Plant clathrin heavy chain: sequence analysis and restricted localisation in growing pollen tubes

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SUMMARY

Clathrin-coated vesicles were isolated from soybean (Glycine max L.) cells in suspension culture and their purity was assessed using SDS-PAGE, peptide sequencing and electron microscopy. Antibodies raised to these coated vesicles were used to immunoscreen a soybean cDNA library in λgt11 and isolate a partial clone of the clathrin heavy chain (HC) gene. Full-length cDNA for soybean clathrin HC was deduced by 5' and 3' cDNA amplification. The cDNA encodes an amino acid sequence of 1,700 residues, which is slightly larger than rat clathrin HC and may account for the reduced mobility of plant clathrin on SDS-PAGE. Insertion of these extra residues is largely confined to the amino and carboxy termini. Other domains within the heavy chain arms, including those implicated in light chain binding and trimerisation, are relatively well conserved between eukaryotes. A computer algorithm to determine α-helical coiled-coil structures reveals that only one domain, aligning to residues 1,460-1,489 in rat clathrin HC, has a high probability for coiled-coil structure in all five eukaryotic clathrin HC sequences. This provides further evidence that the interaction between clathrin heavy and light chains is mediated by three bundles of coiled-coils near to the carboxy terminus. In analysing the role of plant clathrin in endocytotic trafficking, as against trafficking from the Golgi apparatus to the vacuole, our attention was focused on membrane recycling in tip-growing pollen tubes. These rapidly growing cells are highly secretory and require a high level of plasma membrane recycling to maintain the tube tip architecture. Monoclonal antibodies to plant clathrin HC confirmed that coated vesicles are relatively abundant in tip-growing pollen tubes of Lilium longiflorum. This analysis also demonstrated that a high proportion of the clathrin present is in an assembled state, suggesting a highly dynamic trafficking pathway. Immunofluorescence analysis of pollen tubes revealed that clathrin localises to the plasma membrane at the apex of the pollen tube tip, which is consistent with high levels of clathrin-mediated membrane recycling. The use of these reagents in conjunction with tip-growing pollen tubes has created a unique opportunity to examine the basis for constitutive endocytosis, so that the more complex question of receptor-mediated pathways in plants can also be assessed.

Key words: Clathrin coated vesicle, Endocytosis, Monoclonal antibody, Heavy chain sequence, Pollen tube

INTRODUCTION

Clathrin-coated vesicles are responsible for the selective translocation of receptor-ligand complexes in a variety of eukaryotic cells (Brodsky et al., 1991). At the plasma membrane, macromolecules are internalised by receptor-mediated endocytosis, whilst at the trans-Golgi network a distinct population of clathrin-coated vesicles is responsible for the sorting and transport of lysosomal enzymes (Pearse and Robinson, 1990). Our understanding of the structure of clathrin-coated vesicles is largely derived from studies on animal cells. These have revealed that the vesicle coat consists of clathrin triskelions composed of clathrin heavy and light chains, which assemble to form the polygonal lattice characteristic of these organelles. The clathrin light chains provide the recognition site for coated vesicle disassembly by hsc70 and a variety of light chain isoforms have been identified (Jackson and Parham, 1988). Underneath the clathrin shell lie the adaptor complexes, which bind the selected receptor molecules and cross-link them to clathrin. The adaptor complex is a heterotetrameric structure composed of two 100-110 kDa proteins, either α- and β-adaptin at the plasma membrane, or γ- and β'-adaptin at the trans-Golgi network (Pearse and Robinson, 1990). Also present are medium and small chains of 50 kDa (μ2) and 17 kDa (σ2) at the plasma membrane, or 47 kDa (μ1) and 19 kDa (σ1) at the trans-Golgi network. A recent study has demonstrated that the medium chain (μ2) of the plasma membrane-adaptor complex binds to tyrosine-based signals of membrane proteins (Ohno et al., 1995). This suggests that the medium chains of the adaptor complex play a major role in dictating specificity, while the remaining adaptor proteins create an appropriate environment for receptor recruitment and clathrin binding.

There is at present only preliminary evidence for receptor-mediated endocytosis (RME) in plants (Horn et al., 1989) and recent research on plant coated vesicles has concentrated on their role in trafficking from the trans-Golgi network in developing pea cotyledons (Lin et al., 1992; Demmer et al., 1993).
It is nonetheless clear that clathrin also mediates membrane retrieval at the plant plasma membrane (Fowke et al., 1991; Mollenhauer et al., 1991). The use of cationised ferritin has demonstrated that the internalisation of plasma membrane follows essentially the same pathway as in animal cells. Thus, coated pit formation is followed by vesicle budding, clathrin uncoating, transport to the partially coated reticulum and from there to the multivesicular body and the vacuole (Tanchak et al., 1984; Fowke et al., 1991). In addition, the morphology of plant and animal endocytic vesicles appears to be almost identical, although there are subtle differences in the \( M_f \) of clathrin heavy chains and the length of the triskelion arms (Coleman et al., 1987, 1988). While it is tempting to assume that this reflects a common protein composition, the conservation of such vital components as clathrin heavy and light chains and the adaptor complex components has yet to be demonstrated for plant coated vesicles. A deeper understanding of the endocytic process in plants will clearly require the same detailed structural information that is available for animal cells.

Indeed, this may be the only effective means of assessing whether RME occurs in plants, and in examining the molecular basis of selectivity and specificity for coated pit assembly.

Progress in characterising clathrin-mediated endocytosis in plants has been delayed by a variety of factors, including the low recovery of coated vesicles, the barrier presented by the plant cell wall and the lack of endocytic markers, or cross-reacting antibodies (see also Robinson and Hillmer, 1990). Further progress will require a detailed structural analysis of plant coated vesicles, the development of reagents to monitor internalisation and selection of an appropriate plant cell in which to perform this analysis. As a first step to resolving this situation, we have isolated clathrin-coated vesicles from soybean cells in suspension culture and analysed the principal coat components. This has provided vital information on the structure of plant clathrin and generated the necessary immunological and molecular probes to dissect the pathway. The use of these reagents has also allowed us to identify a suitable cell type, the tip-growing pollen tube, which is amenable to further genetic and biochemical analysis. We discuss the implications of these results and possible avenues of further research.

**MATERIALS AND METHODS**

**Plant material**

Suspension cultured soybean \((Glycine max\ L.)\) cells were grown in 400 ml of liquid B5 medium in 1 l flasks on a rotary shaker at 25°C in continuous darkness. Anthers were collected 2 days after anthesis from flowers of \(Lilium longiflorum\) (obtained from a local florists) and air dried for 8 hours followed by 16 hours at ~18°C. Pollen was separated from the anthers with a brush and stored at ~70°C until use.

**Isolation of coated vesicles**

Cell suspension cultures were resuspended in fresh media 24 hours before the start of the experiment. The cells were harvested by filtration through 2 layers of muslin and resuspended in 2 volumes of buffer A composed of 0.1 M Mes-NaOH, pH 6.5, 0.3 M sorbitol, 0.2% BSA, 1 mM EGTA, 3 mM EDTA, 0.5 mM MgCl₂, 0.02% NaN₃, 4 mM DTT, 1 mM PMSF, 1 μM pepstatin and 1 μg/ml leupeptin. The suspension was homogenised using a bead beater for 1 minute per load (15 second pulse; 45 second delay). The homogenate was centrifuged successively at 500 g for 5 minutes, 5,000 g for 10 minutes, 20,000 g for 30 minutes and 120,000 g for 1 hour. The resulting pellets were resuspended in buffer A and layered onto linear gradients of 9% to 90% D₂O and centrifuged at 40,000 g for 30 minutes. The supernatant was decanted, diluted twofold with buffer B and centrifuged at 120,000 g for 1 hour. The pellets were resuspended in buffer B, layered onto a linear gradient of 9% D₂O/2% Ficoll to 90% D₂O/25% Ficoll and centrifuged at 80,000 g for 16 hours. Successive fractions were recovered from the gradient, diluted fourfold with buffer B and centrifuged at 120,000 g for 1 hour. The resulting pellets were resuspended in buffer B and those fractions containing clathrin heavy chain (as determined by SDS-PAGE) were pooled, made up to 5 ml, layered on to a 30 ml gradient of 5% to 25% sucrose in buffer B and centrifuged at 100,000 g for 90 minutes. Fractions corresponding to 10% to 20% sucrose were collected, diluted with 2 volumes of buffer B and centrifuged at 100,000 g for 60 minutes. The resulting clear pellet was resuspended in a small volume of buffer B and stored at either 4°C, or at ~70°C until use.

**Isolation of pig brain coated vesicles**

Coated vesicles were isolated from freshly obtained pig brains by differential centrifugation and gel-filtration on Sephacryl S-1000 as described (Cambell et al., 1984).

**Polyclonal and monoclonal antibodies**

Balb/c mice were immunised with sucrose-purified clathrin-coated vesicles from soybean, using standard procedures (Galfre and Milstein, 1981). Spleen cells of mice showing an immune response were fused with P3-NS1/1-Ag4-1 myeloma cells and screened for antibodies to plant clathrin by western blotting. Following cloning at limiting dilution, antibody was purified by affinity chromatography (HiTrap, Pharmacia) and characterised using an antibody isotyping kit (Pierce). The monoclonal antibody ‘4A8’, is an IgG1 immunoglobulin.

**Gel electrophoresis and immunoblotting**

Gel electrophoresis was carried out on 8% resolving gels using the buffer systems of Laemmli (1970). Gels were either stained with Coomassie Brilliant Blue R-250, or by silver staining using silver stain plus (Bio-Rad). Transfer to Hybond-C (Amersham International) was carried out in 20% methanol, 25 mM Tris, 192 mM glycine and 0.02% SDS at 100 V for 1 hour. After transfer membranes were washed briefly in TBS and incubated in TBS containing 5% low fat milk for 1 hour at room temperature. The blot was added to primary antibody diluted in TBS containing 1% BSA and incubated overnight at 4°C. The membrane was washed for 5 minutes each in TBS, TBS+0.5 M NaCl, TBS+0.1% Triton X-100 and TBS. The blot was then incubated with peroxidase-conjugated secondary antibody in TBS+1% BSA for 1 hour at 4°C. Membranes were washed as before and visualised using either a peroxidase staining kit (Vector Labs.) or ECL (Amersham).

**Isolation of a soybean clathrin HC clone**

A λgt11 soybean stretch library (Clontech no. FL1062b) was immunoscreened according to standard protocols (Sambrook et al., 1989), using immune serum to soybean clathrin HC. Out of 350,000 plaques screened, a single immunoreactive plaque was detected and the 3.2 kb insert excised by EcoRI digestion and subcloned into M13mp18. Sequencing of both strands was performed by dideoxy chain termination (Sanger et al., 1977) using a T7 sequencing kit (Pharmacia) and 35S-dATP (Amerham International). The remaining 5' and 3' coding regions were identified by Marathon™ cDNA amplification (Clontech). Total RNA was isolated from freeze-dried soybean cells using standard plant protocols (Sambrook et al., 1989) and poly(A)+ RNA purified using a PolyATract™ isolation kit (Promega). Marathon™ cDNA synthesis and adaptor ligation was performed as described by Chenchik et al. (1995), with the following exceptions: second strand synthesis was allowed to proceed for 6
hours at 16°C; following the addition of T4 DNA polymerase the sample was incubated for 2 hours at 16°C and the adaptor ligated cDNA was used directly in long distance PCR, as dilution in Tricine-EDTA buffer prevented priming. The complete cDNA of the soybean clathrin HC was deduced using synthetic oligonucleotides to bridge the PCR products. Automated sequencing of both strands of DNA was by dye termination reaction.

**DNA blots**

Genomic DNA was isolated from soybean cells in suspension culture according to the method of Dellaporta et al. (1983) and Southern analysis was performed using the method of Sambrook et al. (1989). Probes labelled with [32P]dCTP were prepared by random priming DNA labelling (Boehringer-Mannheim). Hybridisations were at 42°C in the presence of 50% formamide.

**Sequence analysis**

Sequences were compared using PILEUP (University of Wisconsin Genetics Computer Group analysis software package) with a gap weight of 3.0 and a gap length weight of 0.1. Probabilities of forming coiled-coil structures were determined using the algorithm of Knight and Kendrick-Jones (1993) based on the programme developed by Lupas et al. (1991). Changes to the programmes’ parameters made it necessary to repeat the analysis of the rat, Dictyostelium and yeast clathrin HC sequences performed previously (Nathke et al., 1992), while including the probability plots for soybean and Drosophila clathrin HC sequences.

**Detection of clathrin-coated vesicles in microsomal fractions from pollen tubes**

Pollen from L. longiflorum (10 mg pollen per ml of buffer) was incubated in germination buffer (Kohno et al., 1990) on a rotator for 90 minutes at 30°C. Germinated pollen was collected by centrifugation at 600 g for 2 minutes and resuspended in buffer B. Pollen tubes were homogenised using a Potter homogeniser and centrifuged at 600 g for 1 minute to remove cell debris. The supernatant was re-centrifuged at 14,000 g for 1 minutes, the supernatant decanted and centrifuged at 100,000 g for 10 minutes at 4°C. The resulting pellet was resuspended in a small volume of buffer B and analysed by SDS-PAGE.

**The ratio of assembled/disassembled clathrin during pollen tube growth**

Microsomal extracts were prepared as above using a buffer composed of 10 mM Hepes (pH 7.4), 150 mM NaCl, 1 mM EGTA, 0.5 mM MgCl2, 0.2% Triton X-100, 4 mM DTT and 0.2% azide (Acton et al., 1993). Following centrifugation at 100,000 g, supernatants were decanted and the pellets resuspended in sample buffer. TCA was added to the supernatants to a final concentration of 10% and the samples were left overnight at 4°C. The TCA precipitate was collected by centrifugation at 16,000 g for 10 minutes at 4°C and washed in cold 100% acetone for 30 minutes at 4°C. Following centrifugation at 16,000 g for 10 minutes the pellets were resuspended in sample buffer and aliquots of supernatant and pellet fractions were resolved on SDS-PAGE. The proportion of clathrin HC in each fraction was analysed by probing western blots with the monoclonal antibody 4A8.

**Immunofluorescence confocal microscopy**

Pollen tubes were germinated as above and allowed to settle for 5 minutes. The growth medium was decanted and the pollen tubes resuspended in 100 mM Pipes buffer (pH 7.0) containing 4% formaldehyde and 4 mM EGTA. The samples were left to fix for 1 hour at 20°C. The following solution changes were accomplished by centrifugation at 600 g for 1 minute. After a brief wash in PBS, pollen tubes were permeabilised by addition of PBS containing 0.5% Triton X-100 for 10 minutes. The samples were briefly washed in PBS, resuspended in blocking buffer (PBS containing 5% foetal calf serum, 0.1% Triton X-100, 0.02% SDS, 5% horse serum and 0.02% azide) and left for 1 hour. Pollen tubes were then incubated with 1° antibody in blocking buffer. Following three 5-minute washes in PBS, pollen tubes were resuspended in blocking buffer containing FITC-conjugate of goat anti-mouse IgG and left for 1 hour. The pollen tubes were given three 5-minute washes in PBS, one 5-minute wash in distilled water and allowed to air-dry onto the surface of a microscope slide. Pollen tubes were mounted in 0.2 M Tris-HCl (pH 8.3), 85% glycerol and 3% n-propyl gallate, and viewed using a Bio-Rad MRC-1000 confocal microscope. Control experiments included the use of pre-immune sera, the omission of 1° and 2° antibodies and labelling with a monoclonal antibody to α-tubulin.

**RESULTS**

**Purification and analysis of plant coated vesicles**

We elected to isolate clathrin-coated vesicles from soybean cells in suspension culture, as these are a recognised source of endocytotic coated vesicles (Coleman et al., 1988). The protocol employed was adapted from that developed for soybean protoplasts (Mersey et al., 1985) and included EDTA in the extraction buffers to avoid contamination by ribosomes, and azide to inhibit ATP-dependent uncoating. Protein assays revealed that the recovery of these highly purified coated vesicles was relatively low, with 0.2 µg protein g⁻¹ FW. This value is only 3% to 5% of that reported for developing pea cotyledons and brain tissue, respectively (Harley and Beevers, 1989). However, it appears that coated vesicles from pea cotyledons (in which Golgi-trafficting predominates) are contaminated by phytoferritin, which may lead to an overestimation of their recovery (Hoh and Robinson, 1993). There is no equivalent 28 kDa contaminant in the sucrose-purified coated vesicles from soybean.

The profile of highly purified coated vesicles on SDS-PAGE is strikingly similar to that of animal coated vesicles, with a dominant band of 185 kDa and relatively few other bands, of which those around 110 kDa and a doublet of 50-54 kDa are the most obvious (Fig 1[i]A+B). Immunoblotting confirmed that the 50-54 kDa doublet is tubulin: this is not unexpected as tubulin co-purifies with mammalian clathrin-coated vesicles. Silver staining reveals that other polypeptides are also present with bands at 130-140 kDa, a doublet at 110 kDa and another at 90 kDa (Fig. 1[i]C). Silver staining also reveals the presence of a number of lower molecular mass polypeptides, but it is not clear which, if any, are clathrin light chains. The soybean coated vesicle preparations were judged to be free from contamination following ultrastructural analysis of negatively stained specimens (Fig. 1[ii]).

**Identification and sequence analysis of soybean clathrin HC**

The amino terminus of soybean clathrin heavy chain is blocked, but internal amino acid sequence was obtained by partial digestion with V8 protease (Cleveland, 1983) and peptide sequencing (see legend to Fig. 3). However, we reasoned that the highly degenerate nature of oligonucleotides generated to these sequences would prohibit stringent library screening, and were therefore prompted to raise antibodies to plant clathrin, using sucrose-purified clathrin-coated vesicles from soybean as antigen. A polyclonal antiserum with high affinity for plant clathrin HC on western blots (see Fig. 6a),
was then used to immunoscreen a soybean cDNA library in λgt11. Screening of up to 350,000 plaques gave a single positive plaque containing an insert of 3.2 kb. Sequencing revealed that the 3.2 kb fragment was approximately 60% identical to rat clathrin and the translated sequence spanned amino acid residues 275-1346 (Kirchhausen et al., 1987). Further screening using 32P-labelled cDNA probes of this fragment failed to identify other homologous sequences in the library used. The remaining heavy chain sequences were therefore cloned by PCR using oligonucleotides raised to sequences mapping to the 5′ and 3′ ends in conjunction with Marathon™ cDNA amplification. The resulting cDNA includes an AUG at the start that resides within an authentic translation sequence (Grierson and Covey, 1988) and provides an open reading frame of 5,100 bp. The nucleotide sequence provides a corresponding translated amino acid sequence of 1,700 residues. The calculated size of soybean clathrin HC (193,353 Da) is slightly larger than that calculated for rat clathrin HC (191,597 Da) and may explain the reduced mobility of soybean clathrin HC on SDS-PAGE.

DNA Southern blot analysis at high stringency, using a cDNA probe from the coding region (bases 2,022-3,876), gave two bands in soybean genomic DNA restricted with either EcoRI or BamHI (Fig. 2). The intensity and size of these bands are consistent with the position of restriction sites within this region and the proportion of template available for random priming either side of the restriction site. This suggests that only one gene codes for clathrin and there are no pseudogenes, or highly homologous genes, in the soybean genome.

The previously determined peptide sequence for V8 protease digests of soybean clathrin HC confirmed that the translated amino acid sequence is a legitimate soybean gene product (Fig. 3). The amino acid sequence of the soybean clathrin HC is most closely related to the other eukaryotic clathrin HC sequences in the following order: rat (56% identity; 75% similarity), Drosophila (56% identity; 74% similarity), Dictyostelium (54% identity; 74% similarity) and yeast (44% identity; 68% similarity). Multiple sequence alignments reveal that the greatest degree of divergence is confined to the amino-terminal and carboxy-terminal residues. There are a number of major gaps introduced into the first 250 residues of the eukaryotic sequences, some of which are unique to soybean HC. The fact that extra residues are accommodated in the N-terminal domain of soybean HC may satisfy the point that structural restraints are less vital in these domains than in the extended arms of the HC, which make intimate contact with each other and with clathrin light chains (Lemmon et al., 1991; Nathke et al., 1992).

There is evidence that the light chain binding and trimerisation sites of mammalian HC lie adjacent to each other, spanning residues 1,460-1,489 and 1,490-1,587, respectively (Nathke et al., 1992). Multiple sequence alignments reveal that both these sites are well conserved in soybean clathrin HC. The C-terminal domain (residues 1,590-1,675 in mammals) may form the globular protrusion on top of triskelia at the vertex (Nathke et al., 1992). It is evident that the residues at the extreme C termini are very poorly conserved between species. Analysis of a C-terminal mutant of yeast HC has already demonstrated that this globular protrusion plays no essential role in triskelia assembly or clathrin function (Lemmon et al., 1991).

An analysis of clathrin HC and light chain sequences suggests that there is a high probability of their interacting via...
a-helical coiled-coils (Nathke et al., 1992). This was established using a computer programme to compare residues and their flanking sequences with residues in known coiled-coil structures (Lupas et al., 1991). Equivalent analysis of soybean HC, using a modified form of this programme (Knight et al., 1993), gives a high probability of a-helical coiled-coil from residues 1,475-1,502 (Fig. 4). These residues align to the equivalent domain of residues 1,460-1,489 in the rat and other eukaryotic sequences. This is the only domain that gives a consistently high probability of coiled-coil structures in all five sequences. As previously noted the weak prediction of a heptad repeat pattern for residues 1,151-1,178 in mammalian HC (Kirchhausen et al., 1987) is not shared by yeast HC (Nathke et al., 1992) and the heptad pattern for soybean HC is also weak. The coiled-coil prediction near to the C-terminal domain of soybean HC (residues 1,617-1,644), which may form the protrusion near to the vertex of the triskelia (Nathke et al., 1992), is more pronounced than the equivalent region in rat HC, but similar to that calculated for dictyostelium HC.

**Clathrin-mediated membrane recycling in pollen tubes**

The prominent band of 185 kDa apparent in microsomal extracts from pollen tubes of *L. longiflorum* on SDS-PAGE was blotted and probed with monoclonal antibody 4A8, raised to soybean clathrin HC (Fig. 5A). This revealed that the procedure used to isolate microsomal extracts results in greater recovery of both total protein and clathrin from pollen tubes of *L. longiflorum* than from an equivalent sample of soybean cells. This may merely reflect differences in the degree of vacuolation between the two cell types, but it is a useful indication of the suitability of pollen tubes for coated vesicle analysis. It is also consistent with the high levels of clathrin-mediated endocytosis reported for similarly highly secretory plant cells (Steer, 1988). The monoclonal antibody used specifically recognises clathrin HC in plants and we have also employed it to qualitatively assess the proportion of clathrin that is present in either the assembled, or the unassembled, pool of clathrin. This revealed that a very high proportion of the clathrin HC in growing pollen tubes is present in an assembled state (Fig. 5B). This compares with mammalian cells in which 30-35% of the clathrin heavy chain is assembled in cells with low endocytotic activity and 90% in cells that are endocytotically and/or exocytotically active (Goud et al., 1985).
The distribution of clathrin-coated structures was examined in formaldehyde-fixed and detergent-permeabilised pollen tubes using antibodies raised to soybean clathrin heavy chain. The monoclonal antibodies that we had raised to soybean clathrin HC recognise plant clathrin HC on SDS-PAGE and native protein dot-blotted on to membrane, but failed to cross-react with clathrin in formaldehyde-fixed cells. However, the polyclonal antiserum used in the immunoscreening protocol for clathrin HC was effective at revealing the distribution of clathrin in pollen. Western blots of crude extracts from pollen tubes of *Lilium* revealed that the polyclonal mouse antiserum specifically recognises clathrin HC (Fig. 6A). In cells, the distribution of clathrin HC was restricted to the tube tip apex, with an intense signal extending up to 10 µm from the tip (Fig. 6B). Optical sectioning through the upper face of the pollen tube revealed that this signal was concentrated at the plasma membrane of the tube wall, the logical site for clathrin-coated pits implicated in membrane recycling in pollen tubes (Fig. 6C,D). The intense staining at the tube apex differs from the punctate staining pattern reported for receptor-mediated pathways in endocytotic mammalian cells (Brodsky, 1988). However, in the pollen tube both exocytosis and membrane recycling appear to be restricted to a zone near the apex, while receptor-mediated endocytosis in other eukaryotes occurs in clusters of coated pits dispersed over the surface of the plasma membrane. There is no evidence of a requirement for clathrin-mediated vacuolar trafficking in the vegetative cell of pollen tubes (Noguchi, 1990), but we cannot rule out the possibility that such a mechanism exists in the generative cell as autofluorescence of the generative cell prevented this analysis (results not shown). However, the contribution that clathrin makes to trafficking in the generative cell is likely to be small compared to that at the tip apex, given that there are relatively few organelles within the generative cell cytoplasm. No fluorescence (other than autofluorescence of the germinative cell) was detected in pollen tubes incubated with pre-immune sera (Fig. 6E), with secondary antibodies alone or with no antibodies.

**DISCUSSION**

The results presented here further clarify the structure of plant clathrin HC and provide evidence that endocytotic membrane recycling in pollen tubes proceeds through a clathrin-mediated pathway. The cloning and sequencing of the soybean clathrin HC are the first departures from the traditional biochemical analysis of coated vesicle trafficking in plants. A comparison of the clathrin HC sequence from plants with those of other eukaryotes reveals interesting insights into those domains that are conserved or diverged during speciation. The increased number of residues in soybean HC provides a plausible explanation for the higher $M_t$ of plant clathrin on SDS-PAGE, although the relationship between sequence size and gel mobility does not always apply, as in the case of yeast clathrin HC (Lemmon et al., 1991). Interestingly, plant heavy chains are reported to be approximately 60 nm in length, while those of mammals are 45-55 nm in length (Coleman et al., 1987).
These differences in arm length do not appear to alter the overall dimensions of assembled polygons and hexagons in the coat structure (Coleman et al., 1987). These authors predicted that up to 80-90 amino acids arranged as an α-helix would be required to account for this size difference. We find no direct evidence for this, the 25 extra residues in soybean HC relative to rat HC, are largely accommodated in the terminal domains, which form the large globular protrusions and appear to make little contribution to arm length. Interestingly, gaps that appear in the alignment of soybean with other clathrin HC sequences occur in the region preceding the flexible linker (residues 479-523 in rat), which allows the globular N-terminal domain to project inwards and make contact with the adaptor complex (Kirchhausen et al., 1987). It is possible that these differences in soybean clathrin HC terminal sequences may reflect subtle changes in the association of plant triskelia with the underlying adaptor complex.

Two sequence domains of great structural importance are the trimerisation domain and the light chain binding domain. The unique trimeric structure of clathrin is mediated by non-covalent association of HC monomers and the sequence of rat clathrin HC initially suggested that the proline-rich region of the carboxy terminus (residues 1,631-1,675) could be responsible for this interaction (Kirchhausen et al., 1987). Subsequent analysis of a mutant yeast clathrin HC gene lacking this domain revealed that clathrin was present as trimers and that the mutant was able to complement clathrin HC-deficient yeast (Lemmon et al., 1991). A revised model of the clathrin triskelion now suggests that residues 1,488-1,587 are responsible for trimerisation and residues 1,438-1,481 include sequences required for light chain binding (Nathke et al., 1992). This model was based on peptide mapping, immuno-electron microscopy and computer predictions of coiled-coil structures. The alignments and coiled-coil calculations for soybean HC also highlight the conservation and importance of residues 1,475-1,502. This domain aligns to the sequence 1,460-1,489 in rat HC and is the only coiled-coil site in all five eukaryotic species. The importance of the weakly predicted pattern of heptad repeats spanning residues 1,107-1,184 in rat clathrin HC (Kirchhausen et al., 1987) is further reduced by this comparison. This makes it unlikely that the light chains associate with heavy chain trimers in ‘bundles’ of four coiled-coils as envisaged for mammalian triskelia. The coiled-coil prediction also has a bearing on the identification of plant clathrin light chain homologs, which appear to be poorly conserved between species, and so may be difficult to identify from sequence comparisons alone. In view of the evidence for coiled-coil interactions between clathrin HC and clathrin light chains, one can hypothesise that authentic plant clathrin light chains will have a similar probability of forming a coiled-coil structure.

Endocytotic recycling in plants appears to be closely linked to secretory activity, while evidence of RME remains largely circumstantial and mostly concerns the uptake of fungal elicitors (Low and Chandra, 1994). In order to develop a model of plasma membrane recycling, we have focused on endocytotic recycling during the growth of pollen tubes. These exhibit rapid growth rates (up to 1 cm h⁻¹) and have highly developed trafficking pathways. Previous analysis of pollen biology has revealed that vesicle fusion with the plasma membrane results in excess membrane at the tube tip, up to 80% of which may be recycled (Steer, 1988), and the uptake of FITC-labelled dextran demonstrates that endocytosis is particularly active at the tube tip (O’Driscoll et al., 1993). Our analysis of pollen tubes of *Lilium* revealed that coated vesicles are relatively abundant and that almost all of the clathrin HC present is in an assembled state during tube growth. Our immunofluorescence analysis demonstrated that clathrin is concentrated at the extreme apex of the tube tip. This distribution corresponds to the site of secretory vesicle fusion and the point of entry of FITC-labelled dextran by endocytosis, and fits well with the known organisation of pollen tubes, which has been examined following both chemical fixation, and rapid freeze fixation and freeze substitution. In chemically fixed pollen tubes there appear to be a number of distict zones, in the first 5 μm of the tip there is a cap of tightly packed vesicles, this is followed by a zone rich in ER and mitochondria and then another subapical zone approximately 10-15 μm from the tip where Golgi and other organelles appear (Picton and Steer, 1981; Steer and Steer, 1990). In freeze-substituted pollen tubes this pattern is repeated, but a number of randomly orientated elements of ER can also be seen at the vesicle-rich tip apex (Lancellle et al., 1987; Lancelle and Hepler, 1992). There is evidence that two populations of smooth vesicles are present at the pollen tube tip, the secretory vesicles of 300 nm diameter and unidentified vesicles of 50 nm diameter (Cresti and Tiezzi, 1990). The high levels of assembled clathrin present in pollen tubes are consistent with rapid rates of uncoating and recycling to the plasma membrane, so the 50 nm diameter vesicles may be uncoated vesicles trafficking back from the apex. The fate of this re-internalised membrane is not clear. Perhaps the membrane structures reported to be present at the tip apex in freeze-substituted pollen tubes (Lancellle and Hepler, 1992) serve as an early endosomal compartment for recycling. These results suggest that clathrin is dedicated to selective membrane recycling following secretory vesicle fusion in pollen tubes. This role may be similar to that played by clathrin during synaptic vesicle recycling in nerve terminals, where there is evidence that synaptic vesicle membrane proteins recruit AP-2 to the plasma membrane (Maycox et al., 1992; Zhang et al., 1994). An interesting question that arises from these observations on pollen tubes is: what directs coated pits to assemble at this precise position on the plasma membrane? Currently, there is no information on how plant clathrin is recruited to the plasma membrane, but since adaptins are part of the coat structure it is likely that as in animal cells they interact with specific membrane proteins. The vegetative pollen cell is an ideal system in which to examine the biochemistry of this process. Techniques have already been developed to isolate secretory vesicles and plasma membrane-rich fractions from pollen tubes (Van der Woude et al., 1971), and antibodies to clathrin HC and β-adaptin can be used to immunoprecipitate coated vesicles, so revealing those proteins that are selectively internalised. A similar protocol has been used to demonstrate that immunoprecipitation of the EGF receptor of A-431 cells results in recovery of almost half of the available adaptin pool (Sorkin and Carpenter, 1993). The isolation of vegetative cell-specific pollen promoters (Twell, 1992) has also created the means to perturb clathrin levels within the growing pollen tube, using antisense constructs to the heavy chain and N-terminal mutants to create a variety of clathrin-deficient phenotypes.

In conclusion, antibodies and molecular probes to clathrin are now available and we have described an interesting plant
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