a physical connection between the extracellular matrix and the cytoplasm (He et al., 1996). It is therefore possible that the EGF-like regions in AtELP may also be responsible for interaction with a ligand.

The two Tyr-containing regions in the putative cytoplasmic portion of AtELP may function as signals for targeting the protein. Tyr-based signals are important for the targeting, stability, and function of a number of receptor proteins in both the mammalian and yeast secretory pathways (Bos et al., 1993; Lin Cereghino et al., 1995; Rohrer et al., 1995; Cooper and Stevens, 1996). The cytoplasmic tails of these membrane receptors contain Tyr-based sorting signals (Yxxϕ, where Y represents a Tyr, x any amino acid, and ϕ a hydrophobic amino acid) that allow the recruitment of the adaptor proteins to form the coated pits (for review, see Sandoval and Bakke, 1994).

During receptor-mediated endocytosis in animal cells, cytoplasmic Tyr-based sorting signals of cell-surface receptors associate with a cytosolic adaptor protein complex (AP2, consisting of α-adaptin, β2-adaptin, μ2, and σ2) at the PM. Subsequent interaction of the receptor-adaptin complex with clathrin initiates the formation of CCVs (Pearse and Robinson, 1990). These vesicles then bud from the PM and fuse with the endosomal membrane, where the receptor is delivered for further transport to the lysosome for degradation or recycling back to the PM.

In addition, some Tyr motifs have been found to be important for the retention of membrane proteins at the TGN in mammalian and yeast cells (Bos et al., 1993; Voorhees et al., 1995; Wilcox et al., 1992), and, most recently, a Tyr-based targeting signal has been shown to mediate the sorting of an integral membrane glycoprotein into Golgi-derived CCVs (Höning et al., 1996). These cytoplasmic Tyr motifs interact with other components of the sorting machinery, such as the clathrin-associated AP1 adaptor protein complex (β1-adaptin, γ-adaptin, μ1, and σ1) at the TGN. The requirement for multiple Tyr signals and their interaction with adaptor proteins at the late-Golgi has been shown in both animal and yeast cells during lysosomal or vacuolar targeting (Glickman et al., 1989; Lin Cereghino et al., 1995; Rad et al., 1995; Höning et al., 1996). The first Tyr-containing region (amino acids 589–594) in the cytoplasmic region of AtELP is adjacent to the transmembrane region and shows similarity to the Tyr-based signals required for the targeting and function of the M-6-PR and Vps10p (Glickman et al., 1989; Lin Cereghino et al., 1995). The second cytoplasmic Tyr motif, YMPL (amino acids 606–609), fits the consensus motif YXXϕ. The YMPL motif resembles other Tyr-based motifs implicated in targeting to endosomes or the TGN and interaction with the clathrin-associated adaptor proteins (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993; Ohno et al., 1995). Both of the Tyr-containing regions are conserved between AtELP and the maize EST clone.

Protein transport from both the PM and TGN to the vacuole/lysosome in yeast and animals proceeds via an inter-

mediate endosome-like compartment (Gruenberg et al., 1989; Vida et al., 1993). The presence of multiple intermediate compartments (early, medial, or late endosomes) between the Golgi, PM, and vacuole/lysosome is well documented in both mammalian and yeast cells (for review, see Pryer et al., 1992; Vida et al., 1993). The transport processes between the Golgi or PM to the endosome are thought to be mediated by CCVs. The endosome-to-vacuole lysosome transport may also be vesicle-mediated. Alternatively, the endosome may fuse with the vacuole upon maturation. In plants, the presence of such intermediate compartments between the ER, Golgi, PM, and vacuole have been suggested in recent years. These include the rough ER (for review, see Okita and Rogers, 1996), partially coated reticulum (Hillmer et al., 1988; Griffing, 1991), and a post-Golgi compartment (Conceição et al., 1997). Recently, some plant cells have been shown to contain two functionally different vacuoles at specific developmental stages that may later fuse to become a large vacuole (Hoh et al., 1995; Paris et al., 1996).

The results obtained from biochemical and subcellular fractionation studies of *A. thaliana* indicated that AtELP is an integral membrane protein that resides in at least two different membrane populations. One may correspond to a novel intermediate compartment (fractionating at 26.5% Suc) that is less dense than the ER, Golgi, PM, and more dense than the vacuole. This could be a plant counterpart of the mammalian or yeast endosome. It is interesting that the density at which this compartment fractionates (26.5% Suc) in Suc gradients is similar to that reported for endosomes in animal cells (Beevers, 1996) and yeast (Vida et al., 1993; Becherer et al., 1996), suggesting the localization of AtELP in an endosome-like intermediate compartment in *A. thaliana*. Although some intermediate or post-Golgi compartments (sometimes also referred to as intermediate compartments) have been identified morphologically (Staehelin et al., 1990) or their presence suggested in some recent reviews (Okita and Rogers, 1996), to our knowledge no molecular markers for any of these compartments have been described yet, making it difficult for us to determine whether AtELP is present in any of them. Thus, we refer to the AtELP compartment as a yet undefined, novel intermediate compartment in the plant cell.

The other type of membrane-containing AtELP may represent small transport vesicles such as CCVs. Indeed, AtELP co-fractionated with high-speed pellet fractions containing clathrin in subcellular fractionation studies, and the AtELP homolog in pea was enriched in CCVs prepared from developing cotyledons. Furthermore, these vesicle preparations were enriched for the clathrin-associated β-adaptin-type protein. These results suggest that AtELP may also be associated with CCVs. This would be supported by the presence of a Yxxϕ motif in the putative cytoplasmic domain of AtELP, which may associate with clathrin via their interaction with adaptins during the formation of CCVs. The lack of marker proteins in pea prevented us from carrying out subcellular fractionation studies similar to those performed with *A. thaliana*.

The antibody raised against AtELP recognized three polypeptides in the range of approximately 80 kD. Although we cannot determine at this point whether these polypeptides are all AtELP-like proteins or posttranslational modifications of one (AtELP) protein, the three polypeptides always appeared to co-fractionate in all of our subcellular fractionation studies. Recently, Beevers (1996) and Paris and Rogers (1996) indicated the presence of several 80-kD integral membrane proteins in pea and Arabidopsis with domain structure and subcellular location similar to that of AtELP. A member of this group of proteins isolated from pea CCVs (BP-80; Kirsch et al., 1994b) has been shown to bind a broad range of plant vacuolar targeting signals (Kirsch et al., 1996). Similar to AtELP, the 80-kD protein from pea was not found to localize with ER or Golgi markers but fractionated as a less-dense compartment on Suc density centrifugation experiments (Beevers, 1996; Okita and Rogers, 1996). However, when this less-dense membrane fraction from pea cotyledons was incubated and allowed to associate with clathrin components, it sedimented in Suc density gradients at a density equivalent to that of CCVs (Kirsch et al., 1994a).

To our knowledge, AtELP is the first marker protein described that may reside in both an endosome-like intermediate compartment and CCVs in plants. The presence of AtELP in at least two different membrane populations may indicate that the protein is recycled between the membrane types. It is interesting that both the yeast Vps10p and the mammalian M-6-PR are type I integral membrane proteins required for the transport of multiple vacuolar/lysosomal hydrolases (Brown et al., 1986; Marcusson et al., 1994; Cooper and Stevens, 1996), and that they cycle between the late-Golgi and endosomal compartments in their function as sorting receptors for soluble vacuolar proteins. In addition, many of the membrane proteins (EGFR, LDLR, and transferrin receptor) involved in receptor-mediated endocytosis are cycled between the PM and the endosome (for review, see Pearse and Robinson, 1990).

During the review process of this manuscript, a search of the GenBank identified homologs of AtELP in pea (accession no. U79958), Arabidopsis (accession nos. U79959 and U79960), and maize (accession no. U79961) corresponding to the pea vacuolar sorting receptor BP-80 and its homologs, respectively.

In conclusion, the organization of the predicted functional domains of AtELP, its localization to an intermediate compartment, and association with CCVs are similar to several transmembrane receptor proteins that function at the PM or TGN during protein sorting along the mammalian and yeast endocytic or secretory pathways. We therefore propose that AtELP may play a similar role in intracellular protein trafficking in the plant cell. Studies are currently under way to investigate this possibility, especially the role of AtELP in the trafficking of soluble vacuolar proteins containing the plant vacuolar targeting signals identified thus far (Chrispeels and Raikhel, 1992).

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