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UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

CHARACTERIZATION OF CLATHRIN ADAPTOR PROTEINS FROM COTYLEDONS OF DEVELOPING PEA (*Pisum sativum* L.)

A Dissertation

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

Doctor of Philosophy

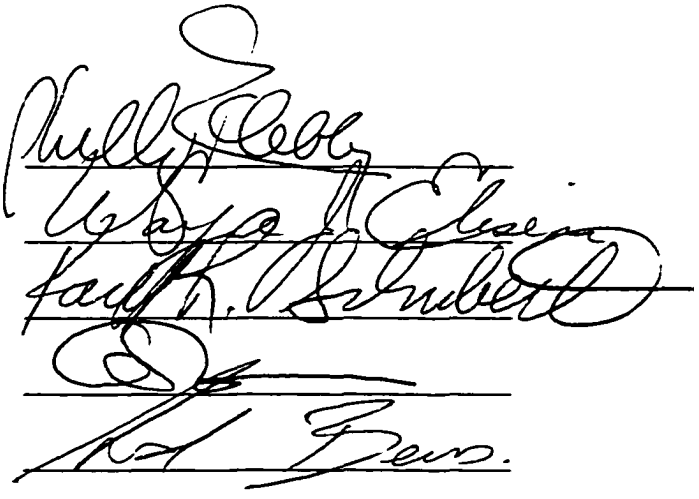
By

Juliet Marie Butler
Norman, Oklahoma
1998

CHARACTERIZATION OF CLATHRIN ADAPTOR PROTEINS
FROM COTYLEDONS OF DEVELOPING PEA (*Pisum sativum* L.)

A dissertation APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY



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CHAPTER I

Introduction: Coated Vesicle-Mediated Protein Trafficking in Eukaryotic Cells

The eukaryotic cell, composed of a complex system of internal membranes and discrete organelles, requires additional spatial and temporal strategies for the movement of proteins other than those utilized by prokaryotic cells. Some newly synthesized proteins must reach, and be integrated within a specific organellar lumen or membrane, others are destined for extracellular secretion. Thus, in addition to the physical requirement for movement, efficient mechanisms for the selective targeting of proteins from the site of synthesis to the site of deposition are required.

Three types of protein transport with distinct targeting mechanisms have been identified: they are the gated, transmembrane and vesicular pathways. Gated transport, characteristic of bi-directional protein movement between the nucleus and cytosol via nuclear pores, allows smaller molecules to diffuse unimpeded while selectively transporting both macromolecules and molecular assemblages. Nuclear localization signals present within the protein sequence are required for import of large protein molecules. It is likely that a selective transport mechanism is also required for the export of ribosomes and messenger RNA into the cytoplasm.

Transmembrane transport, characteristic of import of mitochondrial, plastid, peroxisomal and endoplasmic reticulum proteins, depends upon both membrane-bound translocator proteins specific to the organelle and the presence of targeting signals within the newly-synthesized proteins. Both gated and transmembrane protein transport have been recently reviewed (Hicks and Raikel, 1995; Cline and Henry, 1996; Erdmann et al., 1997) and will not be discussed further in this review.

Trafficking of transmembrane and luminal proteins of and between the organelles of the biosynthetic/secretory and endocytic pathways (endoplasmic reticulum, Golgi, lysosome, endosome, secretory vesicles and plasma membrane), is accomplished via cargo-laden vesicles which bud off a donor membrane and fuse to a topologically equivalent acceptor membrane (Rothman and Wieland, 1996). Over the last two decades, morphological studies have identified four types of vesicles which can be differentiated on the basis of their distinct protein coats. These are the two types of clathrin-coated vesicles (containing either the Golgi-derived AP1 adaptor complex or the plasma membrane-derived AP2 adaptor complex) and the two types of coatomer-coated vesicles (COPI and COPII). Individual protein components of these coats have been characterized and further biochemical and genetic studies have focused on the specific sorting mechanisms (retention/targeting) and recognition mechanisms (docking/fusion), required both to maintain the identity of the donor and acceptor membranes and to provide accurate delivery of the protein cargo. Convergence of these areas of study has produced a general model of vesicular transport, although many details remain unknown. It appears that specific

components of the vesicular protein coat function to mechanically sculpt the vesicle out of the donor membrane (Rothman and Wieland, 1996). Other coat proteins interact with sorting signals present in the cytoplasmic tails of donor membrane proteins. This interaction serves to either concentrate or exclude specific proteins from a specific vesicle. Movement occurs via diffusion or in concert with cytoskeletal elements. Cytoplasmically oriented fusion proteins of the donor and target membranes respectively mediate docking of the vesicle after its protein coat is removed. Throughout the entire transport process, specificity is thought to be provided by the interaction of vesicular coat proteins with molecules such as guanosine triphosphate and associated GTPases, and the phosphorylated derivatives of phophatidylinositol, the phosphoinositides (De Camilli et al., 1996). This review will focus on coated-vesicle mediated transport of the endocytic and secretory/biosynthetic pathways in yeast and mammalian tissues, with final emphasis on our knowledge of clathrin-coated vesicle mediated protein trafficking in plants. Specific and detailed reviews of pertinent literature will preface each chapter in this dissertation.

Part I. COP Coat Proteins in the Early Biosynthetic Pathway

Initially clathrin was identified (Pearse, 1975) as a major constituent of the polygonal coats of vesicles involved in the uptake of yolk proteins (Roth and Porter, 1964). Morphological studies in the 1980's suggested that while clathrin mediated transport from both the *trans*-Golgi and plasma membrane, transport of proteins from

the ER through the *cis* and *medial* Golgi cisternae to the *trans* cisternae appeared not to involve clathrin (Orci et al., 1984; Griffiths et al., 1985). Subsequently, (Orci et al., 1986) demonstrated the production of numerous non-clathrin coated vesicles from isolated Golgi membranes in a cell-free system which reconstitutes, *in vitro*, protein transport between Golgi compartments. Donor Golgi membranes from a VSV (vesicular stomatitis virus) infected mutant cell line of CHO (Chinese hamster ovary) cells deficient in the enzyme N-acetylglucosamine (GlcNac) transferase I were incubated with wild-type Golgi membranes, ATP and cytosol. Incorporation of [^{3}H]GLcNAc into VSV-G (vesicular stomatitis virus glycoprotein) from UDP-[H^3]GLcNAc was taken as a measure of transport. Transport to the acceptor, wild-type Golgi membranes was found to be blocked with addition of GTP-γS to the incubation mixture (Balch et al., 1984). The resulting vesicle buildup in the presence of GTP-γS facilitated purification and characterization of a large 700 kD coat protein complex from brain cytosol (Malhotra et al., 1989; Walters et al., 1991). This large complex, now known as COPI or coatomer, is composed of seven subunits in mammals (α, β, β′, γ, δ, ε and ξ) while Rec1p, Sec23p, Sec27p, Sec21p, and Ret2p compose COP I in *Saccharomyces cerevisiae*. Weak homologies to clathrin coated vesicle adaptor subunits have been shown for β, δ, and ξ COPS (Duden et al., 1991; Cosson et al., 1996).

Since the initial description of the involvement of COPI-coated vesicles in forward (*cis→medial→trans*) intra-Golgi stack transport, conflicting data concerning their role in the endocytic/biosynthetic pathways have emerged. A number of yeast

COPI defective mutants (i.e. *sec21-1* and *ret2-1*) fail to transport carboxypeptidase Y from the ER to the Golgi (Cosson et al., 1996) and antibodies against β-COP block transport of VSV-G protein to the Golgi (Peter et al., 1993). The budding of COPI vesicles directly from the ER has been reported (Bednarek et al., 1995). Most recently, VSV-G, tagged with green fluorescent protein, has been shown to accumulate in tubovesicular structures in living cells (Presley et al., 1997). The authors suggest that these structures are the intermediate compartment(s) shown to be enriched in the COPI subunit β-COP (Peter et al., 1993). A coatomer-binding motif containing two phenylalanine domains, present in certain members of the p24 family of putative cargo receptors, enhanced transport from the ER and between the Golgi stacks when transferred to a reporter molecule (Fiedler et al., 1996) These data implicate COPI-coated vesicles not only in forward intra-Golgi stack transport but also in a pre-Golgi stage in forward transport. However, in addition to this anterograde transport function, the participation of COPI in retrograde transport from (and through) the Golgi has been demonstrated in a number of studies. Retrieval of resident ER luminal proteins containing the HDEL/KDEL ER retention signal from biosynthetic pathway membranes as distant from the ER as the *trans*-Golgi network (TGN) has been reported (Miesenböck and Rothman, 1995) and COPI was shown to interact with transmembrane proteins containing a carboxy-terminal di-lysine (KKXX) retrieval motif (Cosson and Letourneur, 1994). *In vivo*, COPI has been shown to be required not only for retrograde transport of Emp 47p, a t-SNARE containing a di-lysine motif, but also for the HDEL receptor ERD2 (Lewis and

Pelham, 1996). Immunogold labeling of exocrine pancreatic cells using antibodies against β-COP localizes the majority of protein to the *cis*-Golgi side, rather than the *medial* or *trans*-Golgi stacks (Oprins et al., 1993). Since the concentration of escaped ER proteins would be expected to be greatest in the compartments immediately adjoining the ER, this *cis* labeling is consistent with COPI-coated vesicles functioning in retrieval. In addition to a role in retrieval of escaped proteins, data also suggests that COPI functions in the endocytic pathway and enables the pH-dependent formation of vesicles that transport proteins from early to late endosomes (Aniento et al., 1996). In this case, COPI was shown to contain only a subset of the polypeptides of the usual protein complex, with the γ and δ components absent. Finally, COPI may be involved in the vesiculation of the Golgi in mammalian cells during mitosis (Misteli and Warren, 1994).

Recruitment of COPI to Golgi membranes was shown to be dependent on GTP and the cytosolic GTPase, ARF (ADP-ribosylation factor) (Palmer et al., 1993). Up regulation of COPI vesicle assembly appears to involve a PtdIns(4,5)P_2 (phosphatidylinositol-4,5-bisphosphate) stimulated guanine nucleotide exchange activation of ARF, which then activates a Golgi-specific phospholipase D (Liscovitch and Cantley, 1995). This phospholipase D generates phosphatidic acid (PA), which stimulates a PtdIns(4)P-5 kinase to produce additional PtdIns(4,5)P_2. Down regulation of ARF may be provided by a ARF GAP (GTPase activating protein) activated by PA in concert with PtdIns(4,5)P_2 (Donaldson et al, 1992).

COPII proteins were first identified as products of genes shown, by complementation analysis of yeast temperature-sensitive secretion (*sec*) mutants, to be required for budding of ER-to-Golgi transport vesicles. Cloning of these genes and the construction of dihydrofolate reductase fusion genes facilitated purification of functional proteins and protein complexes. Using a transport reconstitution system developed by Rexach and Schekman (1991) which measures packaging of ^{35}S-labeled pro-α-factor (gpaF), a precursor of a secreted yeast mating pheromone, into ER-derived transport vesicles, a number of studies have demonstrated, *in vitro,* the absolute requirement for these proteins in budding of ER-derived vesicles (Salama et al., 1993; Hicke and Schekman, 1989; Hicke et al., 1992; Yoshihisa et al., 1993). While COPI is found assembled as a single cytosolic complex, or protomer, COPII coat proteins are found unassembled in the cytosol as single monomer (Sar1p) and as two heterodimeric complexes (the 400 kD Sec 23p complex composed of Sec23p and Sec 24p, and the Sec13p complex composed of Sec13p and Sec31p). Sar1p, a small (21 kD) GTPase which shares 34% homology with yeast ARF (Nakano and Muramatsu, 1989), has been shown to be activated by the GTPase-activating protein (GAP) Sec23p (Yoshihisa et al., 1993). While the primary role of COPII-coated vesicles appears to be in ER to Golgi transport, at least some COPII components may have multiple functions. Sec13p was shown to be required for the delivery of Gap1p, an amino acid transporter, to the PM (plasma membrane) in cells grown on urea or ammonia, suggesting a role for COP proteins in a novel nitrogen-regulated late secretory pathway (Roberg et al., 1997).

It appears that COPII proteins are conserved throughout eukaryotic organisms. On Western blots, antibodies against yeast Sec23p labeled rat hepatic and rat pancreatic proteins of similar size (Orci et al., 1991), and homologs of Sec13p have been identified in both human and murine tissue. Sar1p and Sec12p (an ER-membrane protein involved in Sar1p binding (Yoshihisa et al., 1993) homologs have been identified in both fission yeast and in the flowering plant *Arabidopsis thaliana* (d'Enfert et al., 1992). In similarity to α and β′-COP, both Sec13p (Pryer et al., 1993) and Sec31p (Salama and Schekman, 1995) have been shown to contain highly conserved eukaryotic protein sequences ending with Trp-Asp, known as WD-40 repeats, which regulate a number of diverse cellular functions (Neer et al., 1994). However, the precise role of these repeats in regulation of both COPI and COPII-coated vesicles is unknown.

Part II. Transport Specificity in COP-Coated Vesicles

The current model of vesicular transport, developed from our knowledge of both clathrin-coated vesicles and synaptic vesicles, requires that cargo proteins are actively selected and concentrated into vesicles while resident proteins of the donor organelle are excluded (Rothman and Wieland, 1996). While research has been limited, data supports active selection and concentration of cargo molecules for COP-coated vesicles as well. An enrichment of cargo and membrane trafficking proteins in COPII vesicles has been demonstrated (Bednarek et al., 1995). In addition, glyco-pro-α-factor has been shown to be selectively bundled into COPII vesicles (but not

COPI vesicles) which bud from the ER (Barlowe et al., 1994). Erv25p and Emp24p, components of COPII coats, are considered candidates for the "adaptors" required by the general vesicular model for packaging of both soluble and membrane-bound proteins (Belden and Barlowe, 1996). A role for lectin-type molecules (e.g. ERGIC-53) in selective cargo transport has also been suggested (Fiedler and Simons, 1995). Specificity in docking of COP-coated vesicles, as was shown originally for synaptic vesicles (Söllner et al., 1993), is provided by the pairing, in a lock-and-key manner, of single representatives from each of two families of integral membrane proteins termed SNAREs (SNAP receptors). SNARE proteins are localized to both vesicle and donor membranes (v-SNAREs) and to the target membranes (t-SNAREs). Also required in the reaction are soluble protein factors including NSF (N-ethylmaleimide-sensitive factor, called Sec18p in yeast) and α-SNAP (α-soluble NSF attachment protein, Sec17p in yeast). In yeast, both COPI and COPII-coated vesicles are known to carry the v-SNAREs Bet1p, Bos1p and Sec22p (Bednarek et al., 1995), and are thought to interact with the Golgi t-SNAREs Sed5p (Banfield et al., 1994) and Ufe1p (Lewis and Pelham, 1996). Mammalian SNAREs involved in early secretory transport include rsec22a, GOS-28, syntaxin 5, rbet 1, rsec22b and membrin (see Hay and Scheller (1997) and references within).

Part III. Clathrin Coat Proteins

In animals, trafficking of proteins in the late biosynthetic/secretory and endocytic pathways is mediated by another class of transport vesicles with distinctive

lattice-like protein coats. These vesicles, observed in electron micrographs in the 1960's (Rosenbluth and Wissig, 1963; Rosenbluth and Wissig, 1964; Roth and Porter, 1964), were first isolated in the mid 1970's. The major coat protein was named clathrin, meaning "lattice-like", in description of the vesicles' appearance in electron micrographs (Pearse, 1975). Clathrin-coated vesicles have also been isolated from budding yeast and plant tissues suggesting they are a universal transport motif in eukaryotes. Most clathrin research has focused on mammalian tissues (primarily brain). Thus, our knowledge of clathrin-mediated mechanisms of protein sorting, budding, and transport in non-mammalian systems is severely limited.

Mammalian clathrin, the structural protein of the vesicle coat, consists of three heavy chains (HCs) of 192 kD and three light chains (LCs) of ~ 30 kD assembled to form the three legged structure, the triskelion, which is the building block of the coat's polygonal lattice. Three extended HC molecules, each bound to a single LC (one of two, LCa or LCb in mammals), are bound through their carboxy termini at the vertex of the triskelion. This trimerization domain, small globular domains at the extreme C-terminus of each HC, and the LCs bound to the proximal portions of the HCs form the central hub of the molecule. The legs, with globular N-terminal domains, are joined to the hub through a protease sensitive bend or "knee". Isolated triskelions, under appropriate conditions, spontaneously reassemble into polyhedral cages. It is thought that, *in vivo*, under appropriate control, triskelion assembly sculpts out the bud from the underlying membrane. Data suggest that the LCs have a role in regulating HC dynamics. *In vivo*, LCs confer both pH sensitivity (Liu et al.,

1995) and cation-dependence to assembly (Ungewickell and Ungewickell, 1991). In addition LCs are subject to phosphorylation, and bind Ca^{2+} and calmodulin (Mooibroek et al., 1987). Recent work using yeast HC mutants demonstrates that LCs bind to the trimerization domain of HC, suggesting regulation of HC conformation through LC modulation (Pishvaee et al., 1997). HC trimerization has been shown to be defective in LC deficient yeast (Huang et al., 1997).

In addition to clathrin, coats of CCVs include a class of proteins which were first shown to stimulate assembly of clathrin, under physiological conditions of pH and ionic strength, into empty "coats" resembling coated vesicles in size and regularity (Zaremba and Keen, 1983). These proteins were termed assembly proteins, or APs. APs exist both as heterotetromeric complexes (AP1 and AP2) and as monomers (AP180 and auxilin) which co-purify during size exclusion chromatography of CCV coats. While the two major AP complexes are expressed ubiquitously in mammals, their relative distribution differs among tissues (Ungewickell et al., 1994). Immunofluorescence microscopy has localized AP2 to the plasma membrane, while AP1 localizes to the *trans*-Golgi (Ahle et al., 1988). The molecular structure and morphology of the two complexes are similar, consisting of two ~100 kD molecular weight subunits ($\alpha/\beta 2$ for AP2, $\gamma/\beta 1$ for AP1) termed "adaptins", and single copies of the ~ 50 kD medium subunit (either $\mu 2$ or $\mu 1$) and the ~20 kD small subunits (either $\sigma 2$ or $\sigma 1$). Sequence homology suggest that while the β-adaptins are highly conserved (Ponnambalam et al., 1990) α and γ are more divergent. Interaction between 14 *armadillo*-gene like repeats (short α-helices

separated by exposed joining loops) in the amino terminal portions of the β-subunits and the μ- and σ-subunits may be important for assembly of the functional heteromeric complex (Traub, 1997). These repeats have been identified in a number of proteins, which are similar only in their common propensity for interaction with other proteins. Sequence homologies of ~50% exist between the μ and σ subunits of AP1 and AP2. By application of a freeze-etch technique and platinum staining, Heuser and Keen (1988) demonstrated that AP2 is composed of a core "brick-like" structure with appendages or "ears" attached by flexible stalks; AP1 is thought to have a similar structure. AP180 and auxilin, specific to neuronal tissues, have structures and sequences unrelated to either AP1 or AP2. By sequence and biochemical analysis, auxilin has been shown to have both a clathrin-binding site and a DnaJ motif. This motif, similar in sequence to that of a cofactor for a bacterial hsc70 homolog, interacts with the CCV-uncoating ATPase, Hsc70. Uncoating of unmodified (native) clathrin requires both the clathrin-binding and DnaJ sites (Holstein et al., 1996). Although AP180 displays the highest specific assembly activity of any known AP and competes with AP2 for binding to intact clathrin cages, its role *in vivo* is unknown (Lindner and Ungewickell, 1992).

While AP1 and AP2 clathrin-coated vesicles were the first identified, and are still the best characterized, recent studies have demonstrated that clathrin, and clathrin/adaptor homologs participate in other types of vesicular structures, both clathrin dependent and clathrin independent. A human gene, HC22, encoding a clathrin heavy-chain-like protein, is expressed predominantly in skeletal muscle.

Involvement of this homolog with muscle cell endocytosis, the formation of unusual lattice morphologies associated with adhesion plaques or postsynaptic membrane recycling, or organization of cytoskeletal elements has been postulated (Brodsky (1997) and references within). Novel clathrin-coated vesicles, which lack both α- and γ-adaptins, have been show to bud from endosomes (Stoorvogel et al., 1996). In addition to the novel clathrin and clathrin-like structures lacking conventional adaptors, a number of adaptor homologs participate in structures independent of clathrin. A cDNA clone encoding a novel 47 kD protein from the electric ray *Discopyge ommata* was used to identify two μ-subunit homologs, p47A and p47B from a rat spinal cord cDNA library. While p47B was shown to be neuron specific, p47A mRNA was expressed in other organs as well as in somatic tissue (Pevsner et al., 1994). Immunoprecipitation using polyclonal anti-p47 showed p47B protein to be associated with three other neuron-specific proteins, including β-NAP, a novel β1/β2 homolog of an adaptor complex found on non-clathrin coated vesicles which bud from the TGN (Simpson et al., 1996). More recently, p47A has been shown to be a likely component of a large, ubiquitously expressed complex, which includes two AP σ-subunit homologs, as well as a 140 kD protein homologous to β-NAP (Dell'Angelica et al., 1997). Finally, the genes for two additional μ-adaptin-related proteins, μ-ARP1 and μ-ARP2 have been cloned and shown to be expressed in a large number of tissues (Wang and Kilimann, 1997).

Part IV. Properties of AP Complexes Determine CCV Function

Clathrin-coated vesicles have been implicated in the endocytosis of transmembrane receptors, membrane recycling, transepithelial transport, and the intracellular transport of Golgi derived secretory and lysosomal proteins. Central to this multifunctional task are the capabilities of APs to bind clathrin and promote coat assembly, as well as to bind membranes, provide targeting information, interact with sorting signals present on transmembrane receptors and interact with soluble and lipid-bound phosphorylated metabolites of inositol.

AP-clathrin binding involves two binding sites on clathrin HC, one on the proximal leg or "hub", and the second on the amino-terminal domain (Murphy and Keen, 1992; Keen et al., 1991). Both the "ear" and core domains of the β-adaptins appear to be required for clathrin polymerization, since elastase-treated APs no longer support coat assembly (Zaremba and Keen, 1985). In addition, when assembled coats are proteolytically treated, the APs dissociate, leaving intact cages, plus cleaved ear and core fragments (Schroder, 1991). Alternatively, Traub et al. (1995), showed that while proteolytically cleaved ear and core fragments of β1-adaptin lacking the hinge region bind to Golgi membranes in the presence of GTPγS, only intact β1 recruits clathrin. This suggests a role for the hinge region in clathrin assembly. Using recombinant β core fragments, the hinge region was shown to be essential for coat assembly *in vitro*, and the clathrin-binding site mapped to a 50-residue region in the center of the hinge which appears to be conserved in all known β-subunits in multicellular organisms (Shih and Gallusser, 1995). While limited information is

available, interactions between the β-adaptins and other AP subunits also seems likely to be required for clathrin assembly onto membranes *in vivo*.

APs serve not only to mediate assembly of clathrin, but also to bind clathrin to specialized regions of donor membranes. The AP2 core region was shown to bind both plasma membrane (of cultured fibroblasts) and triskelions during coat assembly, forming pits which invaginated normally (Peeler et al., 1993). Similarly, Traub et al. (1995) showed that the structural determinants required for binding of AP1 to Golgi membranes also reside in the core region. Further, targeting of the APs to their appropriate membranes is dependent on a 200-residue region within the N-terminal core of both α and γ subunits which interacts with medium and small adaptor subunits. Thus, is seems likely that targeting determinants may reside in the smaller subunits themselves (Page and Robinson, 1995). Numerous homologs of AP medium and small subunits in the mammalian and yeast genomes have been identified by sequence analysis (see above). This heterogeneity suggests a role for these subunits in providing specificity in targeting. Other proteins show homology to coatomer subunits. Thus, it appears that AP1 and AP2 medium and small units may be members of larger families of targeting proteins (Brodsky, 1997 and references within).

Interactions of adaptor proteins with sorting signals present in the cytoplasmic tails of transmembrane receptors are thought to drive cargo loading into CCVs, both at the PM and the TGN. At the PM, endocytosis is mediated by three types of receptors which are either constitutively concentrated, recruited into coated pits only

upon ligand-binding or retained on the PM until released by some signaling event (Schmid, 1997). AP2 binds constituitively concentrated receptors such as the LDL-receptor, the transferrin receptor, the asialoglycoprotein receptor, and the cation independent 276 kD mannose 6-phosphate/insulin-like growth factor II (CI Man-6-P/IGF-II) receptor. The latter is involved in retrieval of escaped lysosomal enzymes as well as transport of lysosomal hydrolases via TGN derived CCVs. Lysosomal acid phosphatase (LAP), an integral membrane protein which appears to exit the TGN via a clathrin-independent "bulk-flow" mechanism, is also internalized constituitively via CCVs. These receptors possess tyrosine-based sorting signals of the form YppØ (tyrosine-polar-polar-hydrophobic), which, through interaction with the μ2-subunit, govern sorting in the endocytic pathway (Boll et al., 1996).

Unlike constitutively internalized nutrient receptors, the epidermal growth factor receptor (EGF-R) requires activation by its ligand (EGF) to be efficiently internalized from the PM via CCVs (Sorkin and Carpenter, 1993). However, in similarity to the nutrient receptors, a tyrosine-sorting motif present in the 48 amino acid cytoplasmic regulatory domain of the EGF-R has been shown to interact with AP2. Additional regulatory mechanisms or co-factors may be required for ligand-activated internalization of EGF-R, since mutant EGF-R lacking a kinase domain was not internalized. Phosphorylation was not required for AP-2 binding, suggesting an indirect effect of the receptor kinase on internalization. Eps15, known to be a phophorylation substrate of the EGF-R kinase, has been shown to bind α-adaptin, and

may be a component of a putative docking complex required for targeting AP-2 to the PM (Benmerah et al., 1996; Tebar et al., 1996).

A novel mechanism for regulation of endocytosis appears to be provided by a class of receptors retained on the PM of certain cell types until released by some signaling event. CD4, a type I transmembrane glycoprotein which recognizes non-polymorphic regions of the class II major histocompatibility complex (MHC), has been shown to be constiutively internalized and recycled in non-lymphoid cells. However, CD4 is actively excluded from coated pits (and thus not endocytosed) in lymphocytic cells by the formation of a stable complex with the protein tyrosine kinase $p56^{lck}$. Upon phosphorylation of CD4 and $p56^{lck}$ by protein kinase C the complex dissociates, CD4 enters coated pits and is internalized (Pelchen-Matthews et al., 1991; Pelchen-Matthews et al., 1992).

Clathrin-mediated sorting of other endocytic receptors, such as the low affinity IgG Fc receptor (FcRII-B2) on macrophages required for targeting of soluble antigen-antibody complexes for lysosomal degradation, appears to utilize a di-leucine motif for internalization and basolateral sorting (Hunziker and Fumey, 1994). Targeting of the lysosomal membrane glycoprotein limp-II requires a similar Leu-Ile motif in its cytoplasmic tail (Ogata and Fukuda, 1994).

The tyrosine-sorting motif, although first shown to be required for endocytosis of PM receptors, also functions as a sorting motif for the TGN, both for transport of endosomal proteins and recycling of TGN proteins. TGN38, a type I integral membrane protein of the TGN thought to play role in sorting of "secretory/exocytic"

proteins, requires a tyrosine-sorting motif present in its cytoplasmic domain for recycling from the PM to the TGN via clathrin-coated pits (Stanley and Howell, 1993). Recently, using the yeast two-hybrid system to evaluate adaptor/sorting signal interactions, the μ2-subunit of AP2 was shown to interact with the tyrosine-sorting motif of TGN38. In addition, the μ1-subunit of AP1 was seen to interact with the tyrosine-sorting signals of lamp-1, CD68, and H2-Mb, all of which are targeted to the endosome at the TGN (Ohno et al., 1995). The cytosolic domains of lamp-1, a lysosomal membrane glycoprotein localized to AP1 positive vesicles and tubules of the TGN , binds both AP1 and AP2 (Höning et al., 1996).

The best characterized vesicle-mediated sorting process of eukaryotic cells is that of the soluble mammalian lysosomal hydrolases which is mediated by the two mannose 6-phosphate receptors, the 46 kD cation dependent (CD Man-6-P) and the cation independent 276 kD mannose 6-phosphate/insulin-like growth factor II (CI Man-6-P/IGF-II). Precursors of lysosomal hydrolases leave the ER bearing N-linked oligosaccharides with terminal mannose residue(s) which are further modified in the *cis*-Golgi by transfer of GlcNAc-phosphate to the mannose residue by GlcNAc phosphotransferase. Cleavage of the GLcNAc by a phosphoglycosidase creates the mannose 6-phosphate marker recognized by the two Man 6-P receptors. Clathrin-mediated interaction with these receptors was first demonstrated by the formation of spherical aggregates capable of binding clathrin to form recognizable coat structures from purified clathrin adaptors and receptors (Pearse, 1985). Subsequent cloning of the two receptors led to the identification of several potential AP binding sites in their

cytoplasmic tails (Lobel et al., 1987; Pohlmann et al., 1987). Binding of both AP1 and AP2 to the immobilized cytoplasmic tail of the CI Man-6-P receptor was shown to be saturable and independent of the binding of the other adaptor complex. Mutational analysis allowed localization of AP2 interaction to the tyrosine containing region of the cytoplasmic tail (Glickman et al., 1989). A His-Leu-Leu site in the CD Man 6-P receptor was shown to be necessary for lysosomal function and the sorting signals of the CI Man 6-P receptor were further characterized (Johnson and Kornfeld, 1992a; Johnson and Kornfeld, 1992b). Phosphorylation of casein kinase sites adjacent to the di-leucine motifs was shown to be important for efficient sorting of lysosomal hydrolases by both Man 6-P receptors (Chen et al., 1993; Mauxion et al., 1996).

While characterization of the binding-site interactions of the Man 6-P receptors and clathrin adaptors proceeds, recent studies have begun to concentrate on cargo proteins, and the functional interaction of vesicle cargo recruitment and coat formation and regulation of vesicular transport *in vivo*. It appears that in mammalian cells, while each Man 6-P receptor transports the same lysosomal proteins neither receptor can substitute for the loss of the other *in vivo*, because each carries a distinct complement of proteins. Evolution of the two receptors is thought to be driven by the heterogeneity of Man 6-P markers and the inability of a single receptor to efficiently transport the full complement of hydrolases required for lysosomal functioning (Pohlmann et al., 1995). Expression levels of the Man 6-P receptors determine both the degree of AP1 binding and the number of TGN-derived clathrin-coated vesicles

present in mouse fibroblasts, demonstrating that the processes of sorting and vesicle formation are coupled (Le Borgne and Hoflack, 1997).

Recent evidence suggests that additional regulatory complexity in cargo recruitment/sorting and vesicle formation will be demonstrated in the next few years. Cytosolic factors such as ARF, a GTPase required for coatomer binding to Golgi membranes, have been shown to be required for binding of AP1 to isolated Golgi membranes (Stamnes and Rothman, 1993), as well as for high affinity binding of AP1 to the CI Man 6-P receptor (Le Borgne et al., 1996). There is increasing evidence that certain phosphoinositides and phosphoinositols are crucial regulatory factors in transport processes involving clathrin-coated membranes. In yeast, temperature sensitive mutants of VPS34 (which encodes a PtdIns-3 kinase) exhibit defective protein sorting from the Golgi to the vacuole at the restrictive temperature. In mammals p85-p110 , a similar PI-3 kinase, is co-internalized with the platelet-derived growth factor (PDGF) receptor. When interaction of the kinase with the PDGF-R is blocked, defective sorting and degredation of the receptor is observed, independent of the actual internalization event, indicating p85-p110 is involved, not in vesicle formation, but later in the endocytic pathway (De Camilli et al., 1996). The α-subunit of AP2 is known to bind proteins with known links to phosphotidylinositol metabolism such as synaptotagmin (Zhang et al., 1994), as well as to phosphoinositides and inositol polyphosphates (Beck and Keen 1991; Timerman et al., 1992; Voglmaier et al., 1992; Gaidarov et al., 1996). Recently, it was shown that phosphoinositides with a phosphate at the D-3 position of the inositol ring enhanced

binding of AP2 to the tyrosine-sorting motif of TGN38, further demonstrating a role for phosphoinositides as regulators in clathrin-mediated vesicular traffic (Rapoport et al., 1997). Other proteins involved in clathrin-mediated trafficking, such as AP180 and dynamin, also bind inositol phosphates or phosphoinositides (De Camilli et al., 1996). In addition, PtdIns(4,5)P_2 has been shown to stimulate the guanine nucleotide exchange activation of ARF, known to be required for binding of AP1 to Golgi membranes (De Camilli et al., 1996).

Part V. Clathrin-Mediated Trafficking in Plants

While our knowledge of CCVs function in recycling of both receptors and membranes and in the intracellular transport of Golgi-derived lysosomal proteins in mammals is substantial, our understanding of the involvement of clathrin-coated vesicles in endocytosis, membrane processing and intracellular protein trafficking in plants is still rudimentary. Early evidence for endocytosis in plants comes from the work of Fowke et al. (1991) and Joachim and Robinson (1984). Cationized ferritin was localized to PM coated pits of soybean protoplasts, and over time, followed through PM buds, structurally distinct coated vesicles, and ultimately, the partially coated reticulum (PCR). The PCR is thought to be the plant equivalent of the TGN. Two additional studies suggest that, in plants, CCVs are active in retrieval of plasma membrane via endocytosis. Smooth secretory vesicles carry Golgi-derived matrix polysaccharides to the cell wall during cell plate formation, but no buildup of plasma membrane is observed (Staehelin and Moore, 1995). Eight times the number of

coated pits are observed in cell-wall forming tobacco protoplasts than in protoplasts incapable of forming cell walls (Fowke et al., 1983). Receptor-mediated endocytosis has been well established in animal systems, but hard supporting evidence in plant systems is limited to a few studies on uptake of fungal elicitor proteins and biotin (Horn et al., 1989; Low and Chandra 1994). A role for CCVs in protein sorting and intracellular protein transport has also been postulated. Developing cotyledons of legumes actively deposit storage proteins in large protein storage vacuoles termed "protein bodies" and early studies suggested that CCVs of garden pea carry precursors of lectin as well as other vacuolar proteins such as hydrolytic enzymes (Harley and Beevers, 1989) and the storage proteins legumin and vicilin (Robinson et al., 1989; Robinson et al., 1991; Hoh et al., 1991). However, recent studies suggest that dense vesicles, not CCVs, transport legumin and vicilin from the trans-Golgi network to protein storage vacuoles (Hohl et al., 1996). Since more than one type of vacuole is present in storage parenchyma (Hohl et al., 1996; Paris et al., 1996) CCVs may well participate in transport of other vacuolar proteins to other types of vacuoles. The isolation of a putative vacuolar transport receptor which binds to the N-terminal vacuolar targeting domain of barley aleurain and sweet potato sporamin (Kirsch et al., 1994) from a CCV-enriched fraction of pea cotyledon supports this sorting/transport role for CCVs.

REFERENCES

Ahle S, Mann A, Eichelsbacher U, Ungewickell E (1988) Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. EMBO J **7:** 919-929

Aniento F, Gu F, Parton RG, Gruenberg J (1996) An endosomal β-COP is involved in the pH-dependent formation of transport vesicles destined for late endosomes. J Cell Biol **133:** 29-41

Balch W, Dunphy W, Braell W, Rothman J (1984) Reconstitution of the transport of protein between successive compartments of the Golgi measured by coupled incorporation of N-acetylglucosamine. Cell **39:** 405-416

Banfield DK, Lewis MJ, Rabouille C, Warren GA, Pelham HRB (1994) Localization of Sed5, a putative vesicle targeting molecule, to the *cis*-Golgi network involves both its transmembrane and cytoplasmic domains. J Cell Biol **127:** 357-371

Barlowe C, Orci L, Yeung T, Hosobuchi M, Hamamoto S, Salama N, Rexach MF, Ravazzola M, Amherdt M, Schekman R (1994) COPII: A membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. Cell **77:** 895-907

Beck KA, Keen JH (1991) Interaction of Phosphoinositide Cycle Intermediates with the Plasma Membrane-associated Clathrin Assembly Protein AP-2. J Biol Chem **266:** 4442-4447

Bednarek SY, Ravazzola M, Hosobuchi M, Amherdt M, Perrelet A, Sheckman R, Orci L (1995) COPI- and COPII-coated vesicles bud directly from the endoplasmic reticulum in yeast. Cell **83:** 1183-1196

Belden WJ, Barlowe C (1996) Erv25p, a components of COPII-coated vesicles, forms a complex with Emp24p that is required for efficient endoplasmic reticulum to Golgi transport. J Biol Chem **271:** 26939-26946

Benmerah A, Bègue B, Dautry-Varsat A, Cerf-Bensussan N (1996) The ear of α-adaptin interacts with the COOH-terminal domain of the Eps15 protein. J Biol Chem **271:** 12111-12116

Boll W, Ohno H, Songyang Z, Rapoport I, Cantley LC, Bonificino JS, Kirchhausen T (1996) Sequence requirements for the recognition of tyrosine-based endocytic signal by clathrin AP-2 complexes. EMBO J **15:** 5789-5795

Brodsky FM (1997) New fashions in vesicle coats. Trends Cell Biol **7:** 175-179

Chen HJ, Remmler J, Delany JC, Messner DJ, Lobel P (1993) Mutational analysis of the cation-independent mannose 6-phosphate insulin-like growth factor II receptor. A consensus casein kinase II site followed by 2 leucines near the carboxyl terminus is important for intracellular targeting of lysosomal enzymes. J Biol Chem **268:** 22338-22346

Cline K, Henry R (1996) Import and routing of nucleus-encoded chloroplast proteins. Annu Rev Cell and Dev Biol **12:** 1-26

Cosson P, Demolliere C, Hennecke S, Duden R, LeTourneur F (1996) δ- and ξ-COP, two coatomer subunits homologous to clathrin associated proteins, are involved in ER retrieval. EMBO J **15:** 1792-1798

Cosson P, Letourneur F (1994) Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. Science **263:** 169-1631

d'Enfert C, Gensse M, Gaillardin C (1992) Fission yeast and a plant have functional homologues of the Sar1 and Sec12 proteins involved in ER to Golgi traffic in budding yeast. EMBO J **11:** 4205-4211

De Camilli P, Emr SD, McPherson PS, Novick P (1996) Phosphoinositides as regulators in membrane traffic. Science **271:** 1533-1539

Dell'Angelica EC, Ohno H, OOi CE, Rabinovick E, Roche KW, Bonifacino JS (1997) AP-3: an adaptor-like protein complex with ubiquitous expression. EMBO J **16:** 917-928

Donaldson JG, Cassel D, Kahn RA, Klausner, RD (1992) ADP-ribosylation factor, a small GTP-binding protein, is required for the binding of the coatomer β-COP to Golgi membranes. Proc Natl Acad Sci USA **89:** 6408-6412

Duden R, Griffiths G, Frank R, Argos P, Kreis TE (1991) β-COP, a 110 kD protein associated with non-clathrin coated vesicles and the Golgi complex shows homology to β-adaptin. Cell **64:** 649-665

Erdmann R, Veenhuis M, Kunau W-H (1997) Peroxisomes: organelles at the crossroads. Trends Cell Biol **7:** 400-407

Fiedler K, Simons K (1995) The role of N-glycans in the secretory pathway. Cell **81:** 309-312

Fiedler K, Veit M, Stammes MA, Rothman JE (1996) Bimodal interaction of coatomer with the p24 family of putative cargo receptors. Science **273:** 1396-1399

Fowke LC, Griffing LR, Mersey BG, Van der Valk P (1983) Protoplasts for studies of plasma membrane and associated cell organelles. Experientia Suppl **46:** 101-110

Fowke LC, Tanchek MA, Galway ME (1991) Untrastructural cytology of the endocytotic pathway in plants. *In* R Hawes, JOD Coleman, DE Evans eds, Endocytosis, Exocytosis and Vesicle Traffic in Plants.: Cambridge University Press, Cambridge, pp15-40

Gaidarov I, Chen Q, Falck JR, Reddy KK, Keen JH (1996) A functional phopatidylinositol 3,4,5-trisphosphate/phosphinositide binding domain in the clathrin adaptor AP-2 α Subunit. J Biol Chem **271:** 20922-20929

Glickman JN, Conibear E, Pearse BMF (1989) Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth factor II receptor. EMBO J **8:** 1041-1047

Griffiths G, Pfeiffer S, Simons K, Matlin K (1985) Exit of newly synthesized membrane proteins from the *trans* Golgi cisterna of the Golgi complex to the plasma membrane. J Cell Biol **101:** 949-964

Harley SM, Beevers L (1989) Coated vesicles are involved in the transport of storage proteins during seed development in *Pisum sativum* L. Plant Physiol **91:** 674-678

Hay JC, Scheller RH (1997) SNAREs and NSF in targeted membrane fusion. Curr Op Cell Biol **9:** 505-512

Heuser JE, Keen J (1988) Deep-etch visualization of proteins involved in clathrin assembly. J Cell Biol **107:** 877-886

Hicke L, Schekman R (1989) Yeast Sec23p acts in the cytoplasm to promote protein transport from the endoplasmic reticulum to the Golgi complex *in vivo* and *in vitro*. EMBO J **8:** 1677-1684

Hicke L, Yoshihisa T, Schekman R (1992) Sec23p and a novel 105-kDa protein function as a multimeric complex to promotes vesicle budding and protein transport from the endoplasmic reticulum. Mol Biol Cell **3:** 667-676

Hicks GR, Raikel NV (1995) Protein import into the nucleus: An integrated view. Annu Rev Cell Devel Biol **11:** 155-188

Hoh B, Schauermann G, Robinson DG (1991) Storage protein polypeptides in clathrin coated vesicle fractions are not due to endomembrane contamination. J Plant Physiol **138:** 309-316

Hohl I, Robinson DG, Chrispeels MJ, Hinz G (1996) Transport of storage proteins to the vacuole is mediated by vesicle without a clathrin coat. J Cell Sci **109:** 2539-2550

Holstein SEH, Ungewickell H, Ungewickell E (1996) Mechanism of clathrin basket dissociation: Separate functions of protein domains of the DnaJ homologue auxilin. J Cell Biol **135:** 925-937

Höning S, Griffith J, Geuze HJ, Hunziker W (1996) The tyrosine-based lysosomal targeting signal in lamp-1 mediates sorting into Golgi-derived clathrin-coated vesicles. EMBO J. **15:** 5230-5239

Horn MA, Heinstein PF, Low PS (1989) Receptor-mediated endocytosis in plant cells. Plant Cell **1:** 1003-1009

Huang KM, Gullberg L, Nelson KK, Stefan CJ, Blumer K (1997) Novel functions of clathrin light chains: Clathrin heavy chain trimerization is defective in light chain-deficient yeast. J Cell Sci **110:** 899-910

Hunziker W, Fumey C (1994) A di-leucine motif mediates endocytosis and basolateral sorting of macrophage IgG Fc receptors in MDCK cells. EMBO J **13:** 2963-2969

Joachim S, Robinson DG (1984) Endocytosis of cationic ferritin by bean leaf protoplasts. Eur J Cell Biol. **34:** 212-216

Johnson K, Kornfeld S (1992a) A His-Leu-Leu sequence near the carboxyl terminus of the cytoplasmic domain of the cation-dependent mannose 6-phosphate receptor is necessary for the lysosomal enzyme sorting function. J. Biol. Chem. **267:** 17110-17115

Johnson K, Kornfeld S (1992b) The cytoplasmic tail of the mannose -6-phosphate/insulin-like growth factor -II has two signals for lysosomal enzyme sorting in the Golgi. J Cell Biol. **119:** 249-257

Keen J, Beck KA, Kirchhausen T, Jarrett, T (1991) Clathrin domains involved in recognition by assembly protein AP-2. J Biol Chem **266:** 7950-7956

Kirsch T, Paris N, Butler JM, Beevers L, Rogers JC (1994) Purification and initial characterization of a plant vacuolar targeting receptor. Proc Nat Acad Sci USA **91:** 3403-3407

Le Borgne R, Griffiths G, Hoflack B (1996) Mannose-6-Phosphate Receptors and ADP-ribosylation Factors Cooperate for High Affinity Interaction of the AP-1 Golgi Assembly Proteins with Membranes. J Biol Chem **271:** 2162-2170

Le Borgne R, Hoflack B (1997) Mannose 6-phosphate receptors regulate the formation of clathrin-coated vesicles in the TGN. J Cell Biol **2:** 335-345

Lewis MJ, Pelham HR (1996) SNARE-mediated retrograde traffic from the Golgi complex to the endoplasmic reticulum. Cell **85:** 205-215

Lindner R, Ungewickell E (1992) Clathrin-associated proteins of bovine brain coated vesicles. An analysis of their number and assembly-promoting activity. J Biol Chem **267:** 16567-16573

Liscovitch M, Cantley LC (1995) Signal transduction and membrane traffic: The PITP/phosphoinositide connection. Cell **81:** 659-662

Liu S-H, Wong ML, Craik CS, Brodsky FM (1995) Regulation of clathrin assembly and trimerization defined using recombinant triskelion hubs. Cell **83:** 257-267

Lobel P, Dahmn NM, Breitmeyer J, Chirwin JM, Kornfeld S (1987) Cloning of the bovine 215-kDa cation independent mannose-6-phosphate receptor. Proc Natl Acad Sci USA **84:** 2233-2237

Low PS, Chandra S (1994) Endocytosis in plants. Annu Rev Plant Physiol Plant Mol Biol **45:** 609-631

Malhotra V, Serafini T, Orci L, Shepherd JC, Rothman JE (1989) Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. Cell **58:** 329-336

Mauxion F, Leborgne R, Lehmann-Munier H, Hoflack B (1996) A casein kinase II phosphorylation site in the cytoplasmic domain of the cation-dependent mannose 6-phosphate receptor determines the high affinity interaction of the AP-1 Golgi assembly proteins with membranes. J Biol Chem **271:** 2171-2178

Miesenböck G, Rothman JE (1995) The capacity to retrieve escaped ER proteins extends to the *trans*- most cisternae of the Golgi stack. J Cell Biol **129:** 309-319

Misteli T,Warren G. (1994) COP-coated vesicles are involved in the mitotic fragmentation of Golgi stacks in a cell-free system. J Cell Biol **125:** 269-282

Mooibroek M, Michiel DF, Wang HH (1987) Clathrin light chains are calcium-binding proteins. J Biol Chem **262:** 25-28

Murphy J-E, Keen JH (1992) Recognition sites for clathrin associated protein AP-2 and protein AP-3 on clathrin triskelia. J Biol Chem **267:** 10850-10855

Nakano A, Muramatsu M (1989) A novel GTP-binding protein, SAR1p, is involved in transport from the endoplasmic reticulum to the Golgi apparatus. J Cell Biol **109:** 2677-2691

Neer EJ, Schmidt CJ, Nambudripad R, Smith TF (1994) The ancient regulatory-protein family of WD-repeat proteins. Nature **371:** 297-300

Ogata S, Fukuda M (1994) Lysosomal targeting of Limp II membrane glycoprotein requires a novel Leu-Ile motif at a particular position in cytoplasmic tail. J Biol Chem **269:** 5210-5217

Ohno H, Stewart J, Fournier M-C, Bosshart H, Rhee I, Miyatake S, Saito T, Gallusser A, Kirchhausen T, Bonifacino JS (1995) Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science **269:** 1872-1875

Oprins A, Duden R, Kreis TE, Geuze HJ, Slot JW (1993) β-COP localizes mainly to the cis-Golgi side in exocrine pancreas. J Cell Biol. **121:** 49-59

Orci L, Ravazzola M, Louvard D, Perrelet A (1984) Distribution asymétrique de la clathrine au niveau de l'appareil de Golgi de cellules sécrétoires á polypeptides. C R Acad Sci **299:** 697-702

Orci L, Ravazzola M, Meda P, Holcomb C, Moore H-P, Hicke L, Schekman R (1991) Mammalian Sec23p homologue is restricted to the endoplasmic reticulum transitional cytoplasm. Proc Nat Acad Sci USA **88:** 8611-8615

Orci L, Glick BS, Rothman JE (1986) A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. Cell **46:** 171-184

Page LJ, Robinson MS (1995) Targeting signals and subunit interactions in coated vesicle adaptor complexes. J Cell Biol **131:** 619-630

Palmer DJ, Helms JB, Beckers CJM, Orci L, Rothman JE (1993) Binding of coatomer to Golgi membranes requires ADP-ribosylation factor. J Biol Chem **268:** 12083-12089

Paris N, Stanley CM, Jones RL, Rogers JC (1996) Plant cells contain two functionally distinct vacuolar compartments. Cell **85:** 563-572

Pearse BMF (1975) Coated vesicles from pig brain: purification and biochemical characterization. J Mol Biol **97:** 93-98

Pearse BMF (1985) Assembly of the mannose-6-phosphate receptor into reconstituted clathrin coats. EMBO J. **4:** 2457-2460

Peeler JS, Donzell W, Anderson RGW (1993) The appendage domain of the AP-2 subunit is not required for assembly or invagination of clathrin-coated pits. J Cell Biol **120:** 47-54

Pelchen-Matthews A, Armes JE, Griffiths G, Marsh M (1991) Differential endocytosis of CD4 in lymphocytic and nonlymphocytic cells. J Exp Med **173:** 575-587

Pelchen-Matthews A, Boulet I, Littman DR, Ragard R, Marsh M (1992) The protein kinase $p56^{lck}$ inhibits CD4 endocytosis by preventing entry of CD4 into coated pits. J Cell Biol **117:** 279-290

Peter F, Plutner H, Zhu H, Kreis TE, Balch WE (1993) β-COP is essential for transport of protein from the endoplasmic reticulum to the Golgi *in vitro*. J Cell Biol **122:** 1155-1167

Pevsner J, Volknandt W, Wong B, Scheller RH (1994) Two rat homologs of clathrin-associated adaptor proteins. Gene **146:** 279-283

Pishvaee B, Munn A, Payne GS (1997) A novel structural mode for regulation of clathrin function. EMBO J. **16:** 2227-2239

Pohlmann R, Boeker M, Wendland C, von Figura K (1995) The two mannose 6-phosphate receptors transport distinct complements of lysosomal proteins. J Biol Chem **270:** 27311-27318

Pohlmann R, Nagel G, Schmidt B, Stein M, Lorkowsky G, Krentler C, Cully J, Meyer HE, Grzeschik K-H, Mersmann G, Hasilik A, Von Figura K (1987) Cloning of a cDNA encoding the human cation-dependent mannose 6-phosphate-specific receptor. Proc Natl Acad Sci USA **84:** 5575-5579

Ponnambalam S, Robinson MS, Jackson AP, Peiperl L, Parham P (1990) Conservation and diversity in families of coated vesicle adaptins. J Bio Chem **265:** 4814-4820

Presley JF, Cole NB, Schroer TA, Hirschberg K, Zaal KJM, Lippincott-Schwartz J (1997) ER-to Golgi transport visualized in living cells. Nature **389:** 81-85

Pryer NK, Salama NR, Schekman RW, Kaiser CA (1993) Cytosolic Sec13 complex is required for vesicle formation from the endoplasmic reticulum *in vitro*. J Cell Biol **120:** 867-875

Rapoport I, Miyazaki M, Boll WDB, Cantley LC, Shoelson S, Kirchhausen, T (1997) Regulatory interactions in the recognition of endocytic sorting signals by AP-2 complexes. EMBO J **16:** 2240-2250

Rexach MF, Schekman RW (1991) Distinct biochemical requirements for the budding, targeting, and fusion of ER-derived transport vesicles. J Cell Biol **114:** 219-229

Roberg KJ, Rowley N, Kaiser CA (1997) Physiological regulation of membrane protein sorting late in the secretory pathway of *Saccharomyces cerevisiae*. J Cell Biol **137:** 1469-1482

Robinson DG, Balusek K, Depta H, Hoh B, Holstein SEH (1991) Isolation and characterization of plant coated vesicles. *In* C Hawes, D Evans, J Coleman eds. Endocytosis, Exocytosis and Vesicle Traffic in Plant Cells,Cambridge: University Press, Cambridge, pp 65-79

Robinson DG, Balusek K, Freundt H (1989) Legumin antibodies recognize polypeptides in coated vesicles from developing pea. Protoplasma **150:** 79-82

Rosenbluth J, Wissig SL (1963) The uptake of ferritin by toad spinal ganglion cells. J Cell Biol. **19:** 91A

Rosenbluth J, Wissig SL (1964) The distribution of exogenous ferritin in toad spinal ganglia and the mechanism of its uptake by neurons. J Cell Biol **23:** 307-325

Roth TF, Porter KR (1964) Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti* L. J Cell Biol **20:** 313-332

Rothman JE, Wieland FT (1996) Protein sorting by transport vesicles. Science **272:** 227-234

Salama NR, Schekman RW (1995) The role of coat proteins in the biosynthesis of secretory proteins. Curr Op in Cell Biol **7:** 536-543

Salama NR, Yeung T, Schekman RW (1993) The Sec 13p complex and reconstiution of vesicle budding from the ER with purified cytosolic proteins. EMBO J **12:** 4073-4082

Schmid SL (1997) Clathrin-coated vesicle formation and protein sorting: an integrated process. Annu Rev Biochem **66:** 511-548

Schroder S (1991) Subunit interaction and function of clathrin-coated vesicle adaptors from the Golgi and the plasma membrane. J Biol Chem **266:** 7910-7918

Shih W, Gallusser AKT (1995) A clathrin-binding site in the hinge of the β2 chain of the mammalian AP-2 complexes. J Biol Chem **270:** 3183-31090

Simpson F, Bright NA, West MA, Darnell RB, Robinson MS (1996) A novel adaptor-related protein complex. J Cell Biol **133:** 749-760

Söllner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Germanos S, Tempst P, Rothman JE (1993) SNAP receptors implicated in vesicle targeting and fusion. Nature **362:** 318-324

Sorkin A, Carpenter G (1993) Interaction of activated EGF receptors with coated-pit adaptins. Science **261:** 612-615

Staehelin LA, Moore I (1995) The plant Golgi apparatus: Structure, functional organization and trafficking mechanisms. Annu Rev Plant Physiol Plant Mol Biol **46:** 261-288

Stamnes MA, Rothman JE (1993) The binding of AP-1 clathrin adaptor particles to golgi membranes requires ADP-ribosylation factor, a small GTP-binding protein. Cell **73:** 999-1005

Stanley KK, Howell KE (1993) TGN38/41: a molecule on the move. Trends Cell Biol **3:** 252-255

Stoorvogel W, Oorschot V, Geuze HJ (1996) A novel class of clathrin-coated vesicles budding from endosomes. J Cell Biol **132:** 21-33

Tebar F, Sorkina T, Sorkin A, Ericsson M, Kirchhausen T (1996) Eps15 is a component of clathrin-coated pits and vesicles and is located at the rim of coated pits. J Biol Chem **271:** 28727-28730

Timerman AP, Mayleitner MM, Lukas TJ, Chadwick CC, Saito A, Watterson DM, Schindler H, Fleisher S (1992) Inositol polyphosphate receptor and clathrin assembly protein AP-2 are related proteins that form potassium-selective ion channels in planar lipid bilayers. Proc Natl Acad Sci, USA **89:** 8976-8980

Traub LM (1997) Clathrin-associated adaptor proteins-putting it all together. Trends Cell Biol **7:** 43-46

Traub LM, Kornfeld S, Ungewickell E (1995) Different domains of the AP-1 adaptor complex are required for Golgi membrane binding and clathrin recruitment. J Biol Chem **270:** 4933-4942

Ungewickell E, Plessmann U, Weber K (1994) Purification of Golgi adaptor protein 1 from bovine adrenal gland and characterization of its β1 (β') subunit by microsequencing. Eur J Biochem **222:** 33-40

Ungewickell E, Ungewickell H (1991) Bovine brain clathrin light chains impede heavy chain assembly *in vitro*. J Biol Chem **266:** 12710-12714

Voglmaier SM, Keen JH, Murphy J-E, Ferris CD, Prestwich GD, Snyder SJ, Theibert AB (1992) Inositol hexakisphosphate receptor identified as the clathrin assembly protein AP-2. Biochem Biophys Res Commun **187:** 158-163

Walters M, Serafini T, Rothman J (1991) Coatomer: a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. Nature **349:** 248-251

Wang X, Kilimann M (1997) Identification of two new μ-adaptin-related proteins, μ-ARP1 and μ-ARP2. FEBS Lett **402:** 57-61

Yoshihisa T, Barlowe C, Schekman R (1993) A Sec protein essential for transport vesicle formation from the ER is a novel GTPase activating protein. Science **259:** 1466-1468

Zaremba S, Keen JH (1985) Limited proteolytic digestion of coated vesicle assembly polypeptides abolishes reassembly activity. J Cellular Biochem **28:** 47-58

Zaremba S, Keen JH (1983) Assembly polypeptides from coated vesicles mediate reassembly of unique clathrin coats. J Cell Biol **97:** 1339-1347

Zhang JZ, Daveltov B, Südhof TC, Anderson RGW (1994) Synaptotagmin 1 is a high affinity receptor for clathrin AP-2: implications for membrane recycling. Cell **78:** 751-760

CHAPTER II

Identification and Partial Characterization of Clathrin-Associated Proteins of Developing Pea (*Pisum sativum* L.) Cotyledons

ABSTRACT

Clathrin-coated vesicles (CCVs) from cotyledons of developing pea have been isolated using an improved protocol which increases yield of CCVs and provides suitable protection against proteolysis by vacuolar hydrolases released during processing. In a comparative study of coat dissociation conditions, pea cotyledon CCVs were found to be more resistant to uncoating than brain CCVs with efficient coat removal requiring treatment with higher concentrations of Tris-HCl, elevated pHs, or use of urea, sodium carbonate, or increased incubation times. Inclusion of 0.75M $MgCl_2$ in the dissociation buffer greatly decreased the level of ferritin contamination found in Tris-HCl solubilized coat proteins, allowing purification of milligram quantities of clathrin-associated proteins from developing pea cotyledons.

Tris-HCl solubilized coat proteins of clathrin coated vesicles isolated from cotyledons of developing pea are shown to contain, in addition to clathrin, proteins which elute together at ~295,000 kD upon Superose-6 molecular sizing chromatography. This elution behavior is similar to that of the clathrin-associated proteins (APs) of bovine brain characterized for their *in vitro* involvement in clathrin coat assembly and ability to interact with (adapt to) both clathrin and the cytoplasmic tails of plasma membrane (AP2) and *trans*-Golgi (AP1) receptors. In further similarity to the mammalian adaptor complexes, proteins present in the pea 295,000-kD (AP) fraction appear as large globular structures on electron microscopic analysis. SDS-PAGE demonstrates enrichment of polypeptides of ~110 and ~100 kD, as well as additional peptides of 80, 50, 47 and 38 kD. The ~110 kD peptide has been demonstrated to be immunoreactive to a monoclonal antibody against the β1/β2 adaptin subunit of bovine brain. Screens with polyclonal antibodies against the μ and σ small subunits of brain and the μ subunit of yeast failed to detect additional immunoreactive peptides in the pea clathrin-associated polypeptide fraction.

The pea AP fraction containing the immunoreactive 110 kD polypeptide elutes as a single peak between 75 and 100 mM phosphate on hydroxylapatite chromatography, identifying the pea cotyledon β-adaptin as the AP1 rather than AP2 type. In further similarity to the bovine brain AP1 complex the AP fraction of pea CCVs also fails to bind clathrin-Sepharose. Sulfolink™ immuno-affinity purification of the AP fraction yielded polypeptides of 110, 100, 50 and 47 kD corresponding

approximately to the large and medium subunits of the heterotetromeric adaptors of mammalian tissues.

INTRODUCTION

Clathrin-coated vesicles (CCVs) are small transport organelles apparently present in all eukaryotic cells. Originating as coated pits at both the plasma membrane and *trans*-Golgi network, in mammalian systems CCVs have been implicated in the recycling of both receptors and membranes, transepithelial transport and the intracellular transport of lysosomal proteins. These vesicles are distinguished by their polygonal lattice-like coats constructed of the clathrin triskelion. Each leg of the three-legged triskelion, which is composed of three clathrin heavy chain (HC) and three light (LC) molecules (Keen, 1990), overlaps legs of adjoining triskelions to form the pentagons and hexagons of which the lattice is constructed. In addition to triskelions, CCVs coats from mammalian sources contain a group of polypeptides which elute together after clathrin on molecular sizing chromatography. These proteins were first characterized as factors that, *in vitro,* promote polymerization of purified clathrin triskelions into polygonal cages closely resembling CCVs and in recognition of this property, named "assembly proteins"(Keen et al., 1979). When other functions were subsequently ascribed to these "assembly proteins", the shorthand "AP" became commonly used.

Two heterotetromeric clathrin "assembly" complexes of mammalian tissues have been isolated (Pearse and Robinson, 1984; Manfredi and Bazari, 1987; Keen et

al., 1987) and have been localized by immunofluorescence to either plasma membrane (AP2) or Golgi (AP1) in Madin-Darby bovine kidney (MDBK) cells (Ahle et al., 1988). Both complexes are thought to interact with clathrin and the cytoplasmic tails of receptors specific to the membrane of origin and thus function as "adaptors". By freeze-etch electron microscopy, the molecular structure of AP2 has been shown to be a large central "brick" with two narrowly stalked globular "ears"; the structure of AP1 is thought to be similar (Heuser and Keen, 1988). Three dimensional images of reassembled coats and cages embedded in vitreous ice localize APs to the innermost layer of the vesicle coat, consistent with the "adaptor" concept in which APs interact both with clathrin and transmembrane receptors (Vigers et al., 1986). Several monomeric clathrin-associated proteins (auxilin and AP180) specific to neuronal tissues have also been isolated (Ahle and Ungewickell, 1986; Ahle and Ungewickell, 1990; Lindner and Ungewickell, 1992). Of these, auxilin is thought to participate in the uncoating process *in vivo* by binding clathrin in the presence of ATP and recruiting hsp70, the CCV uncoating ATPase (Ungewickell et al., 1995). AP180, together with the AP2 complex have been shown to be responsible for most of the assembly of clathrin coats observed *in vitro* (Lindner and Ungewickell, 1992). *In vivo*, however, additional co-factor(s) and/or mechanisms may be required to provide selectivity since coat assembly must be restricted to the appropriate membranes (plasma membrane or *trans*-Golgi). This has already been shown in the requirement for ARF (ADP-ribosylation factor), a small GTP binding protein, for binding of AP1 adaptors to Golgi membranes of rat liver (Stamnes and Rothman, 1993).

Several studies indicate that phosphorylation of CCV coat proteins may be involved in regulation of clathrin coat assembly (Georgieva-Hanson et al., 1988; Pypaert et al., 1991). *In vitro*, under appropriate conditions, all four of the 100 kD adaptor subunits (α and β2 of AP2 and γ and β1 of the AP1 complex) can be phosphorylated, as well as both the medium subunits μ1 and μ2 (Méresse et al., 1990; Pauloin and Thurieau, 1993). In addition, clathrin LCb but not LCa has been shown to be phosphorylated by a casein kinase II in the presence of polylysine. (Bar-Zvi and Branton, 1986). While these CCV coat proteins have been shown to be phosphorylated *in* vitro, the physiological role of this phosphorylation is unclear. Recently, however, (Wilde and Brodsky, 1996) have shown that adaptors with phosphorylated 100 kD subunits are predominantly cytosolic and that in the phosphorylated state lose their ability to bind clathrin. This suggests that adaptor phosphorylation plays a role in regulating CCV assembly *in vivo*.

CCVs have been observed in electron micrographs of both non-vascular plants (Weiss 1983; Zhang et al., 1993) and vascular plants (Fowke et al., 1991; Barroso and Pais, 1987). In addition, CCVs have been isolated from a number of vascular plant sources including pea cotyledon (Harley and Beevers, 1989a; Lin et al., 1992), carrot and soybean suspension culture cells (Coleman et al., 1987; Wiedenhoeft et al., 1988), zucchini hypocotyls (Holstein et al., 1994) and wheat germ (Chapter 4, this dissertation). Nevertheless, our knowledge of CCV components in plants is still quite limited. As in mammalian cells, triskelions form the characteristic polygonal coat of plant CCVs. Clathrin HC has been identified, and molecular weights of 185-190 kD

reported (Mersey et al., 1985; Depta and Robinson, 1986; Harley and Beevers, 1989a). A full length cDNA of soybean HC, which codes for an amino acid sequence of 1700 residues, has been sequenced and the calculated size determined to be 193,353 kD, approximately 2000 kD larger than that of rat. This difference in size may explain the larger apparent M_r observed on SDS-PAGE (Blackbourn and Jackson, 1996). Potential clathrin LCs have been identified (Coleman et al., 1987; Lin et al., 1992). Putative plant AP proteins were first isolated from pea cotyledon (Butler and Beevers, 1993), followed by the identification of a β-type adaptin from CCVs of zucchini hypocotyl (Holstein et al., 1994). Recently, cloning of plant homologs of the small subunit of the Golgi-associated clathrin assembly protein AP19 from *Camptotheca acuminata* (Maldonado-Mendoza and Nessler, 1996) and *Arabidopsis thaliana* (Maldonado-Mendoza et al., 1996) have been reported.

A number of different functions have been ascribed to CCVs of plant tissues. Several studies suggest that CCVs are active in retrieval of plasma membrane via endocytosis (Tanchak et al., 1884; Staehelin and Moore, 1995; Fowke et al., 1983) but evidence supporting receptor-mediated endocytosis in plant systems is extremely limited (reviewed in (Horn et al., 1989; Low and Chandra, 1994)). A role for CCVs in protein sorting and intracellular transport has also been postulated. The cotyledons of legumes actively deposit storage proteins in large specialized vacuoles termed "protein bodies"or protein storage vacuoles. Early studies suggested that CCVs of garden pea carry precursors of lectin, other vacuolar proteins such as hydrolytic enzymes (Harley and Beevers, 1989b) and the storage proteins legumin and vicilin

(Robinson et al., 1989; Robinson et al., 1991; Hoh et al., 1991). Current work, however, suggests large non-clathrin coated dense vesicles are responsible for storage protein transport from the *trans*-Golgi to protein storage vacuoles, with CCVs involved in transport of other vacuolar proteins destined for other types of vacuoles (Robinson, 1996). Supporting evidence for this sorting/transport role for CCVs is provided by the purification from a CCV enriched fraction of pea cotyledon (Kirsch et al., 1994; Kirsch et al., 1996) of BP-80, a putative vacuolar targeting receptor which binds to the N-terminal vacuolar targeting domain of barley aleurain and sweet potato sporamin. Clathrin-associated proteins from developing pea have been shown to interact with the BP-80 receptor protein (Chapter 4).

In this paper we describe an improved method for the isolation of CCVs from developing pea cotyledons and have optimized conditions for coat disassembly. Further, we report the partial purification and characterization of a clathrin-associated protein complex containing a ~110 kD protein which, through immunoblotting and hydroxylapatite chromatography, has been identified as a β-type adaptin. The plant complex has been shown to be a globular protein approximately 30 nm in diameter similar in appearance and size to the AP2 heterotetromeric complex of bovine brain.

MATERIALS AND METHODS

Vesicle Isolation

Coated vesicles were isolated from both pea cotyledons and bovine brain using a fusion of the methods of (Lin et al., 1992) and (Campbell et al., 1984) which utilizes both intermediate Ficoll/Sucrose and final Ficoll/D_2O purification steps. This method provides a substantial improvement in purity of CCV preparations over either of the individual methods and allows more rapid processing of larger quantities of tissue.

The following buffers were used for isolation of CCVs. Buffer A: 0.1 M Mes (2-(N-morpholinomethane sulfonic acid), 0.1 M EGTA, 0.5 mM $MgCl_2$, 0.02% sodium azide pH 6.5; Buffer B: Buffer A containing 0.025 mg/ml trypsin inhibitor, 0.5 mM PMSF, and 1 mM DTT; Buffer C: Buffer B plus 0.3 M sorbitol. All buffers were adjusted to final pH at RT.

Field grown peas (*Pisum sativum* L. cv. Burpeeana), picked 24-27 days post anthesis, were mechanically shelled, frozen by submersion in liquid nitrogen, and stored at -80°C. Frozen peas in 500 gram lots were thawed for 15 minutes at room temperature, and homogenized for 2 min in a Waring blender in equivalent amounts (weight/vol) Buffer C. Trypsin inhibitor, DTT and PMSF were added immediately prior to homogenization. The homogenate was squeezed through 4 layers of cheesecloth, and centrifuged at 200 x g (all values are calculated as r^{avg}) for 5 min followed by 17,000 x g for 30 minutes in a Sorvall RC5B centrifuge (GSA rotor) at 4°C. All subsequent centrifugations were conducted at 4°C in either a Beckman L2 or

L8-80 high speed centrifuge. The supernatant was collected by aspiration and centrifuged at 120,000 x g for 40-50 minutes in either a Beckman 45Ti or Beckman 42.1 rotor. Pellets from a total of 1500 gms cotyledons were resuspended using a glass hand homogenizer outfitted with a teflon pestle in Buffer B to a final volume of 10 mls/150 gm cotyledons. Contaminating ribosomes were removed by incubating this suspension with 1 mg/ml pancreatic ribonuclease A for 1 hr at 4°C with gentle shaking.

Following the ribonuclease digestion, an equal volume of 12% Ficoll/12% Sucrose in Buffer A was mixed with the suspension and centrifuged at 20,000 x g for 30 minutes in a Beckman 42.1 rotor. The supernatant was collected by Pasteur pipette, diluted with 3 volumes Buffer A, and centrifuged at 120,000 x g for 50 min as above.

Pellets were then resuspended in 60 ml Buffer A (25g initial cotyledon weight/ml), and could be frozen at -80°C without apparent loss of coated vesicle yield. Finally, the resuspended pellets were layered (10 mls/gradient) onto 20 ml linear gradients consisting of 5% Ficoll/10% D_2O to 25% Ficoll/90% D_2O in Buffer A and centrifuged at 55,000 x g for 16 hrs in a Beckman SW28 swinging bucket rotor. The location of CCVs within the gradients after centrifugation was determined by SDS-PAGE analysis of fractionated gradients and determined to be highly reproducible. In subsequent vesicle preparations regions containing coated vesicles were recovered by Pasteur pipette and pooled, diluted with 6 volumes Buffer A and centrifuged for 50-60 min at 120,000 x g. Pellets containing coated vesicles were resuspended in a small amount of the same buffer and frozen at -80°C until use. Normally, 12-15 mg highly

purified CCVs were obtained from 3 kg cotyledon using this method, although some year-to-year variation in yield was observed.

Clathrin-coated vesicles were isolated from bovine brain using essentially the same method. However, sorbitol was not included in the homogenization buffer and the initial cheesecloth filtration and ribonuclease digestion were also omitted. Typically, 1 Kg brain yielded 50 mg highly purified CCVs.

Standard Dissociation of Pea Cotyledon CCV Coat Proteins

To obtain pea coat proteins CCVs from 3 kg cotyledons were pelleted, resuspended in 1.75 ml dissociation buffer containing 0.05 M Mes, 0.5 M Tris, 0.75 M $MgCl_2$ (Holstein et al., 1994), augmented with 0.02% sodium azide, 0.025 mg/ml trypsin inhibitor, 0.05 mM PMSF and 1 mM DTT, incubated on ice for 60 min with gentle shaking and ultracentrifuged at 170,000 x g in T65 rotor for 50 min. Pellets containing partially uncoated CCVs were resuspended in 1.0 ml dissociation buffer, extracted as above for 30 min, spun as above and supernatants combined. Coat proteins (6-8 mg in 2.5 ml) were loaded on a 1x94 cm Superose-6 column equilibrated in Buffer D (a 1:1 mixture of Buffer A: 1 M Tris-HCl pH 7.0) and eluted downward at 0.2 ml/min at 10°C. One ml fractions were collected. After SDS-PAGE and immunoblotting (see below) to locate APs within the elution profile fractions 36-44 containing dissociated clathrin triskelions and fractions 50-55 containing APs were pooled separately and concentrated using Amicon Centri-Prep10™ concentrators.

Preparation and Fractionation of Brain Coat Proteins

Coat proteins of bovine brain were dissociated from CCVs by addition of 0.33 vol of 2 M Tris-HCl, pH 7.0, 8mM EDTA, 0.4 mM PMSF incubated for 15 min on ice (Lindner and Ungewickell, 1992), followed by ultracentrifugation at 170,000 x g for 50 min in a T65 rotor. Extractions were repeated at least once and supernatants combined. Coat proteins (15-30 mg in 3-6 ml) obtained from 1-2 kg brain were loaded on a 2.5 x 66 cm Superose-6 gel filtration column, equilibrated in Buffer D, and eluted downward at ~ 0.6 ml/min at 10°C. Four ml fractions were collected. After SDS-PAGE and immunoblotting (see below) to locate APs within the elution profile fractions 37-42 containing dissociated clathrin triskelions and fractions 50-58 containing APs were pooled separately and concentrated using Amicon Centri-Prep10™ concentrators.

Hydroxylapatite chromatography

Hydroxylapatite chromatography of clathrin-associated proteins was conducted according to the method of (Manfredi and Bazari, 1987). Superose column fractions containing APs (6 mls total) were dialyzed 2 x 8 hours against starting buffer containing 10mM K_2PO_4, 0.1 M NaCl, 10% glycerol, 0.2mM DTT augmented with 50µg/ml PMSF, pH 8.4. The sample was briefly centrifuged (10 minutes in Sorvall SS-34, 10K), loaded on a 1 x 5 cm HA Ultrogel (Sigma Biochemicals) column equilibrated in above buffer minus PMSF at 0.2ml/min. One ml fractions were collected. After sample loading, the column was first washed with starting buffer and bound protein eluted with a 30 ml 10 to 500 mM K Phosphate gradient.

Protein concentration was monitored at 280 nm using a Gilson Model 240 Spectrophotometer, and conductivities determined using a Cole Palmer Analog Conductivity Meter, calibrated with a commercially available standard.

Protein Electrophoresis, Western Blotting and Immunoprobing

All SDS-PAGE was conducted using either single concentration (12%) or gradient gels (8-18%) with an acrylamide:bis-acrylamide ratio of 37.5:1 (Laemmli, 1970) with a discontinuous buffer system (Maizel, 1971). Gels were stained either with Coomassie Brilliant Blue-G (Neuhoff et al., 1988) or silver (Blum et al., 1987). For immunoprobing of AP, proteins were first electroblotted onto Millipore Immobilon-P transfer membrane at 100 mA overnight using transfer buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3 (Towbin et al., 1979). Immunoreactive bands were subsequently detected using the Amersham ECL system. The primary antibody used was the monoclonal anti-β_1/β2 AP subunit obtained from Sigma (A-4450).

Electron Microscopy

For negative staining, 10 µl drops of samples containing 0.1-1.0 mg/ml protein were placed on Formvar/Carbon coated 75 mesh hex grids, allowed to stand for 1.5 min, wicked off, and fixed for 45 sec with 1% glutaraldehyde. After washing, grids were stained with 2% aqueous uranyl acetate for 4 min, dried, and viewed in a Zeiss-10 transmission electron microscope (TEM) operated at 80 kV. For rotary shadowing, samples of dissociated coat proteins or Superose-6 purified clathrin and adaptors were

mixed with equal volumes of ice cold glycerol and sprayed onto freshly cleaved mica chips which were placed into the JEOL JFD-9000 Freeze-Etching/Fracture Equipment and dried under vacuum for 3 minutes. Chips were rotary shadowed with platinum at a 6% angle for 30 seconds and carbon for 12 seconds. The carbon/platinum film was floated off the mica onto deionized water, picked up on 600 mesh hex grids, and viewed as above.

RESULTS

Purification of CCVs from pea cotyledons

Two factors suggested that successful isolation and characterization of plant adaptor proteins from cotyledons of developing pea would require maximizing the amount of CCVs isolated and minimizing contamination by non-relevant proteins. A protocol for isolation of CCVs had previously been developed in our laboratory, but only very limited amounts of cotyledon could readily be processed using this method. Extending the hours of centrifugation to accommodate larger quantities of cotyledon would increase the possibility that protein degradation caused by the release of vacuolar hydrolases during homogenization might occur. In addition, since the mixed adaptor fraction from brain CCVs represents only 25-30 % of total coat proteins (Keen, 1987), it was expected that processing large quantities of pea cotyledon would be required. Initially, two sequential sucrose step gradients, followed by centrifugation through a 8% (w/v) sucrose cushion in D_2O, were used to purify CCVs from crude microsomal pellets (Harley and Beevers, 1989a). In the present study, the

amount of cotyledons processed was doubled by increasing the ratio of cotyledon/homogenization buffer from 0.5g/ml to 1g/ml. Upon molecular sizing chromatography of coat proteins of CCVs isolated using this method, no protein(s) recognizable as APs could be identified (data not shown). In addition, large peaks in UV-absorbance were observed in the void volume and late in the included volume which were shown by SDS-PAGE not to contain protein (Fig. 1). Substitution of a linear 9-90% D_2O gradient (Lin et al., 1992) for the sucrose step gradients made no improvement in the elution profile (data not shown). However, when a 12% Ficoll/12%Sucrose cushion (Campbell et al., 1984) was substituted for the intermediate purification step, and a final isopycnic centrifugation through a linear ficoll/D_2O gradient was added to the isolation protocol, significant improvement in purification was observed. This hybrid isolation protocol was used for all subsequent isolations of pea cotyledon CCVs. Fig. 2 shows a typical Ficoll/D_2O gradient from the hybrid isolation protocol. The protein composition of the regions marked CV-II, CV-III, CV-IV and LDM (see Fig. 5) proved highly reproducible by SDS-PAGE analysis, with the majority of clathrin coated vesicles found in the regions marked CV-II and CV-III. CV-II and CV-III were pooled for all subsequent experiments.

Molecular sizing chromatography of pea CCV coat proteins

Instead of many peaks, as had been seen initially, the Superose-6 elution profile of coat proteins dissociated from CCVs isolated using the improved, hybrid protocol, showed only three peaks (Fig. 3A). SDS-PAGE analysis of the first

Figure 1. Separation of dissociated coat proteins of pea cotyledon CCV by molecular sizing chromatography. A Tris-HCl extract of a coated vesicle prepared by using sucrose step gradients was applied to Superose-6 and eluted with buffer D. Fractions 21-24 and fractions 54-60 were shown by SDS-PAGE not to contain protein. No proteins recognizable as APs were found.

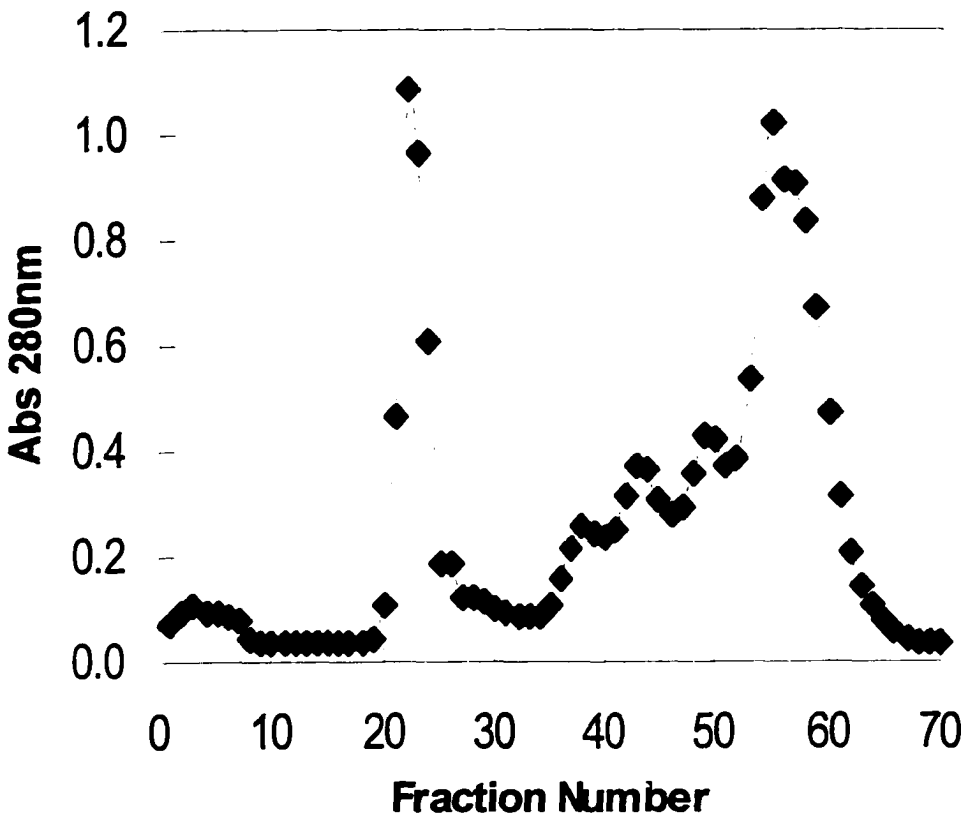

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Abs 280nm
0
10
20
30
40
50
60
70
Fraction Number

Figure 2. Typical Ficoll/D_2O gradient purification of pea cotyledon CCV. Crude CCVs were loaded onto 20 ml linear gradients consisting of 5%Ficoll/10% D_2O to 25% Ficoll/90% D_2O in Buffer A and subjected to isopycnic centrifugation. Regions marked CVII and CVIII containing most of the CCVs were recovered separately by Pasteur pipette, diluted with buffer A and subjected to high-speed centrifugation. Pelleted CCVs were resuspended in buffer A and frozen at -80°C until use.

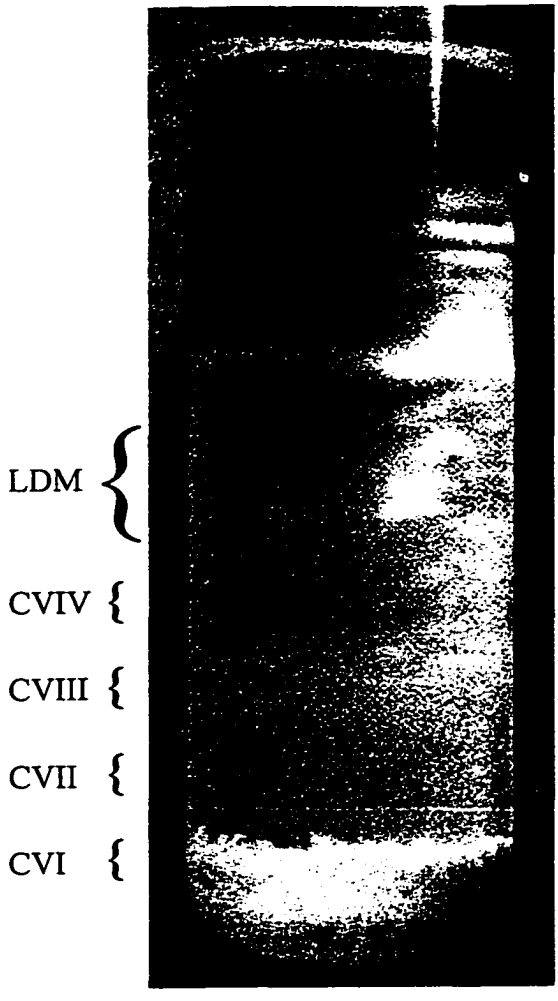
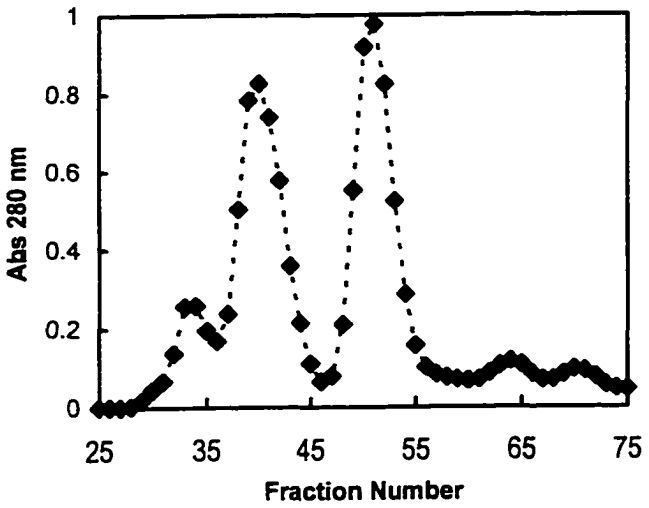

LDM
CVIV
CVIII
CVII
CVI

Figure 3. Separation (A-B) and electrophoretic analysis (C) of dissociated coat proteins of pea cotyledon CCVs. (A) A Tris-HCl extract of CCVs prepared using the improved hybrid isolation protocol was applied to Superose-6 and eluted with buffer D. Partially undissociated coat proteins were found in fractions 32-35 and dissociated clathrin in fractions 37-44. Putative APs were found in fractions 49-54 along with large amounts of a 28kD protein. (B) Coat proteins of CCVs isolated using the improved hybrid protocol were dissociated using a Tris-HCl buffer containing 0.75M $MgCl_2$, applied to Superose-6 and eluted with buffer D. Dissociated clathrin was found in fractions 37-44 and putative APs in fractions 49-54. This peak was much smaller than that seen in equivalent fractions in figure 3a. (C) Electrophoretic analysis of proteins found in peak fraction of third peak in figure 3a. Lane 1, molecular mass standards (200, 116, 97, 66, 45, 31, 21, 14 and 6.5 kD from top to bottom); lane 2, fraction 51 containing putative pea cotyledon AP (*) and the 28kD polypeptide (✧).

A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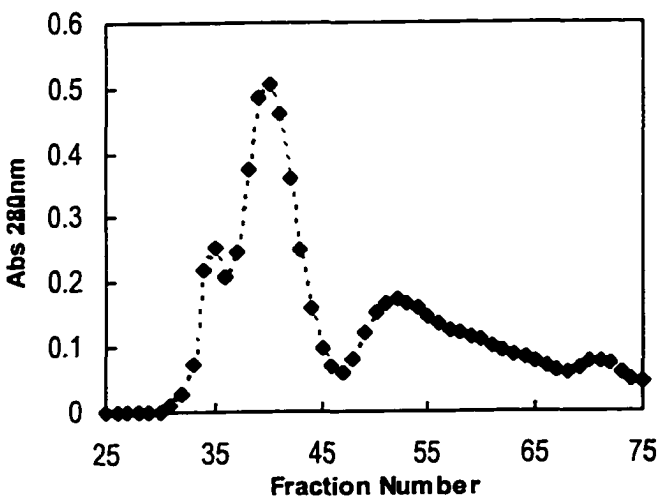
0.6
0.4
0.2
0
25
35
45
55
65
75
Fraction Number

B


Abs 280nm
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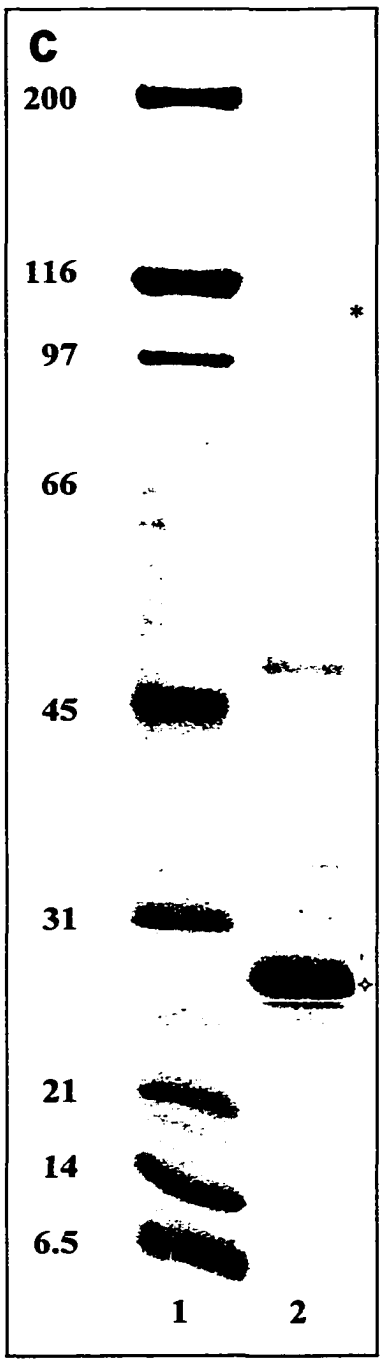
0.5
0.4
0.3
0.2
0.1
0
25
35
45
55
65
75
Fraction Number

C


200
116
97
66
45
31
21
14
6.5
1
2
*

two peaks identified undissociated (peak 1) and dissociated (peak 1) clathrin (data not shown). The third peak contained proteins of ~110, 100, and 50 kD, similar to those of brain AP, as well as 35 and 28 kD (Fig. 3C). Strikingly, the amount of the 28 kD protein appeared to be at least two times greater than the amount of the other proteins combined. The 28 kD protein was subsequently identified as ferritin (Hoh and Robinson, 1993) which had previously been shown to co-elute with APs from placenta (Pearse, 1982). $MgCl_2$ precipitation had previously proven useful in purification of plant ferritin (Hyde et al., 1963; Sczekan and Joshi, 1987). Inclusion of 0.75M $MgCl_2$ in the CCV dissociation buffer removed the yellowish tinge visible in the supernatant containing dissociated CCV coat proteins, and reduced the size of the putative AP peak (Fig. 3B), producing an elution profile virtually indistinguishable from that observed for brain (Keen, 1987).

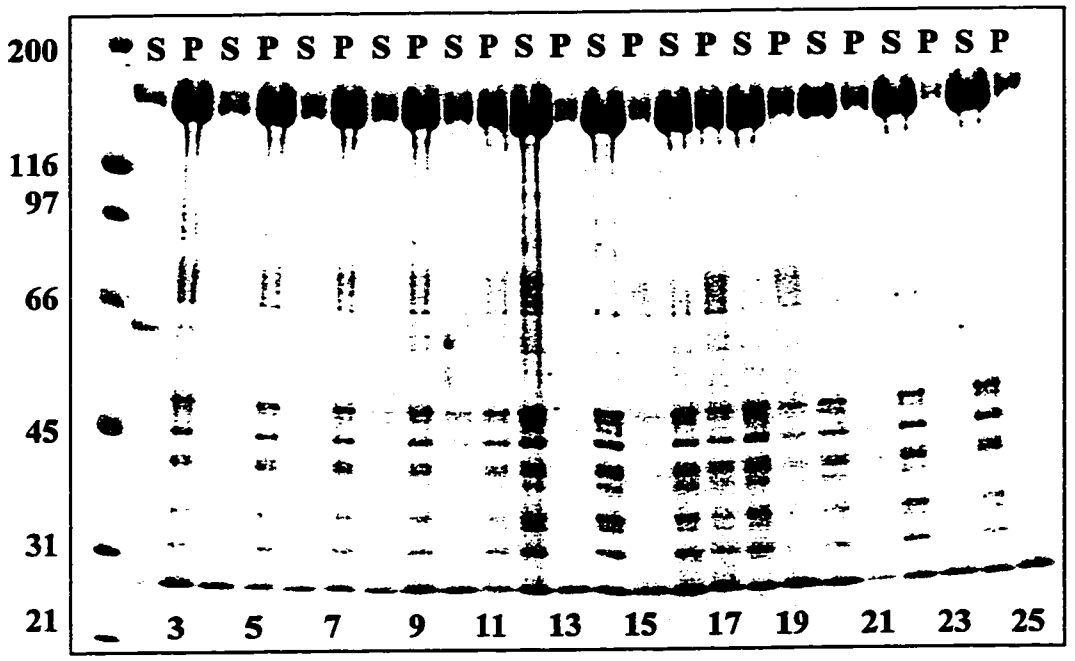
Optimization of dissociation conditions

Having developed an improved procedure for isolation of CCVs and removal of major contaminants, we sought to identify the conditions under which pea coat proteins were most efficiently dissociated. Resuspended CCVs (~75 μg total protein/aliquot) were centrifuged and high-speed supernatants discarded. Pelleted CCVs were resuspended and incubated under the conditions summarized in Table 1. After incubation, the samples were again pelleted and the distribution of clathrin HC between pellet (undissociated material) and high-speed supernatants (dissociated coat proteins) examined by SDS-PAGE as shown in Fig. 4. After incubation of CCVs

Table 1. Buffers used in comparative study of pea CCV dissociation

Lane	Buffer, Incubation Times, Temperature	Formulation
2-3	A, 30 min, 4°C	0.1M Mes, 1.0mM EGTA, 0.5mM $MgCl_2$, 0.02% NaN_3, pH 6.5 @ RT
4-5	D, 30 min, 4°C	Buffer A, 1.0M Tris-HCl, pH 7.0, 1:1 mixture
6-7	E, 30 min, 4°C	0.1M Mes, 0.5M Tris, 1mM EGTA, 0.5mM $MgCl_2$, 0.02% NaN_3, pH 7.0 @ RT
8-9	E, 30 min, RT	0.1M Mes, 0.5M Tris, 1mM EGTA, 0.5mM $MgCl_2$, 0.02% NaN_3, pH 7.0 @ RT
10-11	E, O/N, 4°C	0.1M Mes, 0.5M Tris, 1mM EGTA, 0.5mM $MgCl_2$, 0.02% NaN_3, pH 7.0 @ RT
12-13	F, 30 min, 4°C	Buffer E plus 2% Chaps
14-15	G, 30 min, 4°C	0.1M Mes, 0.5M Tris, 1mM EGTA, 0.5mM $MgCl_2$, 0.02% NaN_3, pH10.0 @ RT
16-17	H, 30 min, 4°C	0.1M Mes, 1.0M Tris, 1mM EGTA, 0.5mM $MgCl_2$, 0.02% NaN_3, pH 7.0 @ RT
18-19	H, 30 min, RT	0.1M Mes, 1.0M Tris, 1mM EGTA, 0.5mM $MgCl_2$, 0.02% NaN_3, pH 7.0 @ RT
20-21	H, O/N, 4°C	0.1M Mes, 1.0M Tris, 1mM EGTA, 0.5mM $MgCl_2$, 0.02% NaN_3, pH 7.0 @ RT
22-23	I, 30 min, 4°C	2.0 M Urea, 20mM Tris, 1mM EDTA, 0.5mM $MgCl_2$, 0.02% NaN_3, 1mM DTT, 0.5mM PMSF, 0.5µg/ml trypsin inhibitor, pH 7.5@RT
24-24	J, 15 min, 4°C	25mM sodium carbonate, pH 10.0@ RT

Figure 4. Electrophoretic analysis of dissociation of pea cotyledon CCVs by various buffers. Proteins were separated on an 8-18% acrylamide gel and stained with Coomassie blue G. Lane 1, molecular mass standards in kD. Lanes 2-25, proteins found in supernatants (S) and pellets (P) after dissociation and high-speed centrifugation. All buffer designations follow that of Table 1. Lanes 2-3, isolation buffer A, 30 min, 4°C; lanes 4-5, buffer D, 30 min, 4°C; lanes 6-7, buffer E, pH 7.0, 30 min, 4°C; lanes 8-9, buffer E, pH 7.0, 30 min, at room temperature; lanes 10-11, buffer E , pH 7.0, overnight at 4°C; lanes 12-13, buffer F, 30 min, 4°C; lanes 14-15, buffer G, 30 min, 4°C; lanes 16-17, buffer H, 30 min, 4°C; lanes 18-19, buffer H, 30 min, room temperature; lanes 20-21, buffer H, overnight at 4°C; lanes 22-23, buffer I, 30 min, 4°C and lanes 24-25, buffer J, 15 min, 4°C.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with the isolation buffer A control (lanes 2-3), most clathrin HC remained in the pellet, indicating little dissociation had occurred. No improvement in dissociation occurred with Superose-6 molecular sizing buffer containing a 1:1 mixture of isolation buffer A at pH 6.5 and 1M Tris-HCl, pH 7.0 (lanes 4-5), nor with the same buffer titrated to pH 7.0, even with room temperature incubation (lanes 6-9). Overnight dissociation increased the amount of clathrin HC found in the supernatant only slightly (lanes 10-11). Addition of 2% Chaps, a non-ionic detergent, however, resulted in a large shift of clathrin HC from the pellet into the supernatant (lanes 12-13), as did buffers containing either 2M Urea or 25mM Na Carbonate, pH 10.0 (lanes 22-25). 0.5M Tris buffer titrated to pH 10 also efficiently dissociated coat proteins from CCVs (lanes 14-15). At pH 7.0, increasing the Tris-HCl concentration to 1.0M increased the proportion of clathrin HC present in the supernatant (compare lanes 16-17 to lanes 2-3). This ratio was improved when incubation was conducted at room temperature or extended overnight at 4°C (lanes 18-21).

Influence of protease inhibitors

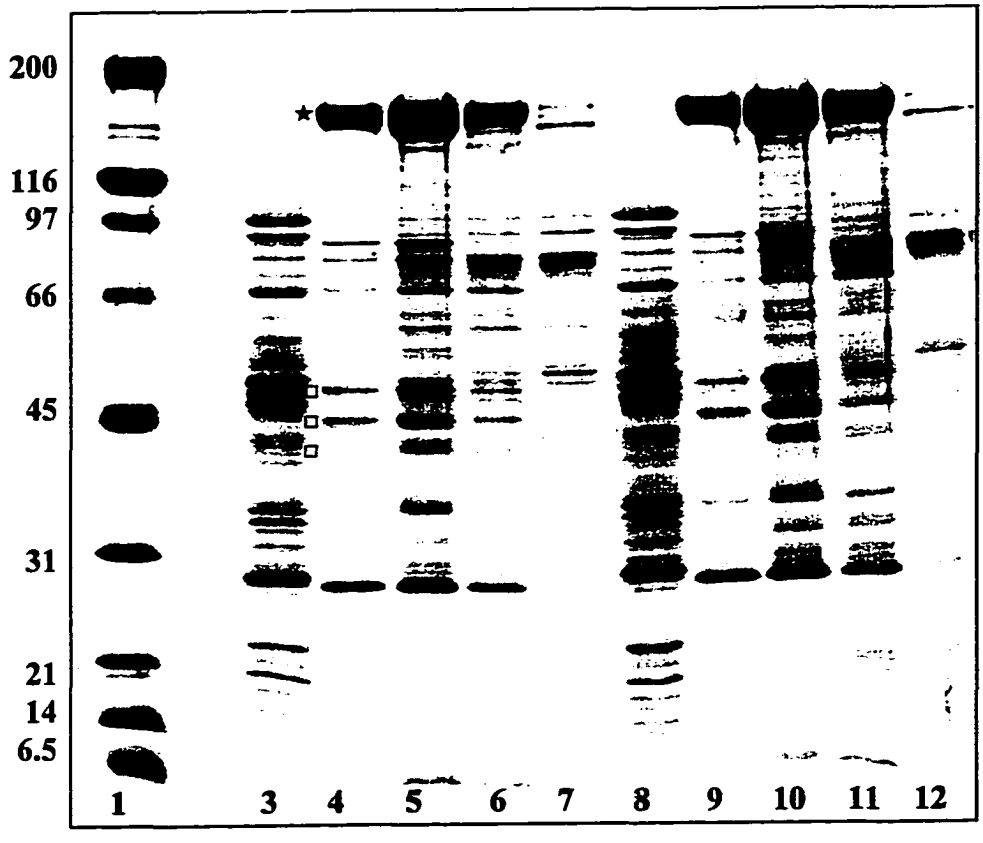
Vacuolar rupture occurs when plant tissues are homogenized, releasing soluble hydrolases. In order to protect against proteolysis, the homogenization buffer (buffer C, see methods) used for CCV isolation contained 1mM DTT (dithiolthreitol), 0.025mg/ml trypsin inhibitor, and 0.5mM PMSF (phenylmethylsulfonyl fluoride). In a comparison of two isolation buffers, replicate CCV preparations were conducted simultaneously. One preparation used Buffer C containing the standard inhibitor

regime described above; in the second, the homogenization buffer contained, in addition to the above, an "inhibitor cocktail" consisting of 1μ/ml leupeptin and 1μ/ml apoprotinin (against serine proteases), 2μ/ml pepstatin (against acid proteases), 1μ/ml chymostatin, 1μ/ml antipain and 1mM benzamidine (Phan et al., 1994). The remainder of the isolation procedure was carried out as described in Methods, maintaining complete separation of the two inhibitor regimes. After the final Ficoll/D_2O gradients, identical regions of each gradients were collected by Pasteur pipette, diluted, pelleted, and pellets resuspended in Buffer A. For each inhibitor regime, aliquots of both the supernatant obtained by low speed centrifugation following homogenization, and each Ficoll/D_2O gradient fraction were analyzed by SDS-PAGE (Fig. 5). No differences were seen in the polypeptide composition for any sample pair.

Identification and characterization of a β-type AP present in pea cotyledon CCV

When a Tris-HCl extract of bovine brain CCVs was applied to a Superose-6 column, clathrin was resolved from a protein peak containing prominent polypeptides with molecular weights of 100-115 kD and 50-55 kD (Keen, 1987). When a similar extract of pea cotyledon CCVs (prepared using the "standard dissociation" described in Methods) was fractionated (see elution profile, Fig. 3B), clathrin was also resolved from a protein peak containing proteins of similar molecular weights to those of brain including proteins of ~110 and 100 kD. Using standards prepared for chromatography (Sigma Chemical Co., St. Louis, MO), the calculated molecular weight of the non-

Figure 5. CCV isolation using an enhanced inhibitor cocktail provides no improvement over the standard inhibitor regime. Fifty μg aliquots of the diluted, pelleted and resuspended ficoll/D_2O gradient pools and the initial low-speed supernatant were separated by SDS-PAGE in an 8-18% acrylamide gel and visualized by Coomassie G. Lane 1, molecular mass standards; lanes 3 and 8, low-speed supernatant; lanes 4 and 9, 5 and 10, 6 and 11 and 7 and 12 represent gradient regions CVII, CVIII, CVIV and LDM respectively. Lanes 3-7, standard inhibitor regime, lanes 8-12, enhanced inhibitor cocktail containing 1 mM benzamidine, 1 μg/ml leupeptin, 2 μg/ml pepstatin, 1 μg/ml chymostatin, 1 μg/ml apoprotinin and 1 μg/ml antipain. Position of clathrin HC indicated by (★), clathrin LCs by (□).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clathrin peak fraction was determined to be ~295 kD, slightly smaller than the 343 kD reported for brain (Keen, 1987). Samples of the pea extract containing dissociated coat proteins (the column load), the clathrin peak, and the ~295 kD peak were prepared by a spray/etch/rotary shadow technique and examined by electron microscopy. While the Tris-HCl extract of coat proteins contained a mixture of both triskelions and globular proteins (Fig. 6A), these components were resolved by Superose-6 chromatography. The clathrin peak contained only triskelions (Fig. 6B) while the ~295 kD peak contained globular proteins with an approximate diameter of 13 nm (Fig. 6C).

Using western blotting and immunoprobing we sought to confirm the identify of the 100-110 kD components of the ~295 kD peak as β-adaptins. Aliquots of fractionated coat proteins were subjected to SDS-PAGE and silver stained (Fig. 7B). A replicate gel was transferred to Immobilon-P, and immunoprobed using two different antibodies raised against β-adaptins. The commercially prepared monoclonal antibody against the brain β1/β2 adaptor subunits (mAb-100/1, (Ahle et al., 1988)) recognized a single band at ~110 kD, which coincided with the ~295 kD peak. When the same blot was probed with a polyclonal antibody against the AP2 large subunits (Timerman et al., 1992), an immunoreactive band at ~80 kD, which also coincided with the ~295 kD peak, was observed (Fig. 7A). In another experiment, mAb-B1/M_6, a monoclonal antibody against the amino-terminal portion of the bovine brain β1/β2 adaptor (Robinson, 1987) also recognized the 110 kD protein (data not shown).

Figure 6. Electron micrographs of rotary shadowed dissociated coat proteins of pea cotyledon CCV (A), and Superose-6 fractions containing pea triskelions (B) and pea APs (C). Samples were diluted with equal volumes of glycerol, sprayed onto mica chips, dried under vacuum and rotary shadowed with platinum and carbon. (A) Mixture of clathrin triskelions (large arrow) and globular proteins (small arrow) present in dissociated coat proteins. (B) Negative image of Superose-6 separated pea clathrin triskelions. (C) Superose-6 separated pea cotyledon APs. Bars represent 100 nm (A and B) and 25 nm (C).

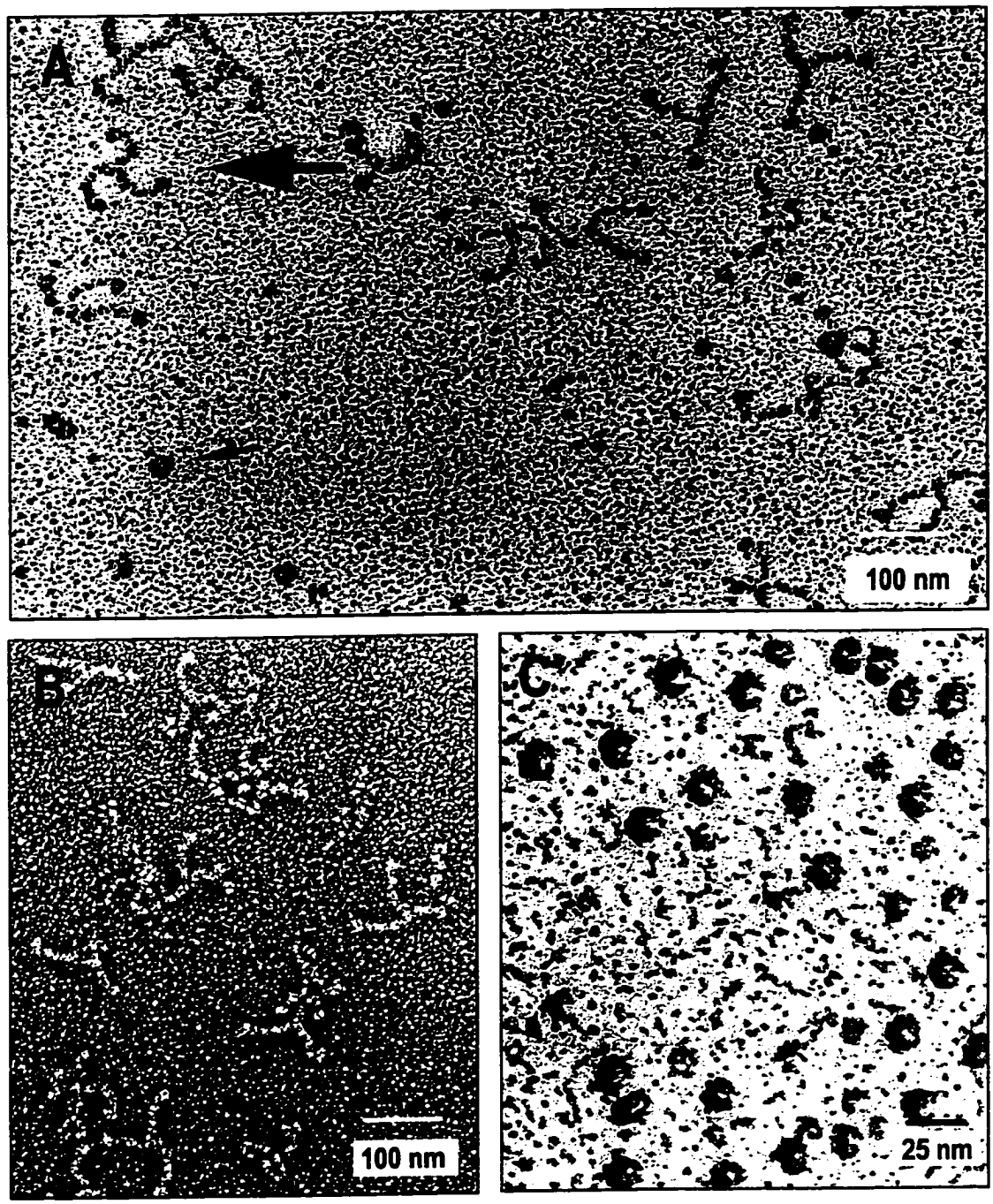
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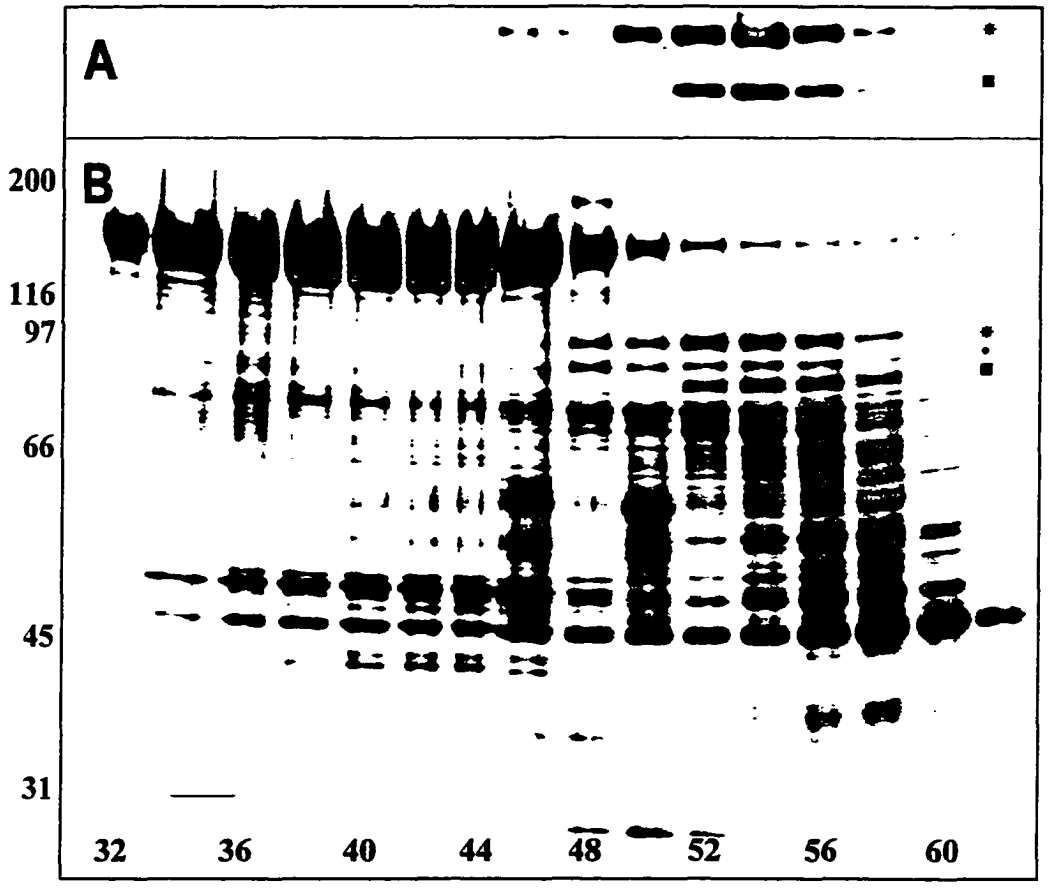
Figure 7. Identification of β-type adaptin in dissociated coats of pea cotyledon CCV fractionated by Superose-6 chromatography. (B) Ten μg aliquots of Superose-6 column fractions were separated by SDS-PAGE in an 8-18% acrylamide gel and proteins visualized by silver staining. Polypeptides of 100 (•) and 110 (*) kD in fractions 49-58 resolved from clathrin containing fractions (36-46). (A) Western blot of replicate gel probed with (*) monoclonal antibodies against the brain β1/β2 AP subunits and polyclonal antibodies against AP2 (▪). ECL detection.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Hydroxylapatite chromatography (HA) of AP proteins purified by Sepharose CL-4B chromatography demonstrated that two distinct complexes, which eluted at 120mM (AP1) and ~260mM PO_4 (AP2), are present in bovine brain CCVs (Manfredi and Bazari, 1987). Having identified potential APs in the Tris-HCl extract of pea CCV coat proteins, we attempted HA chromatography of the putative pea cotyledon AP. When peak adaptor fractions from the Superose-6 column were applied to a HA column, only a small amount of A_{280} absorbance was seen in flow-through fractions, and washing failed to elute any additional proteins. Bound protein eluted as a single peak early in the gradient, between 50 and 75mM Phosphate (Fig. 8). Replicate chromatography produced nearly identical results (data not shown). In comparison, when a mixed brain adaptor pool was applied under the same conditions to the same column, two peaks, which eluted at ~100mM and ~200mM PO_4, were resolved. SDS-PAGE analysis and immunoblotting using antisera against bovine adaptors (see above) identified immunoreactive proteins of 80 and 110 kD within the single pea adaptor HA peak (Fig. 9A). The 80 kD protein, identified with the polyclonal anti-AP2 antibody (Timerman et al., 1992), was seen to elute slightly earlier in the gradient, along with residual ferritin and a strong band at 45 kD. The 110 kD protein, recognized by the monoclonal anti-β1/β2 antibody (Ahle et al., 1988), eluted somewhat later. SDS-PAGE analysis showed another protein at ~100 kD which co-eluted with the 110 kD polypeptide (Fig. 9B).

Figure 8. Hydroxyapatite chromatography of pea cotyledon AP. Pooled, dialyzed, AP containing fractions 49-58 from Superose-6 chromatography were applied to a HA-Ultrogel column, the column washed, and eluted with a 30ml 10-500 mM K Phosphate gradient. One ml fractions were collected.

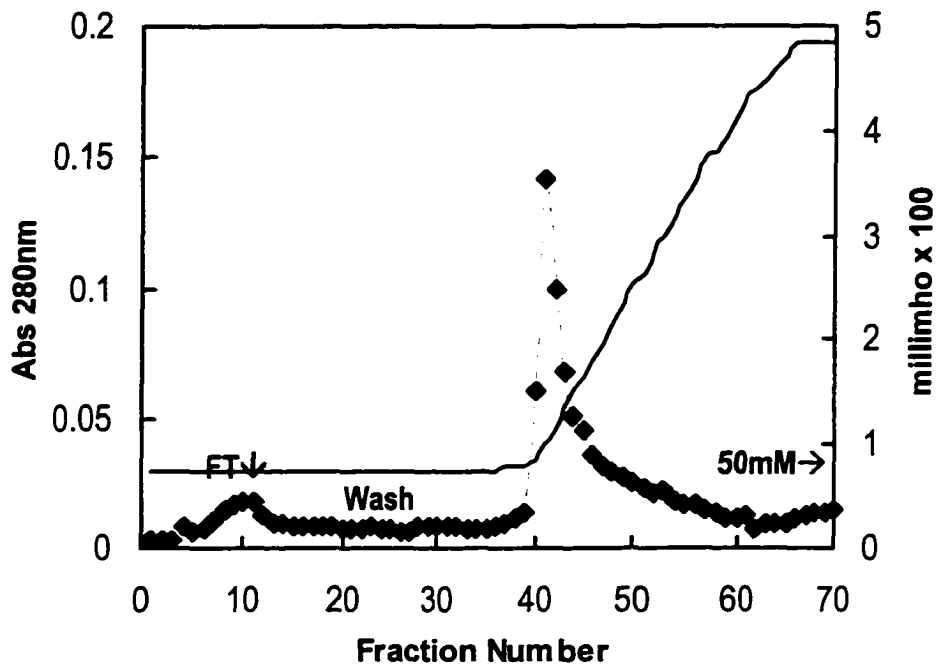
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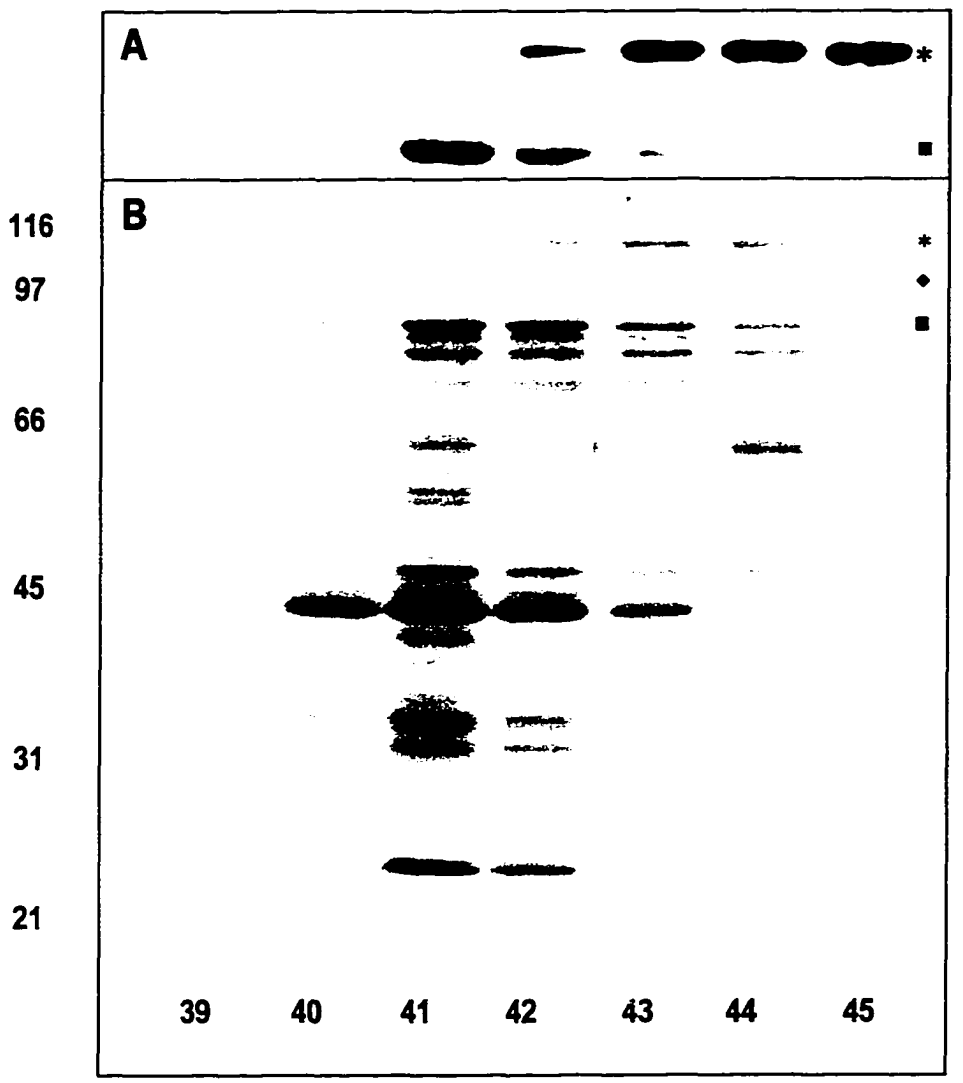
Figure 9. Electrophoretic analysis of hydroxyapatite fractions. Fractions from the HA-Ultrogel profile shown in Figure 8 containing eluted protein (39-45) were divided equally and run on replicate 8-18% acrylamide gels as described in methods. (A) Composite Western blots probed with (*) monoclonal antibodies against the brain β1/β2 AP subunits and polyclonal antibodies against AP2 (▪) using ECL detection. (B) Electrophoretic analysis of HA-Ultrogel fractions, numbered according to Figure 8, stained with Coomassie G. Position of immunoreactive 110 kD (*) and 80 kD (▪) polypeptides identified in (A). A 100 kD protein (♦) is seen to co-elute with the 110kD immunoreactive polypeptide.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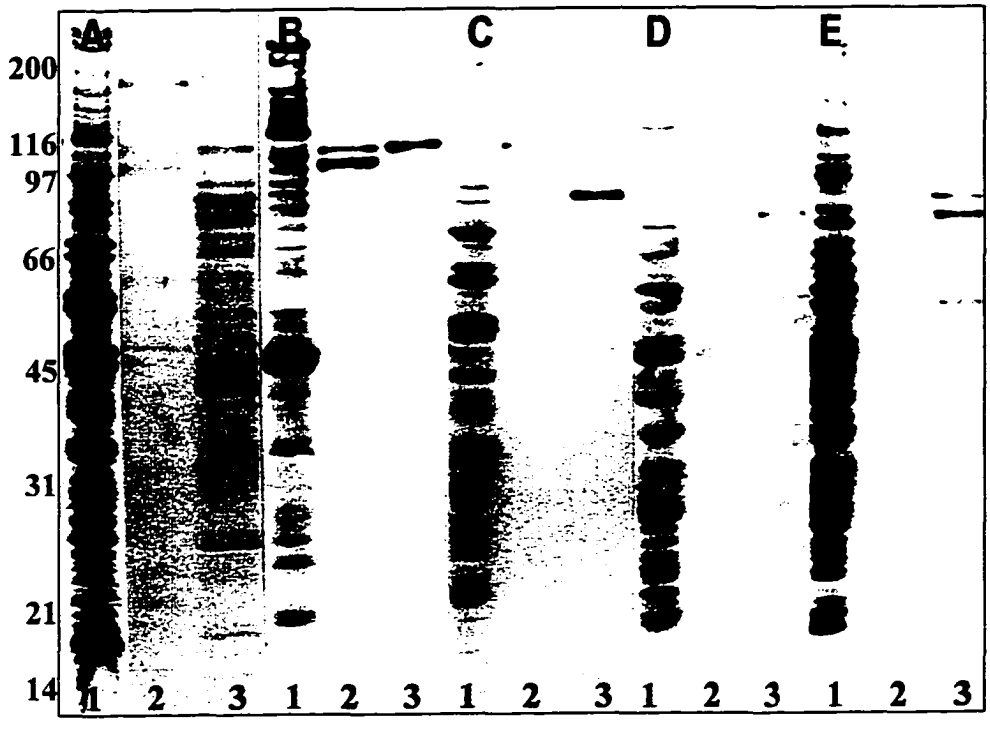
Identification of small and medium subunit equivalents from pea AP

In an attempt to identify other components of the pea AP complexes, western blots of purified brain and pea AP were probed with a variety of polyclonal antibodies against adaptor subunits of yeast and brain. Blots probed with polyclonal antisera against fusion proteins of the yeast small subunits Aps1p (Fig. 10D) and Aps2p (Fig. 10E) (Phan et al., 1994) identified no polypeptides of equivalent size in either pea or brain AP. Polyclonal antisera (Page and Robinson, 1995) against the brain μ-subunits (AP47 and AP50) and σ-subunits (AP17 and AP19) also failed to detect equivalent polypeptides in pea cotyledon AP (data not shown).

In an alternative approach, we attempted to identify the smaller molecular weight components of the pea AP complex by affinity chromatography. Pea APs failed to bind to pea clathrin bound to CN BR-activated Sepharose 4B (data not shown). Commercially available monoclonal antibodies against the β1/β2 large subunits of brain AP (Ahle et al., 1988) were coupled to Pierce Sulfolink™ affinity resin after reduction of the disulfide linkage of the IgG dimer to produce two fragments (each composed of a single heavy and light chain) with free sulfhydryl groups. Activated Sulfolink contains a terminal iodoacetal group, preferentially reactive with free sulfhydryls, which covalently links the resin to antibody fragments, leaving the antigen binding site unaffected. Binding and chromatography were conducted according to manufacturers' recommendations. Polypeptides of 110, 100, 47 and 50 kD bound specifically to the column and were eluted with 0.1 M glycine at

Figure 10. Polyclonal antibodies again the yeast AP small (σ) subunits Aps1 and Aps2 fail to identify equivalent polypeptides in pea and brain AP. (A-E); Lane 1, control GPY1100α, lane 2, 3μg brain AP, lane 3, 25μg pea cotyledon AP. (A) SDS-PAGE analysis, Coomassie G staining. (B-E); Western blot analysis of replicate gel probed with (B) monoclonal antibodies against the brain β1/β2 AP subunits, (C) polyclonal antibodies against AP2. D. polyclonal αAps1, and E. polyclonal αAps2. All primary antibodies used at 1:1000 dilution with ECL detection. Immunoreactive polypeptides of appropriate molecular mass were observed in (B) and (C) while no bands equivalent in size to the yeast small subunits were present in (D) or (E).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pH 2.8 (Fig. 11, lanes 6 and 7), while other proteins of 80 and 70, 45, 38, and 36 kD did not bind and were found in the flow-through fractions (Fig. 11, lanes 2 and 3).

DISCUSSION

CCV coat proteins of bovine brain and other animal tissues contain a predominant polypeptide of 180 kD, two of ~110 and 100 kD and single copy polypeptides of 50, 47, 38 and 33 kD (Keen et al., 1979). The 180 and the two polypeptides of ~ 35 kD have been shown to be clathrin HC and the two clathrin LCs, while the remainder are components of two large heterotetromeric complexes which also include less predominant peptides of 17 and 20 kD. In contrast, in our initial studies, a greater number of polypeptides, with different molecular masses, were found in the coats of pea cotyledon CCVs. Major bands of 110, 50, 46, 40, 33, 31 and 28 kD are present in addition to the 190 kD clathrin HC, although none are as prominent in relationship to clathrin HC as those of brain. Of these, the 50, 46 and 40 kD bands are the strongest. The polypeptides of 50, 46, 40 and 31 kD have previously been demonstrated to possess clathrin LC-like properties of heat stability and calcium binding (Lin et al., 1992) and the 28 kD protein has been identified as ferritin (Hoh and Robinson, 1993). In addition to the above identified proteins, minor bands of 180, 70, 52, 48, 42 and 38 kD were also present. If the APs of pea cotyledon CCVs dissociate from the vesicle and its contents in a manner analogous to those of brain, then pea AP proteins should be found among the remaining unidentified polypeptides.

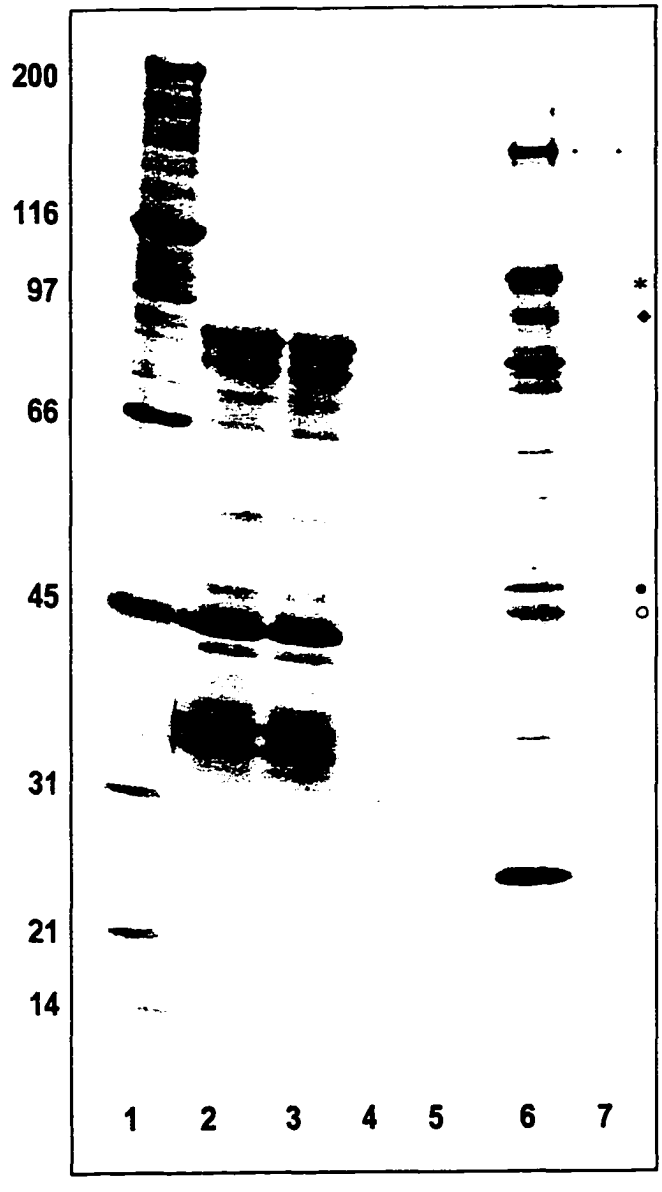
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Figure 11. Electrophoretic analysis of Sulfolink fractions. Monoclonal antibodies against the β1/β2 large subunits of brain AP were coupled to Sulfolink according to manufacturer's recomendations. 200 μg Superose-6 purfied pea cotyledon AP was applied. Lane 1, molecular mass standards. Lanes 2-5, wash fractions. Lanes 6-7, bound proteins sequentially eluted with 0.1 M glycine, pH 2.8. Positions of the bound 110 (*), 100 (◆), 50 (●)and 47 (○) kD proteins are marked.

None of the potential candidates for pea AP proteins, even the 110 kD protein, are prominently represented in the coats of pea cotyledon CCVs. Therefore, any identification and/or purification would necessitate the preparation of significant quantities of highly purified coated vesicles. Since established methods for the isolation of CCVs were inadequate for this task, development of an improved isolation protocol was required. Combination of elements of several different CCV isolation protocols provided both the capacity for processing larger amounts of cotyledon and some of the necessary improvement in purity.

One of the major contaminants in clathrin-coated vesicle preparations from developing pea cotyledon is the iron-storage protein ferritin, present in plastids as a multimeric complex, which is released during homogenization. In legumes, ferritin is found primarily in reproductive tissues including cotyledons, where it is selectively accumulated (Lobreaux and Briat, 1991) for mobilization during seedling development (Hyde et al., 1963). The ferritin complexes present in pea cotyledons, presumably due to their density, co-purify with clathrin coated vesicles, even through the final isopycnic Ficoll/D_2O gradient centrifugation. Then, during coat extraction with Tris-HCl, ferritin complexes fail to sediment with vesicular components on high-speed centrifugation, remaining as a major contaminant in the supernatant containing dissociated coat proteins. Upon Superose-6 molecular sizing chromatography of the extracted coat proteins, the complexed ferritin not only elutes in the same fractions as the putative AP complex, but is also the major protein present (Fig. 3B). This degree of contamination rendered the AP fraction unquantifiable and unusable for any

studies of adaptor interaction with other coat proteins or for further characterization. To minimize ferritin contamination we have capitalized on published schemes which use near molar concentrations of $MgCl_2$ to selectively precipitate ferritin from solution (Hyde et al., 1963) (Sczekan and Joshi, 1987). While it would be possible to treat the partially purified CCVs with $MgCl_2$ at some stage during the isolation procedure itself, any additional step would increase the time required to complete the preparation and might have undesired effects on the vesicles themselves. Instead, we chose to minimize the exposure of CCVs to high concentrations of $MgCl_2$ until the vesicles were to be dissociated. When we added 0.75M $MgCl_2$ to the coat extraction buffer, the yellow coloration previously present in the supernatant after dissociation disappeared, the yellow contamination in the Superose-6 fractions containing the putative APs was decreased and only a small amount of residual ferritin contamination was observed by SDS-PAGE analysis, even with silver staining. A large reduction in the size of the ferritin/AP peak in the elution profile was observed. In addition, we observed that the residual ferritin actually elutes several fractions earlier than does the AP complex. Thus, by using a $MgCl_2$ precipitation during dissociation, and careful selection of fractions, it is possible to nearly eliminate ferritin contamination in pea cotyledon adaptor preparations.

To purify sufficient pea cotyledon AP for both the characterization and interaction studies, maximizing yield at two points was required. The improved CCV isolation protocol significantly increased the amount of CCVs which could be isolated during a given time period, but it was also crucial to strip the maximum quantity of

coat proteins from the CCVs once they were isolated. Previous studies have suggested that dissociation of coat proteins from pea cotyledon CCVs requires harsher conditions than those reported for CCVs isolated from bovine brain (Lin et al., 1992) (Robinson et al., 1991). While efficient dissociation of brain CCV coat proteins occurs within 10 minutes at RT in 0.5M Tris, pH 7.0 (Keen, 1987), we have shown that quantitative coat removal from pea cotyledon CCVs requires elevated pHs, high concentration of urea, use of sodium carbonate or greatly increased incubation times. Although effective, none of these conditions are desirable since with each comes increased risk of protein aggregation, inactivation, or degradation (or a combination of effects). We have found, however, that addition of 0.75M $MgCl_2$ to a 0.5 M Tris-containing dissociation buffer at physiological pH not only effectively precipitates contaminating ferritin, but also increases the recovery of coat proteins from pea cotyledon CCVs when sequential incubations are conducted (see methods). We are thus able to avoid harsher dissociation conditions. Near molar $MgCl_2$ has also been used effectively for coat removal from CCVs of mosquito oocytes (Roth and Porter 1964) and zucchini hypocotyl (Holstein et al., 1994).

It has been suggested that protein degradation caused by hydrolases released during tissue homogenization might be responsible for the weakness of bands in the light chain (30-40 kD) and AP (50 and 100 kD) regions in plant CCVs in comparison to those of brain (Robinson et al., 1991). We wondered whether the measures designed to provide protection against proteolysis in the standard isolation protocol were effective in comparison with a more extensive, but much more expensive,

inhibitor cocktail (Phan et al., 1994). When replicate CCV isolations were conducted simultaneously using either the standard inhibitor regime or this inhibitor cocktail, careful pairwise SDS-PAGE comparison of protein bands from both the initial low speed supernatant after homogenization, and of each pool from the isopycnic Ficoll/D_2O centrifugation, showed few differences between any sample pair. Clearly, the inhibitor cocktail provided no improvement over the standard regime. Consequently, owing to the lack of apparent effect and financial considerations, use of the inhibitor cocktail was not implemented.

Since we observed no difference in the effectiveness of the two inhibitor regimes, the question of whether significant proteolysis occurs during isolation has yet to be resolved. However, we feel that our AP preparations are substantially protease free for the following reason. On western blot analysis of Superose-6 fractionated pea CCV coat proteins, a 110 kD band, but not others, was shown to be immunoreactive against monoclonal antibodies directed against the β1/β2 adaptin subunits of brain. Upon treatment of brain AP with trypsin, brain adaptins are cleaved into two fragments of ~70 and 30-40 kD (Zaremba and Keen, 1985). Plant AP from zucchini exhibits the same behavior (Holstein et al., 1994), with the larger fragment retaining its immunoreactivity to the monoclonal antisera against bovine brain AP. The appearance of the ~70 kD fragment is at the expense of the 108 kD adaptin (Holstein et al., 1994). If significant proteolysis was occurring during either the isolation of pea CCVs or Superose-6 fractionation, we would expect to find immunoreactive proteins of similar molecular weight range to the zucchini fragments

in the AP fractions from pea cotyledon. However, only when AP fractions are repeatedly frozen and thawed over a number of weeks do fragments in the ~70 kD range appear. These fragments, like those of zucchini, are identifiable immunoreactively with the same monoclonal antibody used in zucchini (data not shown).

It appears that a single β-type adaptin is present in plants. Evidence for this is provided by immunoprobes of fractionated coat proteins of pea cotyledon CCVs with monoclonal antisera directed against the mammalian AP β1/β2 subunits (mAb-100/1, (Ahle et al., 1988), mAb-B1/M_6 (Robinson, 1987)). Both antibodies recognize a single immunoreactive band of ~110 kD which elutes on Superose-6, along with other polypeptides, at ~295 kD. The co-elution of the ~110 kD polypeptide with other proteins at a molecular mass similar to that of the brain AP complexes suggests that APs in plants are present as multimeric complexes as in mammalian tissues. Polyclonal antisera which recognizes both the 110 and 100 kD subunits of AP2 from brain CCVs (Timerman et al., 1992), recognizes a single protein of ~80 kD, which co-elutes with the pea ~110 kD AP protein in the ~295 kD fraction, but the antibody fails to recognize the 110 kD protein. The polyclonal antibody may be recognizing a degradation product of the β-adaptin, or alternately, interacting with a differently sized plant homolog of a brain large subunit. It is also conceivable that the antibody is simply recognizing a common epitope on a totally unrelated protein. Of these interpretations, the first is the least possible, since the polyclonal antibody does not recognize the 110 kD adaptin, we have little evidence of

proteolysis in our CCV isolation and the monoclonal antibodies recognize no degradation products present in the same pea Superose-6 fractions. The second is the most attractive, and is supported by the slightly smaller observed size of the AP complex in comparison to brain (~295 versus ~343 kD). No supporting or conflicting evidence for the third interpretation exists. Only additional research can determine which of these alternatives in the most accurate. As a result, we conclude that pea CCVs have only one β-type adaptin, in similarity to yeast (Kirchhausen, 1990) and *Drosophila* (Camidge and Pearse 1994), in contrast to the two present in mammalian CCVs. CCVs isolated from zucchini hypocotyls also appear to contain only a single β-type adaptin (Holstein et al., 1994), suggesting that this configuration may be universal in flowering plants.

Attempts to identify the medium and small subunits of the pea APs by their reactivity to antisera against equivalent subunits of brain and yeast APs using Western blots were unsuccessful. Although the medium (μ) subunits of AP1 and AP2 are thought to be highly conserved between mammalian species (Thurieau et al., 1988) the failure of polyclonal antibodies against bovine brain μ1 and μ2 (Page and Robinson, 1995), to recognize any appropriately size polypeptides in pea cotyledon AP suggest that the plant AP μ-subunits may show little sequence homology to their mammalian counterparts. In other studies polyclonal antibodies against Aps1p and Aps2p, the yeast σ1/σ2 AP subunits (Phan et al., 1994), failed to identify appropriately sized polypeptides in either bovine brain or pea cotyledon APs. This observation is consistent with amino acid sequence analysis which has shown that the

yeast small subunits, APS1 and APS2, have only 53% (Nakai et al., 1993) and 50% (Kirchhausen et al., 1991) identity, respectively, to their murine equivalents. In addition, genomic clones that encode homologs of mammalian AP19, recently isolated from the flowering plants *Camptotheca acuminata* and *Arabiopsis thaliana* (Maldonado-Mendoza and Nessler 1996a; Maldonado-Mendoza et al., 1996b) also show identities of approximately 50% to APS1. This level of sequence conservation, reflecting the evolutionary distance between plants and animals, may prove typical for angiosperm equivalents of many mammalian genes. Clathrin HC from soybean, for example, also exhibits only 50% identity to HC from rat, *Drosophila* and yeast ((Blackbourn and Jackson, 1996).

Two AP complexes are present in mammalian cells, AP2, which localizes to the PM, and AP1, which is found in the *trans*-Golgi. While these two complexes co-elute on molecular sizing chromatography, they can be separated on hydroxylapatite (Manfredi and Bazari, 1987). In contrast, HA chromatography of Superose-6 fractions of pea cotyledon AP produced only a single peak. Within this peak, the 110 kD β-adaptin was found to co-elute with another polypeptide of ~100 kD which is not recognized by the monoclonal β1/β2 antibody. We consider this protein a candidate for the non-β adaptin component of the AP complex. Interestingly, within the single HA peak, the 80 kD protein identified by the polyclonal antibody against AP2 elutes several fractions earlier than the 110 kD protein. If the 80 kD protein were simply a degradation product of the 100 kD β-type adaptin, it should co-elute with the remainder of the complex. It also seems unlikely that a totally unrelated protein

would have so nearly identical behavior on hydroxylapatite. It is conceivable, then, that the 80 kD polypeptide is a component of a second AP complex present in pea cotyledon CCV. Brain clathrin, bound to CNBR-activated Sepharose 4B, has been successfully used to affinity purify AP2 from Tris-HCl dissociated coat proteins (Keen, 1987). AP1, however, failed to bind to the immobilized clathrin and eluted in the flow through. In mammals AP1 has been localized exclusively to the *trans*-Golgi (Ahle et al., 1988). In similar experiments with pea clathrin-CNBR-activated Sepharose 4B, we observed no binding of pea AP to immobilized pea clathrin (data not shown). The lack of binding of pea cotyledon AP on clathrin-Sepharose supports an AP1-like designation and Golgi origin for the adaptor components of clathrin vesicles from pea cotyledon. A Golgi origin for CCVs was also suggested by the presence of a vacuolar receptor in CCVs prepared from pea cotyledon (Kirsch et al., 1996) and immunolocalization studies which co-localize this receptor with the Golgi (Paris et al., 1997). In contrast to the lack of binding of polypeptides from the pea AP preparation to pea clathrin-CNBR-activated Sepharose 4B, polypeptides equivalent in size to brain AP subunits bound specifically to Sulfolink™ resin coupled with a monoclonal antibody against the β-subunits of bovine brain (Ahle et al., 1988). Bound polypeptides of 110 and 100 kD may be homologs of the mammalian large (β and α/γ) subunits. Although we have limited evidence of other non-adaptin subunits from our Western blot and hydroxylapatite data, the specific binding of both a 50 and a 47 kD polypeptide to the Sulfolink column does suggest that two μ-type subunits (and presumably two AP complexes) are present in plants as well as in mammalian

CCVs. Until sequence data is available, however, it is only speculation that these μ-type subunits are components of two distinct AP complexes. Taken together, the Sulfolink and hydroxylapatite data is consistent with the presence of another AP complex in pea cotyledon. However, until the 80 kD protein is characterized, we cannot determine the actual number of AP complexes present in pea cotyledon CCVs. In addition, while it is tempting to identify the complex containing the immunoreactive 110 kD polypeptide as of the β1-type by its early elution on HA, such determination ultimately depends upon convincing immunolocalization, preferably at the electron microscopic level.

REFERENCES

Ahle S, Mann A, Eichelsbacher U, Ungewickell E (1988) Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. EMBO J **7:** 919-929

Ahle S, Ungewickell E (1990) Auxilin, a newly identified clathrin-associated protein in coated vesicles from bovine brain. J Cell Biol **111:** 19-29

Ahle S, Ungewickell E (1986) Purification and properties of a new clathrin assembly protein. EMBO J **5:** 3143-3149

Bar-Zvi D, Branton D (1986) Clathrin-coated vesicles contain two protein kinase activities: phosphorylation of clathrin ß-light chain by casein kinase II. J Biol Chem **261:** 9614-9621

Barroso J, Pais MSS (1987) Coated vesicles in the cytoplasm of the host cells in *Ophrys lutea* Cav. mychorrhizas (Orchidaceae). New Phytol **105:** 67-70

Blackbourn HD, Jackson AP (1996) Plant clathrin heavy chain: sequence analysis and restricted localization in growing pollen tubes. J Cell Sci **109:** 777-787

Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant - proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8:** 93-99

Butler JM, Beevers L (1993) Putative adaptor proteins of clathrin coated vesicles from developing pea. *In* DJ Morré, KE Howell, JJM Bergeron, eds. Molecular Mechanisms of Membrane Traffic, Vol H74. Springer-Verlag, Berlin-Heidelberg, pp 331-332

Camidge DR, Pearse BFM (1994) Cloning of *Drosophila* β-adaptin and its localization on expression in mammalian cells. J Cell Sci **107:** 709-718

Campbell C, Squicciarini J, Shia M, Pilch PF, Fine RE (1984) Identification of a protein kinase as an intrinsic component of rat liver coated vesicles. Biochemistry **23:** 4420-4426

Coleman J, Evans D, Hawes C, Horsley D, Cole L (1987) Structure and molecular organization of higher plant coated vesicles. J Cell Sci **88:** 35-45

Depta H, Robinson DG (1986) The isolation and enrichment of coated vesicles from suspension-cultured carrot cells. Protoplasma **130:** pp162-170

Fowke LC, Griffing LR, Mersey BG, Van der Valk P (1983) Protoplasts for studies of plasma membrane and associated cell organelles. Experientia **Suppl. 46:** 101-110

Fowke LC, Tanchek MA, Galway ME (1991) Untrastructural cytology of the endocytotic pathway in plants. *In* R Hawes, JOD Coleman, DE Evans eds, Endocytosis, Exocytosis and Vesicle Traffic in Plants. Cambridge University, Cambridge, pp15-40

Georgieva-Hanson V, Schook WJ, Puszkin S (1988) Brain CV destabilization and phosphorylation of coat proteins. J Neurochem **50:** 307-315

Harley SM, Beevers L (1989a) Isolation and partial characterization of clathrin-coated vesicles from pea (*Pisum sativum* L.) cotyledons. Protoplasma **150:** 103-109

Harley SM, Beevers L (1989b) Coated vesicles are involved in the transport of storage proteins during seed development in *Pisum sativum* L. Plant Physiol. **91:** 674-678

Heuser JE, Keen J (1988) Deep-etch visualization of proteins involved in clathrin assembly. J Cell Biol **107:** 877-886

Hoh B, Robinson DG (1993) The prominent 28kDa polypeptide in clathrin coated vesicle fraction from developing pea cotyledons in contaminating ferritin. Cell Biol Int **17:** 551-557

Hoh B, Schauermann G, Robinson DG (1991) Storage protein polypeptides in clathrin coated vesicle fractions are not due to endomembrane contamination. J Plant Physiol **138:** 309-316

Holstein SEH, Drucker M, Robinson DG (1994) Identification of a β-type adaptin in plant clathrin-coated vesicles. J Cell Sci **107:** 945-953

Horn MA, Heinstein PF, Low PS (1989) Receptor-mediated endocytosis in plant cells. Plant Cell **1:** 1003-1009

Hyde BB, Hodge AJ, Kahn A, Birnsteil ML (1963) Studies on phytoferritin .1. identification and localization. Ultrastructural Res **9:** 248-258

Keen JH (1987) Clathrin assembly proteins: affinity purification and a model for coat assembly. J Cell Biol **105:** 1989-1998

Keen JH, Chestnut MH, Beck KA (1987) The clathrin coat assembly polypeptide complex. J Biol Chem. **262:** 3864-3871

Keen JH (1990) Clathrin and associated assembly and disassembly proteins. Annu Rev Biochem **59:** 415-438

Keen JH, Willingham MC, Pastan IH (1979) Clathrin-coated vesicles: isolation, dissociation and factor-dependent reassociation of clathrin baskets. Cell **16:** 303-312

Kirchhausen T (1990) Identification of a putative yeast homolog of the mammalian ß chains of the clathrin associated protein complexes. Mol Cell Biol **10:** 6089-6090

Kirchhausen T, Davis AC, Frucht S, Greco BO, Payne GS, Tubb B (1991) AP17 and AP19, the mammalian small chains of the clathrin-associated protein complexes show homology to Yap17p, their putative homolog in yeast. J Biol Chem **266:** 11153-11157

Kirsch T, Paris N, Butler JM, Beevers L, Rogers JC (1994) Purification and initial characterization of a plant vacuolar targeting receptor. Proc Nat Acad Sci USA **91:** 3403-3407

Kirsch T, Saalbach G, Raikel N, Beevers L (1996) Interaction of a potential vacuolar targeting receptor with amino- and carboxyl-terminal targeting determinants. Plant Physiol **111:** 469-474

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:** 680-685

Lin H, Harley SM, Butler JM, Beevers L (1992) Multiplicity of clathrin light-chain-like polypeptides from developing pea (*Pisum sativum* L.) cotyledons. J Cell Sci. **103:** 1127-1137

Lindner R, Ungewickell E (1992) Clathrin-associated proteins of bovine brain coated vesicles. An analysis of their number and assembly-promoting activity. J Biol Chem. **267:** 16567-16573

Lobreaux S, Briat J-F (1991) Ferritin accumulation and degradation in different organs of pea (*Pisum sativum*) during development. Biochem J **274:** 601-606

Low PS, Chandra S (1994) Endocytosis in plants. Annu Rev Plant Physiol Plant Mol Biol **45:** 609-631

Maizel JV (1971) Polyacrylamide gel electrophoresis of viral proteins. Methods Virol **5:** 179-246

Maldonado-Mendoza IE, Nessler CL (1996a) Cloning and expression of the plant homolog of the small subunit of the Golgi-associated clathrin assembly protein AP19 from *Camptotheca acuminata.* Plant Mol Biol **32:** 1149-1153

Maldonado-Mendoza IE, Vincent R, Nessler CL (1996b) Molecular characterization of the AP19 gene family in *Arabidopsis*: component of the clathrin assembly protein complex AP-1 (abstract no. 170). Plant Physiol **111:** S-61

Manfredi JJ, Bazari WL (1987) Purification and characterization of two distinct complexes of assembly polypeptides from calf brain coated vesicles that differ in their polypeptide composition and kinase activities. J Biol Chem **262:** 12182-12188

Méresse S, Ludwig T, Frank R, Hoflack B (1990) Phosphorylation of the cytoplalsmic domain of the bovine cation-independent mannose 6-phosphate receptor. J Biol Chem **31:** 18833-18842

Mersey BG, Griffing LR, Rennie PJ, Fowke LC (1985) The isolation of coated vesicles from protoplasts of soybean. Planta **163:** 317-327

Nakai M, Takada T, Toshiya Endo (1993) Cloning of the YAP19 gene encoding a putative yeast homolog of AP19, the mammalian small chain of the clathrin-assembly proteins. Biochim Biophys Acta **1174:** 282-284

Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis **9:** 255-262

Page LJ, Robinson MS (1995) Targeting signals and subunit interactions in coated vesicle adaptor complexes. J Cell Biol **131:** 619-630

Paris N, Rogers SW, Jiang L, Kirsch T, Beevers L, Phillips TE, Rogers JC (1997) Molecular cloning and further characterization of a probably plant vacuolar sorting receptor. Plant Physiol **115**: 29-39

Pauloin A, Thurieau C (1993) The 50 kDa protein subunit of assembly polypeptide (AP) AP-2 adaptor from clathrin-coated vesicles is phosphorylated on threonine-156 by AP-1 and a soluble AP50 kinase which co-purifies with the assembly polypeptides. Biochem J **296:** 409-415

Pearse BMF, Robinson MS (1984) Purification and properties of 100-kD proteins from coated vesicles and their reconstitution with clathrin. EMBO J **3:** 1951-1957

Pearse BMF (1982) Coated vesicles from human placenta carry ferritin, transferrin, and immunoglobulin G. Proc Nat Acad Sci USA **79:** 451-455

Phan HL, Findlay JA, Chu DS, Tan PK, Kirchhausen T, Payne GS (1994) The *Saccharomyces cerevisiae* APS1 gene encodes a homolog of the small subunit of the mammalian clathrin AP-1 complex: evidence for functional interation with clathrin at the Golgi complex. EMBO J **13:** 1706-1717

Pypaert M, Mundy D, Souter E, Labbe J-C, Graham W (1991) Mitotic cytosol inhibits invagination of coated pits in broken mitotic cells. J Cell Biol **114:** 1159-1166

Robinson DG, Balusek K, Freundt H (1989) Legumin antibodies recognize polypeptides in coated vesicles from developing pea. Protoplasma **150:** 79-82

Robinson DG, Balusek K, Depta H, Hoh B, Holstein SEH (1991) Isolation and characterization of plant coated vesicles. *in* C Hawes, D Evans, J Coleman eds. Endocytosis, Exocytosis and Vesicle Traffic in Plant Cells, Cambridge University Press, Cambridge, pp 65-79 .

Robinson DG (1996) Clathrin-Mediated Trafficking. Trends Plant Sci **1:** 349-355

Robinson DG, Balusek K, Demmer A, Holstein SEH (1991) Some current problems in plant coated vesicle research. Giornale Botanico Italiano **125:** 55-61

Robinson MS (1987) 100-kD coated vesicle proteins: Molecular heterogeneity and intracellular distribution studied with monoclonal antibodies. J Cell Biol **104:** 887-895

Roth TF, Porter KR (1964) Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti* L. J Cell Biol **20:** 313-332

Sczekan SR, Joshi JG (1987) Isolation and characterization of ferritin from soyabeans (*Glycine max*)*. J Biol Chem **262:** 13780-13788

Staehelin LA, Moore I (1995) The plant golgi apparatus: structure, functional organization and trafficking mechanisms. Annu Rev Plant Physiol Plant Mol Biol **46:** 261-288

Stamnes MA, Rothman JE (1993) The binding of AP-1 clathrin adaptor particles to Golgi membranes requires ADP-ribosylation factor, a small GTP-binding protein. Cell **73:** 999-1005

Tanchak MA, Griffing LR, Mersey BG, Fowke LC (1984) Endocytosis of cationized ferritin by coated vesicles of soybean protoplasts. Planta **162:** 481-486

Timerman AP, Mayleitner MM, Lukas TJ, Chadwick CC, Saito A, Watterson DM, Schindler H, Fleisher S (1992) Inositol polyphosphate receptor and clathrin assembly protein AP-2 are related proteins that form potassium-selective ion channels in planar lipid bilayers. Proc Natl Acad Sci USA **89:** 8976-8980

Thurieau C, Brosius J, Burne C, Jolles P, Keen JH, Mattaliano RJ, Chow EP, Ramachandran KL, Kirchhausen T (1988) Molecular cloning and complete amino acid sequence of AP50, an assembly protein associated with clathrin-coated vesicles. DNA **7:** 663-669

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA **76:** 4350-4354

Ungewickell E, Ungewickell H, Holstein SEH, Lindner R, Prasad K, Barouch W, Martin B, Greene LE, Eisenberg E (1995) Role of auxilin in uncoating clathrin-coated vesicles. Nature **378:** 632-635

Vigers GPA, Crowther RA, Pearse BMF (1986) Location of the 100kD - 50kD accessory proteins in clathrin coats. EMBO J **5:** 2079-2085

Weiss RL (1983) Coated vesicles in the contractile vacuole/mating structure region of *Chlamydomonas*. J Untrastructure Res **85:** 33-44

Wiedenhoeft RE, Schmidt GW, Palevitz BA (1988) Dissociation and reassembly of soybean clathrin. Plant Physiol **86:** 412-416

Wilde A, Brodsky FM (1996) *In vivo* phosphorylation of adaptors regulates their interaction with clathrin. J Cell Biol **135:** 635-645

Zaremba S, Keen JH (1985) Limited proteolytic digestion of coated vesicle assembly polypeptides abolishes reassembly activity. J Cell Biochem **28:** 47-58

Zhang XQ, Dubacq J-P, Alfsen A (1993) Biochemical and cytological evidence for the stimulation of clathrin-coated pit (vesicle) formation by exogenous folic acid in *Dunaliella salina* (Chlorophyta). J Phycol **29:** 203-209

Chapter III

Assembly of Clathrin Coat Components of Pea and Brain:

A Comparative Study

ABSTRACT

Dialysis of total coat proteins of clathrin coated vesicles (CCVs) prepared from developing cotyledons of field grown peas against buffer of moderate ionic strength and purification by sucrose density gradient centrifugation produced a single population of coats with mean diameters of ~80 nm. After redissociation and separation by Superose-6 molecular sieve chromatography, SDS-PAGE analysis showed the coats to be enriched in proteins of 114, 110, 80, 52, 50, 48, 47, 46 and 40 kD in addition to the 190 kD clathrin heavy chain. These clathrin binding proteins are considered as potential plant homologs for the clathrin associated proteins thought to drive CCV coat formation in brain.

In a comparative study of isolated coat proteins of clathrin coated vesicles prepared from developing cotyledons of field grown peas and bovine brain, triskelions of pea CCVs reassemble, on dialysis, into two distinct populations of polyhedral cages with means of 91.22nm (±15.724 SD) and 71.60 (±7.74 SD). In contrast, the triskelions from brain CCVs failed to reassemble into lattices under these conditions. Reassembly of triskelions from both bovine brain and peas into cages is stimulated by the incorporation of 2 mM free Ca^{++} into the dialysis buffer. Under these assembly

conditions, two populations of pea clathrin cages with means of 97.94 (±12.87 SD) and 69.5 nm (±8.87 SD) are formed, while only a single population of cages with a mean of 80.42 nm (±9.52 SD) form from brain triskelions.

In a search for potential cytosolic clathrin assembly proteins not identified by the clathrin binding experiments, pea cytosol was fractionated by sequential ammonium sulfate precipitations and included in pea assembly assays. No stimulation of assembly occurred, nor was assembly stimulated by the nucleotides ATP or GTP. Pea clathrin self-assembly was shown to be concentration dependent, however, this was much less apparent at pH 7.2 (a more cytosolically relevant pH). Maximal self-assembly occurred at pH 6.5, however, 30-40% self-assembly continued to pH 7.5.

At neutral pH without addition of free Ca^{++}, reassembly of bovine brain clathrin into regular polyhedral coats is dependent upon addition of adaptor proteins recovered from the molecular sieve chromatography. While pea clathrin readily self-assembles without addition of adaptor proteins, dialysis of pea triskelions in the presence of potential pea adaptors identified as clathrin binding proteins, produces coats of significantly smaller diameter than self-assembled cages or those formed in the presence of calcium. This suggests that these potential adaptors while not required, do exert control over coat reassembly *in vitro*.

Reciprocal mixing of pea adaptor proteins with brain triskelions and brain adaptors with pea triskelions produced quite different results. When pea adaptors were dialyzed with brain clathrin very little assembly occurred and fragments formed were not significantly different than those formed from brain clathrin alone. Pea adaptors

however, do bind to pre-formed brain cages. With the addition of brain adaptors to pea triskelions, however, most clathrin assembled into recognizable coats. SDS-PAGE analysis demonstrates incorporation of brain adaptors into these structures. The average diameter of the cages (70nm±5.95 SD) was intermediate between those formed from pea clathrin/pea adaptors and brain clathrin/brain adaptors suggesting that brain adaptors not only can bind to pea clathrin but can interact to effect the geometry of the resulting coats.

INTRODUCTION

Clathrin coated vesicles (CCVs), subcellular structures with characteristic lattice-like coats formed from the protein clathrin, constitute a universal organelle of eukaryotic cells. These organelles, first described in the 1960's, were postulated to have a role in the uptake of exogenous ferritin in cells of toad spinal ganglion (Rosenbluth and Wissig, 1963) (Rosenbluth and Wissig, 1964) and in the uptake of yolk proteins in the mosquito oocyte (Roth and Porter, 1964). After numerous descriptions in the microscopy literature, coated vesicles were finally isolated from porcine brain (Pearse 1975)and the major protein associated with their coats named clathrin from the Latin "clathrate" meaning "lattice-like". Subsequently, CCVs have been isolated from a variety of mammalian tissues (e.g. bovine brain and adrenal gland, porcine brain and human placenta), yeast, and a number of plant sources

including pea cotyledon, carrot and soybean suspension culture cells, zucchini hypocotyls, soybean epicotyls and wheatgerm.

While in mammalian systems CCVs have been implicated in the recycling of both receptors and membranes, transepithelial transport, and the intracellular transport of Golgi derived secretory and lysosomal proteins, our understanding of the involvement of clathrin coated vesicles in endocytosis, membrane processing and intracellular protein trafficking in plants is still rudimentary. Early evidence for endocytosis in plants comes from the localization of cationized ferritin (Tanchak et al., 1884; Joachim and Robinson, 1884) to plasma membrane (PM) coated pits of soybean protoplasts. The exogenous ferritin was followed through PM buds, structurally distinct coated vesicles, and ultimately, the partially coated reticulum (PCR). Two additional studies suggest that, in plants, CCV are active in retrieval of plasma membrane via endocytosis. Smooth secretory vesicles carry Golgi-derived matrix polysaccharides to the cell wall during cell plate formation, yet no buildup of plasma membrane is observed (Staehelin and Moore, 1995). In addition, eight times the number of coated pits are observed in cell-wall forming tobacco protoplasts, in comparison with protoplasts incapable of forming cell walls ((Fowke et al., 1983). Receptor-mediated endocytosis has been well established in animal systems, but hard supporting evidence in plant systems is limited to a few studies on uptake of fungal elicitor proteins and biotin (reviewed in (Horn et al., 1989; Low and Chandra, 1994)). A role in protein sorting and intracellular protein transport has also been postulated for CCVs. Developing cotyledons of legumes actively deposit storage proteins in

large protein storage vacuoles termed "protein bodies" and early studies suggested that CCVs of garden pea carry precursors of both lectin as well as other vacuolar proteins such as hydrolytic enzymes (Harley and Beevers, 1989b) and the storage proteins legumin and vicilin (Robinson et al., 1989; Robinson et al., 1991; Hoh et al., 1991). However, recent studies suggest that dense vesicles, not CCVs, transport legumin and vicilin from the *trans*-Golgi network to protein storage vacuoles (Hohl et al., 1996). Since more than one type of vacuole is present in storage parenchyma of peas (Hohl et al., 1996; Paris et al., 1996), CCVs may well transport vacuolar hydrolases to lytic vacuoles. The isolation of a putative vacuolar transport receptor which binds to the N-terminal vacuolar targeting domain of barley aleurain, sweet potato sporamin and 2S albumin of Brazil nut ((Kirsch et al., 1994; Kirsch et al., 1996) from a CCV-enriched fraction of pea cotyledon supports this sorting/transport role for CCVs.

In mammalian tissues, the characteristic polygonal lattice of the CCV coat is assembled from triskelions, molecules named for their distinctive three-legged appearance when viewed by electron microscopy. Each leg of the triskelion is composed of a heavy chain (Mr~180 kD) bound to a light chain (33-38 kD) and is divided into two sections by a kink. These sections are labeled proximal and distal according to their distance from the triskelion center. The carboxy termini of three heavy chains form a common hub (the triskelion center) which lies at the vertex of a polyhedron whose edges are formed by the overlapping proximal portion of legs of adjacent triskelions. In addition to triskelions, CCV coats from mammalian sources

contain additional proteins variously described as associated, assembly or adaptors proteins (commonly designated simply as APs) isolated both as heterotetromeric complexes and as monomers. In addition to the heterotetromers, three monomeric proteins designated AP180, auxilin, and p140, present in CCV coats of bovine brain, co-purify with the AP1 and AP2 complexes on molecular sieving of Tris-Cl dissociated coat proteins (Lindner and Ungewickell, 1992). Using electron microscopic imaging of bovine brain CCVs in vitreous ice, the three-dimensional structure of clathrin coats was shown to be three nested shells with the outermost layer formed of triskelions with overlapping legs packed to form lattices of hexagons and pentagons, the intermediate layer consisting of clathrin terminal domains, and the innermost layer consisting of APs (Vigers et al., 1986)

Using immunofluorescence microscopy, the heterotetromeric complexes, AP1 and AP2 (originally termed HA_1 and HA_2 for their elution order on hydroxyapatite chromatography) have been specifically localized to either plasma membrane (AP2) or Golgi (AP1) in Madin-Darby bovine kidney (MDBK) cells (Ahle et al., 1988). Each AP2 complex is thought to contain two ~100-110 kD large subunits (α/β_2) and single copies of the medium and small subunits, μ_2 (50 kD) and σ_2 (17 kD) while AP1 contains two 100 -110 kD subunits (γ/β_1), and single copies of the medium and small subunits, μ_1 (47 kD) and σ_1 (20 kD). Using highly purified AP2 from bovine brain, the molecular structure was shown to be a central "brick-like" structure with ears by freeze-etch electron microscopy (Heuser and Keen, 1988). AP1 is thought to have a similar structure. AP1 and AP2 interact both with clathrin and the

tails of cytoplasmic receptors (Pearse, 1988; Glickman et al., 1989) and AP2 has been shown to promote coat assembly (Zaremba and Keen, 1983; Keen, 1987; Keen et al., 1987) *in vitro*. Of the three additional proteins which co-purify with the AP1 and AP2 complexes on gel filtration of dissociated coat proteins, AP180 (Ahle and Ungewickell, 1986) and auxilin (Ahle and Ungewickell, 1986)but not AP140 (Lindner and Ungewickell, 1992) have been shown to promote coat assembly *in vitro*.

Our knowledge of the molecular composition of CCV coat components, like our knowledge of the function of CCVs in plants is limited. Triskelions, as in mammalian CCVs, form the characteristic polygonal coat of plant CCVs. The major structural component of this coat, clathrin HC, has been identified. Molecular weights of 185-190 kD, slightly larger than that of mammalian HC, have been reported (Mersey et al., 1985; Depta and Robinson, 1986; Balusek et al., 1988; Harley and Beevers, 1989a). Antibodies raised against bovine brain clathrin heavy chain react with plant heavy chain (Cole et al., 1987). However, others have failed to demonstrate similar results. Most recently, a full length cDNA of soybean HC, which codes for an amino acid sequence of 1700 residues, has been sequenced. The calculated size of soybean HC (193,353) is approximately 2000 kD larger than that of rat, and may explain the larger apparent M_r observed on SDS-PAGE (Blackbourn and Jackson, 1996). A number of potential light chains candidates have been identified (Coleman et al., 1987; Lin et al., 1992), and putative plant AP complexes identified and partially characterized in pea cotyledon ((Butler and Beevers, 1993) and Chapter 2, this dissertation) and in zucchini hypocotyl (Holstein et al., 1994).

In mammalian cells CCVs have been shown to undergo a dynamic assembly and disassembly cycle, in which clathrin triskelions polymerize on the donor membrane to form coated pits which then bud and release vesicles. The architecture of the polymerizing coat itself is thought to be the driving force producing curvature in the attached segment of the membrane. *In vivo,* mammalian CCVs are uncoated by a cytosolic uncoating ATPase prior to their fusion with the acceptor membrane. Coat components released are then recycled. An uncoating ATPase has been partially purified from plant sources (Kirsch and Beevers, 1993) and in similarity to the mammalian uncoating ATPase has been shown to be a member of the heat shock family of proteins. *In vitro,* CCVs can be dissociated into coat components and vesicles by incubation with chaotropic reagents such as Tris-HCl, urea and other protonated-polyamines. The vesicle components are then removed by high speed centrifugation. Subsequent partitioning of the coat components by gel chromatography resulted in the identification of the assembly properties of the non-clathrin adaptor peaks. This led to much of our current knowledge of the process of protein sorting by transport vesicles.

Knowledge of vesicular budding processes of CCVs in plants is rudimentary, limiting our understanding of vesicular protein transport. In this paper the requirements for reassembly of plant coats (clathrin triskelions and APs) and cages (clathrin triskelions alone) *in vitro* have been investigated and demonstrated to differ in AP-dependence from those of mammalian sources. While under appropriate conditions assembly of plant triskelions into coats incorporating APs can be

demonstrated, no stimulation of assembly itself occurs. However, assembly of plant triskelions into polyhedral lattices at neutral pH is stimulated by APs isolated from bovine brain. The incorporation of brain AP into hybrid polyhedric structures indicates conservation of binding sequences across the animal and plant kingdoms.

MATERIALS AND METHODS

Buffers

The following buffers were used. Buffer A: 0.1 M NaMes (2-(N-morpholinomethane sulfonic acid), 0.1 M EGTA, 0.5 mM $MgCl_2$, 0.02% sodium azide pH 6.5; Buffer B: Buffer A containing 0.025 mg/ml trypsin inhibitor, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM dithiolthreitol (DTT); Buffer C: Buffer B plus 0.3 M sorbitol. Gel filtration buffer (D) was a 1:1 (vol/vol) mixture of Buffer A : 1.0 M Tris, pH 7.0. Buffer E: 0.1M Pipes, 0.1 M EGTA, 0.5 mM $MgCl_2$, 0.02% Sodium azide, pH 7.2 containing 0.025 mg/ml trypsin inhibitor, 0.5 mM PMSF, and 1 mM DTT. All buffers were adjusted to final pH at RT.

Vesicle Isolation

Coated vesicles were isolated from both pea cotyledons and bovine brain using a fusion of the methods of (Lin et al., 1992) and (Campbell et al., 1984) which utilizes both Ficoll/Sucrose and Ficoll/D_2O purification steps. This method provides a

substantial improvement in purity of CCVs preparations over either of the individual methods.

Field grown peas, (*Pisum sativum* L. cv. Burpeeana), picked 24-27 days post anthesis, were mechanically shelled, frozen by submersion in liquid nitrogen, and stored at -80°C. Frozen peas in 500 gram lots were thawed for 15 minutes at room temperature, and homogenized for 2 min in a Waring blender in equivalent amounts (weight/vol) Buffer C. Trypsin Inhibitor, DTT and PMSF were added immediately prior to homogenization. The homogenate was squeezed through 4 layers of cheesecloth, and centrifuged at 200 x g (all values are calculated as r^{avg}) for 5 min followed by 17,000 x g for 30 minutes in a Sorvall RC5B centrifuge (GSA rotor) at 4°C. All subsequent centrifugations were conducted at 4°C in either a Beckman L2 or L8-80 high speed centrifuge. The supernatant was collected by aspiration and centrifuged at 120,000 x g for 40-50 minutes in either a Beckman 45Ti or Beckman 42.1 rotor. Pellets from a total of 1500 gms cotyledons were resuspended using a hand homogenizer in Buffer B to a final volume of 10 mls/150 gm cotyledons. Contaminating ribosomes were removed by incubating this suspension with 1 mg/ml pancreatic ribonuclease A for 1 hr at 4°C with gentle shaking.

Following the ribonuclease digestion, an equal volume of 12% Ficoll/12% Sucrose in Buffer A was mixed with the suspension and centrifuged at 16K for 30 minutes (20,000 x g) in a Beckman 42.1 rotor. The supernatant was collected by Pasteur pipette, diluted with 3 volumes Buffer A, and centrifuged at 120,000 x g for 50 min as above.

Pellets were then resuspended in 60 ml Buffer A (25g initial cotyledon weight/ml), and could be frozen at -80°C without apparent loss of coated vesicle yield. Finally, the resuspended pellets were layered (10 mls/gradient) onto 20 ml linear gradients consisting of 5% Ficoll/10% D_2O to 25% Ficoll/90% D_2O in Buffer A and centrifuged at 55,000 x g for 16 hrs in a Beckman SW28 swinging bucket rotor. The location of CCVs within the gradients after centrifugation was determined by SDS-PAGE analysis of fractionated gradients and determined to be highly reproducible. In subsequent vesicle preparations regions containing coated vesicles were simply pooled, diluted with 6 volumes Buffer A and centrifuged for 50-60 min at 120,000 x g. Pellets containing coated vesicles were resuspended in a small amount of the same buffer and frozen at -80°C until use. Normally, 12-15 mg highly purified CCVs were obtained from 3 kg cotyledon using this method, although some year-to-year variation in yield was observed.

Clathrin coated vesicles were isolated from bovine brain using essentially the same method. However, sorbitol was not included in the homogenization buffer and the initial cheesecloth filtration and ribonuclease digestion were also omitted. Typically, 1 Kg brain yielded 50 mg highly purified CCV.

Preparation and Fractionation of Coat Proteins

Brain coat proteins were dissociated from CCVs by addition of 0.33 vol of 2 M Tris-HCl, pH 7.0, 8mM EDTA, 0.4 mM PMSF incubated for 15 min on ice (Lindner and Ungewickell, 1992) followed by ultracentrifugation at 170,000 x g for 50 min in a T65

rotor. Extractions were repeated at least once and supernatants combined. Coat proteins (15-30 mg in 3-6 ml) obtained from 1-2 kg brain were loaded on a 2.5 x 66 cm Superose-6 gel filtration column, equilibrated in Buffer D, and eluted downward at ~ 0.6 ml/min at 10°C. Four ml fractions were collected. After SDS-PAGE and immunoblotting (see below) to locate APs within the elution profile fractions 37-42 containing dissociated clathrin triskelions and fractions 50-58 containing APs were pooled separately and concentrated using Amicon Centri-Prep10™ concentrators.

To obtain pea coat proteins CCVs from 3 kg cotyledons are pelleted, resuspended in 1.75 ml dissociation buffer containing 0.05 M Mes, 0.5 M Tris, 0.75 M $MgCl_2$ (Holstein et al., 1994) augmented with 0.02% Sodium azide, 0.025 mg/ml trypsin inhibitor, 0.05 mM PMSF and 1 mM DTT, incubated on ice for 60 min with gentle shaking and ultracentrifuged at 170,000 x g in T65 rotor for 50 min. Pellets containing partially uncoated CCVs were resuspended in 1.0 ml dissociation buffer, extracted as above for 30 min, spun as above and supernatants combined. Coat proteins (6-8 mg in 2.5 ml) were loaded on a 1x94 cm Superose-6 column equilibrated in Buffer D and eluted downward at 0.2 ml/min at 10°C. One ml fractions were collected. After SDS-PAGE and immunoblotting (see below) to locate APs within the elution profile fractions 36-44 containing clathrin triskelions and fractions 50-55 containing APs were pooled separately and concentrated using Amicon Centri-Prep10™ concentrators.

Cage/Coat Reassembly

Initially for the identification of clathrin-binding proteins, coats were reassembled by dialysis of total coat proteins extracted from CCVs as for gel-filtration chromatography

(above) by Tris-HCl against buffer B and purified on sucrose gradients essentially as for large scale assembly (see below). Subsequently, cage and coat structures were reassembled by dialysis of the Superose-6 purified fractions against Buffer B. For some experiments, 2mM $CaCl_2$ was included in the dialysis buffer. Large scale assembly experiments used Pierce Slide-A-Lyzer™ cassettes according to the manufacturer's directions. For assembly, samples containing ~ 0.75-1mg/ml clathrin ± 0.3-0.5 mg/ml AP were dialyzed 24 hours against Buffer B ± 2mM $CaCl_2$. Cages/coats were purified by sucrose density centrifugation following the method of (Lindner and Ungewickell 1992). One ml samples containing dialyzed clathrin triskelions ± AP were layered on 4.5 ml linear 5 to 30% (wt/wt) sucrose gradients in Buffer B ± 2mM $CaCl_2$ and spun in a Beckman SW41 rotor at 26K (90,000 x g) for 60 min. Gradients were pumped from the bottom and 0.4 ml fractions collected. Sucrose concentrations were determined by refractometry and approximate protein concentrations determined spectrophotometically by absorbance at 280 nm.

Small scale dialysis was accomplished using individual microdialysis cups constructed from microcentrifuge tubes (Lindner and Ungewickell, 1992). The bottom one-half of the tube was removed, and a 7mM hole drilled in the cap, which was then closed over a $15mm^2$ piece of dialysis membrane (8 to 10K MWCO). Cups were pushed through holes in a Styrofoam flotation disc to prevent capsizing during stirring of dialysis buffer in a flat bottomed, straight sided glass container. Volumes of samples (containing clathrin triskelions ± AP) were kept constant at 0.1 ml in size. After dialysis against either buffer B or E (depending on pH) samples from small scale experiments

were centrifuged for exactly 4 minutes in a Eppendorf Model 5412 microcentrifuge to pellet aggregated material, and the supernatants recentrifuged in a Beckman 70Ti rotor at 90,000 x g for 20 minutes. Low and high speed pellets were dissolved in SDS sample buffer directly, high speed supernatants were precipitated in 10% TCA before addition of sample buffer. The degree of clathrin assembly was determined by the relative distribution of clathrin heavy-chain between low and high speed pellets and the high speed supernatant by densitometric analysis of Coomassie-G stained 8-18% SDS-PAGE gels (Maizel, 1971).

Electrophoresis and Densitometic Analysis of Gels

All SDS-PAGE was conducted using 8-18% standard (Laemmli, 1970) gels with an acrylamide:bis-acrylamide ratio of 37.5:1. Gels were stained either with Coomassie Brilliant Blue-G (Neuhoff et al., 1988) or silver (Blum et al., 1987). Wet gels (or black and white photographs of gels) were scanned using an Envisions ENV 6100 flat-bed scanner with Adobe Photoshop (150 dpi, 8-bit, 256 grey scale) and densitometic analysis performed on scans using the image analysis software Sigma Scan/Image from Jandel Scientific Inc. run on a Gateway PS-120 computer. Clathrin heavy-chain (HC) bands were outlined and area and average intensities obtained for each. After grey scale inversion, areas and corrected intensities were multiplied to provide a figure for total protein present/band The relative distribution of clathrin HC between low speed pellets (aggregated material), high speed supernatants (unassembled clathrin), and high speed pellets (assembled clathrin) was determined. The accuracy of

this method for protein quantification was determined using bovine serum albumin (BSA) loaded and run on gels as above. Between 2.5 and 120 μg, the relationship between the actual amount of BSA loaded, and the calculated protein present/band was linear, with r^2=0.98.

Electron Microscopy and Image Analysis

For negative staining, 10 μl drops of samples containing 0.1-1.0 mg/ml protein were placed on Formvar/Carbon coated 75 mesh hex grids, allowed to stand for 1.5 min, wicked off, and fixed for 45 sec with 1% glutaraldehyde. After washing, grids were stained with 2% aqueous uranyl acetate for 4 min, dried, and viewed in a Zeiss-10 TEM operated at 80 kV. Micrographs of at least three randomly chosen, non-overlapping fields were taken at either 25 or 50K magnification (using the monostable switch to insure magnification accuracy), printed, sizing bars inserted, and scanned as above. Bars on micrographs were used to calibrate Sigma Scan/Image (see above). Two perpendicular diameters (relative to each other) for each non-overlapping structure were obtained and these measurements were first averaged to reduce variability. Average diameters/condition/fraction were calculated and the data set further analyzed using the SAS (Stanford Analysis System, SAS Institute Inc.) GLM procedure required for unequal sample sizes.

Other Procedures

For immunoprobing of AP, proteins were first electroblotted onto Millipore Immobilon-P transfer membrane at 100 mA overnight using tranfer buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3 (Towbin et al., 1979). Immunoreactive bands were subsequently detected using the Amersham ECL system. The primary antibody used was the monoclonal anti-$\beta_1/\beta 2$ AP subunit obtained from Sigma (A-4450), first produced by (Ahle et al., 1988).

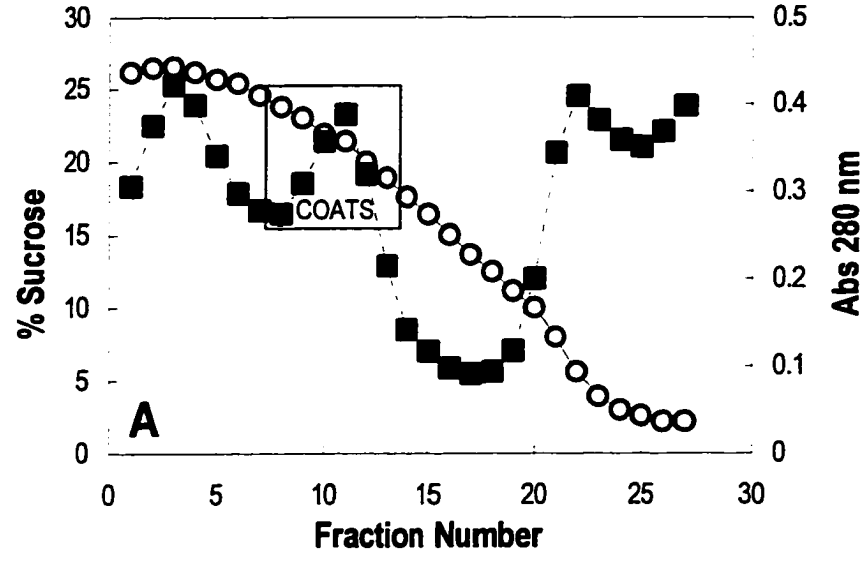
RESULTS

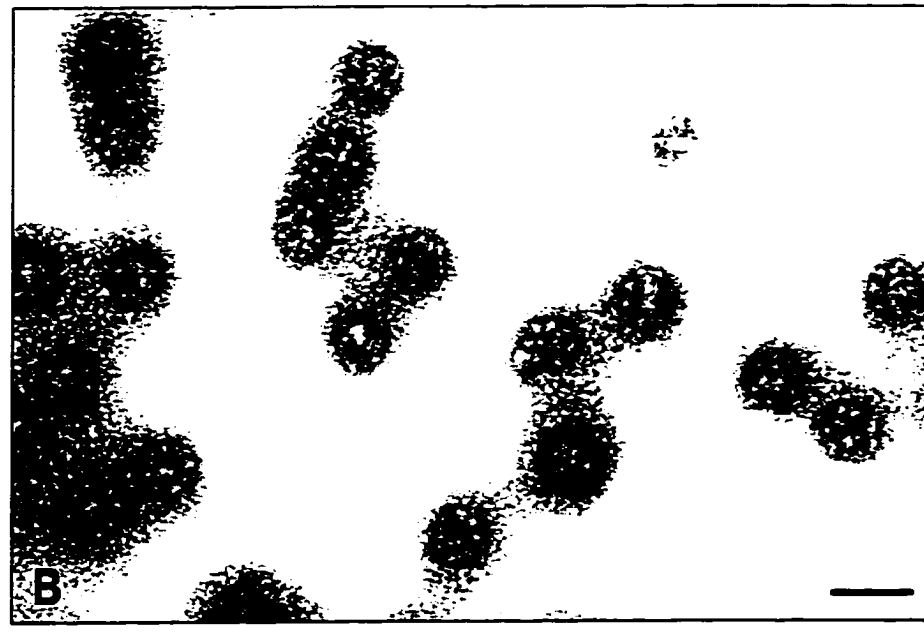
Identification of Clathrin-binding Proteins in Pea CCV

Polyacrylamide gel electrophoresis of pea cotyledon CCVs reveals a protein profile which is more complex than that of coated vesicles of brain. Only clathrin itself is readily identifiable (data not shown). Since the *in* vivo assembly of pea CCVs would require the binding of other proteins to clathrin to form the characteristic polygonal coats, an *in vitro* assembly assay was used to identify which of the many other proteins present in CCVs are clathrin-binding and thus presumably CCV coat proteins, rather than cargo, membrane or other contaminating proteins. Similar assays were successfully utilized to identify clathrin-binding proteins of bovine brain CCVs (Keen and Black, 1986; Lindner and Ungewickell, 1992). Tris-HCl extractable proteins were obtained by incubation of pea cotyledon CCVs with 1M Tris-HCl, pH 7.0 and cleared of contaminating membrane by ultracentrifugation. The pellet was extracted twice, and supernatants combined. To ensure the high concentration of

clathrin shown necessary for efficient assembly in brain, the extraction volume was kept small. The combined supernatants containing 2.5 mg/ml coat proteins were dialyzed for 36 hours against isolation buffer B and assembled coats were purified by sucrose density gradient centrifugation. After fractionation, three peaks at 280 nm were observed (Fig. 1A). The first peak at 25 to 26% sucrose, contained aggregated material while the third peak at the top of the gradient contained unassembled material (data not shown). Electron microscopy of negatively stained material from the second peak (17-23% sucrose) showed regularly shaped polyhedral structures similar in appearance to CCVs approximately 80 nm in diameter (Fig. 1B). This material represented approximately 25% of the total protein applied to the gradient. When subjected to SDS-PAGE, coat fractions showed little simplification over the protein profile of CCVs (data not shown). However, it was observed that ferritin was absent from coat fractions. To maximize the amount of assembled material available for identification of constituent proteins, gradient fractions containing unassembled material (fractions 21-28) were combined, concentrated by Amicon ultradialysis, redialyzed under the same conditions (as above) for an additional 24 hours and reassembled cages again separated from aggregated and unassembled material by sucrose density gradient centrifugation. Gradient fractionation again identified 3 peaks, however, the second absorbance peak containing coats centered slightly lower in the gradient, at 22.7 % versus 21.4% sucrose. To identify clathrin-associated proteins, coat containing fractions from both gradients were combined, concentrated

Figure 1. Reassembly of Tris-HCl dissociated pea cotyledon CCV coat proteins. A, Sucrose gradient fractionation after dialysis (■) absorbance at 280nm; (○) sucrose concentration (wt/wt). Polyhedric structures were found between 17-23% sucrose (boxed region). B, Electron microscopy of reassembled clathrin coats present in fraction 11 (22.7% sucrose), uranyl acetate staining, bar = 100 nm.


30
25
20
15
10
5
0
% Sucrose
0.5
0.4
0.3
0.2
0.1
0
Abs 280 nm
COATS
A
0
5
10
15
20
25
30
Fraction Number


B

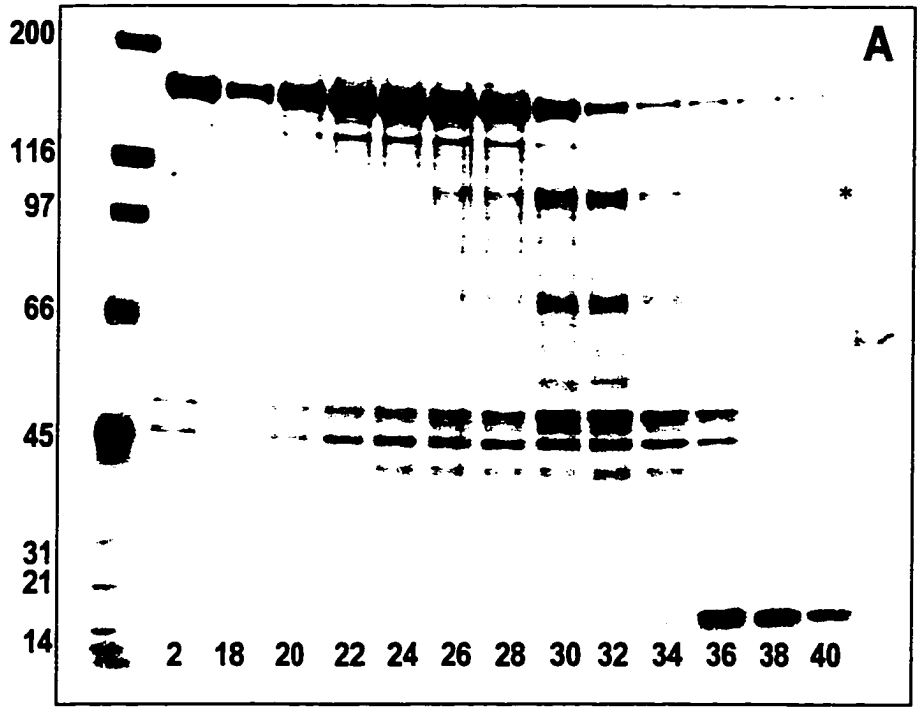
by ammonium sulfate precipitation, redissociated with Tris-HCl and fractionated on Superose-6. SDS-PAGE analysis of the chromatographic fractions showed clathrin and a number of clathrin-binding proteins which elute behind the clathrin. The polypeptide composition of these fractions showed a sizable simplification over that of sizing chromatography of non-reassociated Tris-HCL coat proteins. In addition to clathrin HC and LC, major bands are seen only at 114, 70, 60, 50, 48, and 46 kD. Ferritin, a major 28 kD contaminant of pea cotyledon CCVs whicn normally co-elutes with the clathrin-associated proteins on Superose-6 is absent in the reassembled coats (Figs. 2A and 2B).

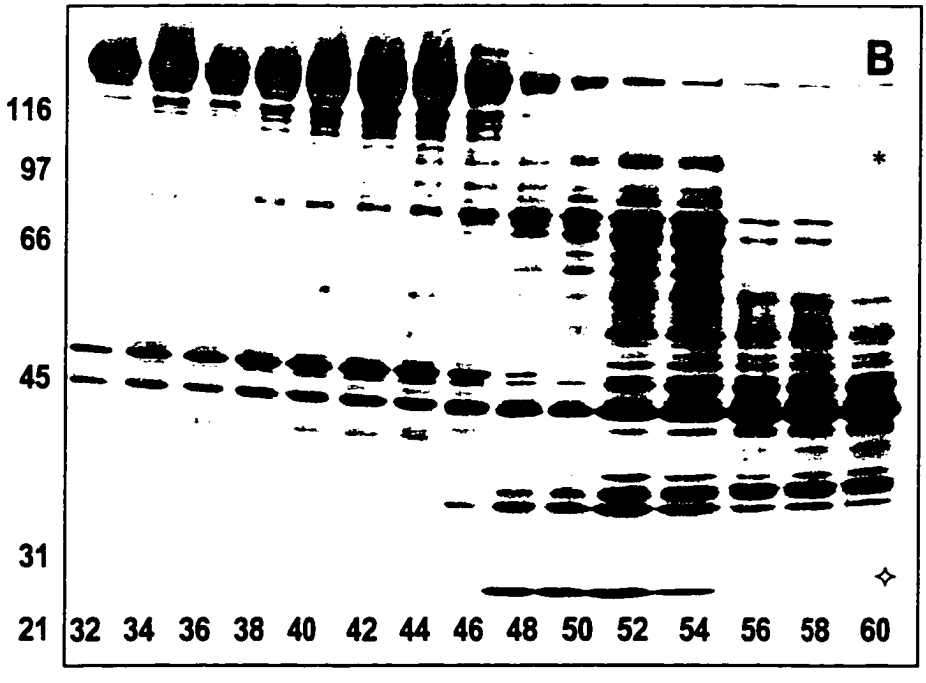
Cage Assembly of Pea and Brain Clathrin Without the Addition of AP

A number of investigators have reported reassembly of purified clathrin from bovine brain into cage or "basket" structures in the absence of any clathrin associated proteins under a number of different conditions such as low pH and low ionic strength, and the presence of divalent cations (Keen et al., 1979; Kirchhausen and Harrison, 1981; Nandi et al., 1980; Zaremba and Keen, 1983). These cages were shown to differ in size and sedimentation from the coats reassembled in the presence of AP proteins (Zaremba and Keen, 1983).

When isolated pea or brain clathrin at approximately 1 mg/ml (from Superose-6 fractionated coat proteins pooled and concentrated as described in methods) were dialyzed against 0.1M Mes dialysis buffer at pH 6.5 (buffer B) and fractionated on sucrose density gradients a striking difference in the degree of cage

Figure 2. Electrophoretic analysis of Superose-6 fractionated pea cotyledon CCV coat proteins. Ten μg aliquots were separated by SDS-PAGE on 8-18% acrylamide gels and silver stained. A, Simplified coat protein composition present in reassembled coat structures. Lane 1, molecular mass standards; lane 2, column load; lanes 18-40, fraction 18-40 from Superose-6 chromatography. B, Polypeptide composition of dissociated total coat proteins without prior reassembly. Lanes 32-60, fractions 32-60 from Superose-6 chromatography. Position of 110 kD β-adaptin (⋆); residual contaminating ferritin (✧).


A
200
116
97
66
45
31
21
14
2 18 20 22 24 26 28 30 32 34 36 38 40
*

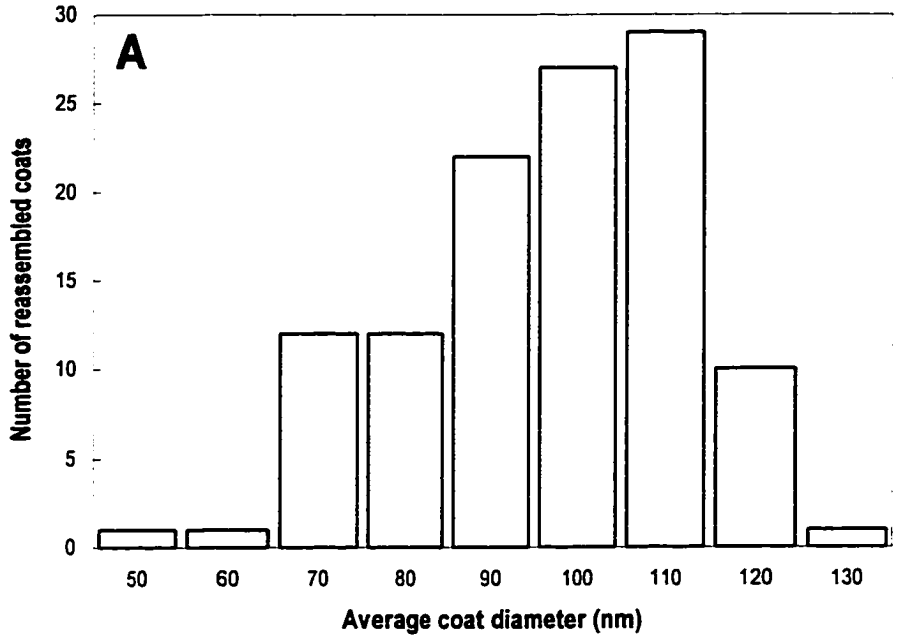

B
116
97
66
45
31
21
32 34 36 38 40 42 44 46 48 50 52 54 56 58 60
*
✧

assembly was observed. In contrast to brain clathrin, significant self-assembly of cages occurs with pea clathrin. After reassembly and separation on sucrose gradients, 53% of the total pea clathrin applied to the gradient was present in peaks at 20.4% sucrose (29% of clathrin applied) and 15.2% sucrose (24% of clathrin applied). Morphological differences between fractions were detected by electron microscopy and image analysis (see methods). Fraction 13 (20.4% sucrose) contained cages with a wide range of diameters (Figs. 3A and 3B) from 46 to 124 nm (mean = 91.22 nm ± 15.73 SD, n = 115). In contrast, in the second peak at fraction 19 (15.2% sucrose), reassembled cages were smaller and more uniform in diameter ranging from 55.8 to 95.85 nm (mean = 71.6 nm ± 7.74 SD, n = 101) (Figs. 4A and 4B). Under the same assembly conditions, essentially 100% of the brain clathrin applied remained at the top of the gradient as unassembled triskelions (Fig. 5).

Properties of Cage Reassembly as a Function of Clathrin Concentration, Time, pH and the Presence of Divalent Cations

Having established that in contrast to brain, pea clathrin readily self-assembled we wondered if the degree of pea clathrin assembly observed *in vitro* might be dependent on factors such as protein concentration, dialysis time, buffer pH, or the presence of divalent cations as has been demonstrated for brain. Using a small scale assembly assay which allows rapid processing of large numbers of samples and quantitative recovery of protein, the concentration dependence of both AP-mediated and calcium-mediated assembly of brain clathrin was demonstrated (Lindner and

Figure 3. Reassembled cages recovered at 20.4% sucrose after gradient fractionation of pea cotyledon clathrin (1.0 mg/ml) dialyzed against buffer B without the addition of AP or calcium. A, Histogram of diameters of coats after reassembly (n=115). Markers on x-axis represent cage sizes below, but not including the data point. B, Electron microscopy of negatively stained cages found at 20.4% sucrose. Uranyl acetate staining. Bar = 100 nm.


A
Number of reassembled coats
30
25
20
15
10
5
0
50
60
70
80
90
100
110
120
130
Average coat diameter (nm)

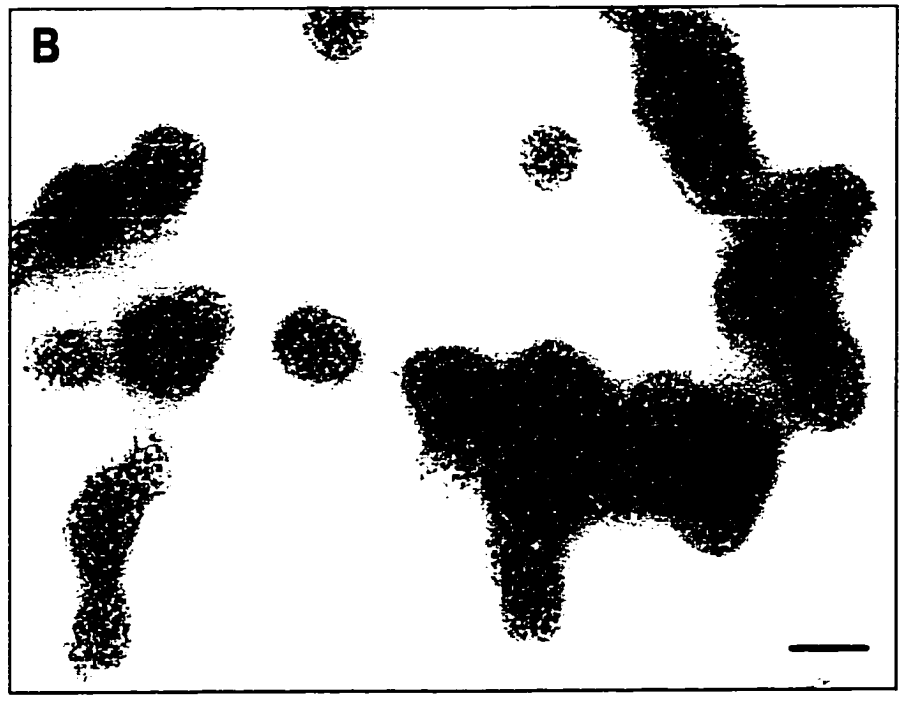
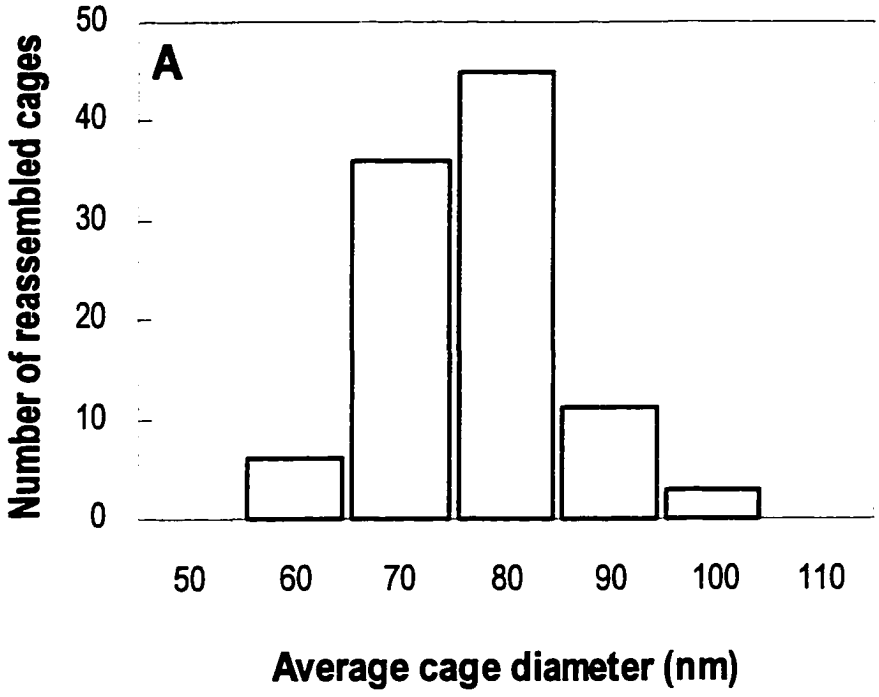

B

Figure 4. Reassembled cages recovered at 15.2% sucrose after gradient fractionation of pea cotyledon clathrin (1.0 mg/ml) dialyzed against buffer B without the addition of AP or calcium. A, Histogram of diameters of coats after reassembly (n=101). Markers on x-axis represent cage sizes below, but not including the data point. B, Electron microscopy of negatively stained cages present at 15.2% sucrose. Uranyl acetate staining. Bar = 100 nm.


A
Number of reassembled cages
50
40
30
20
10
0
50
60
70
80
90
100
110
Average cage diameter (nm)

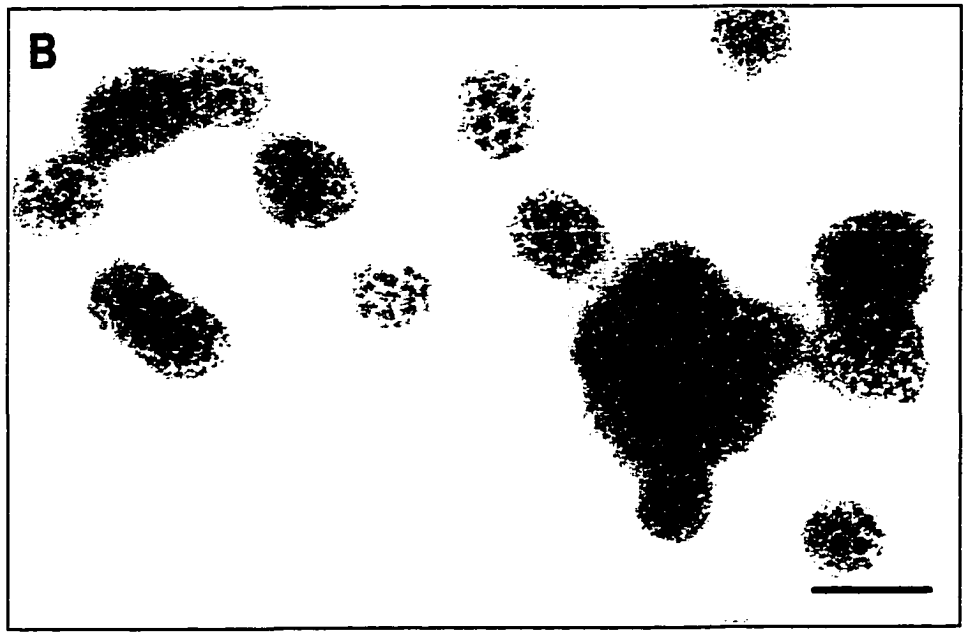
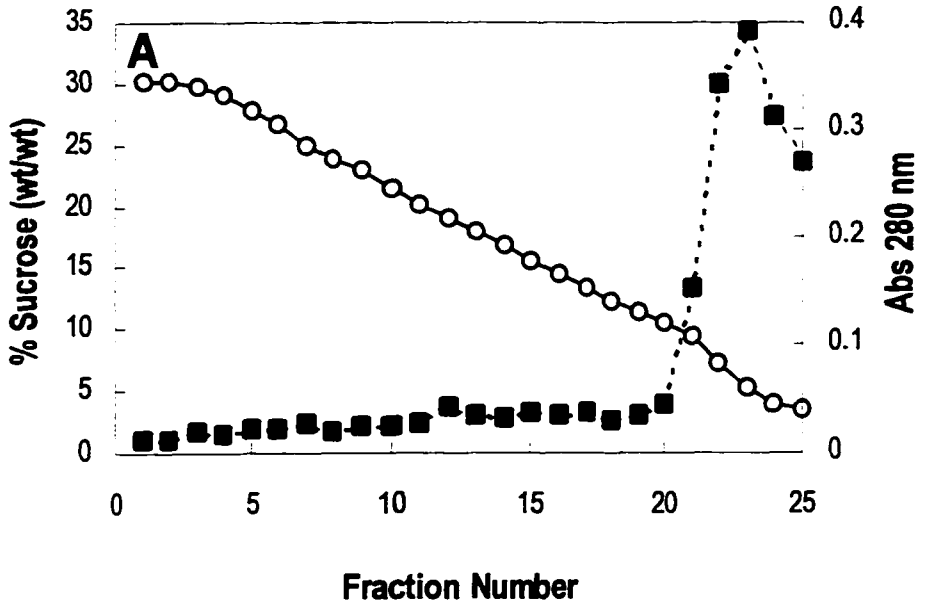
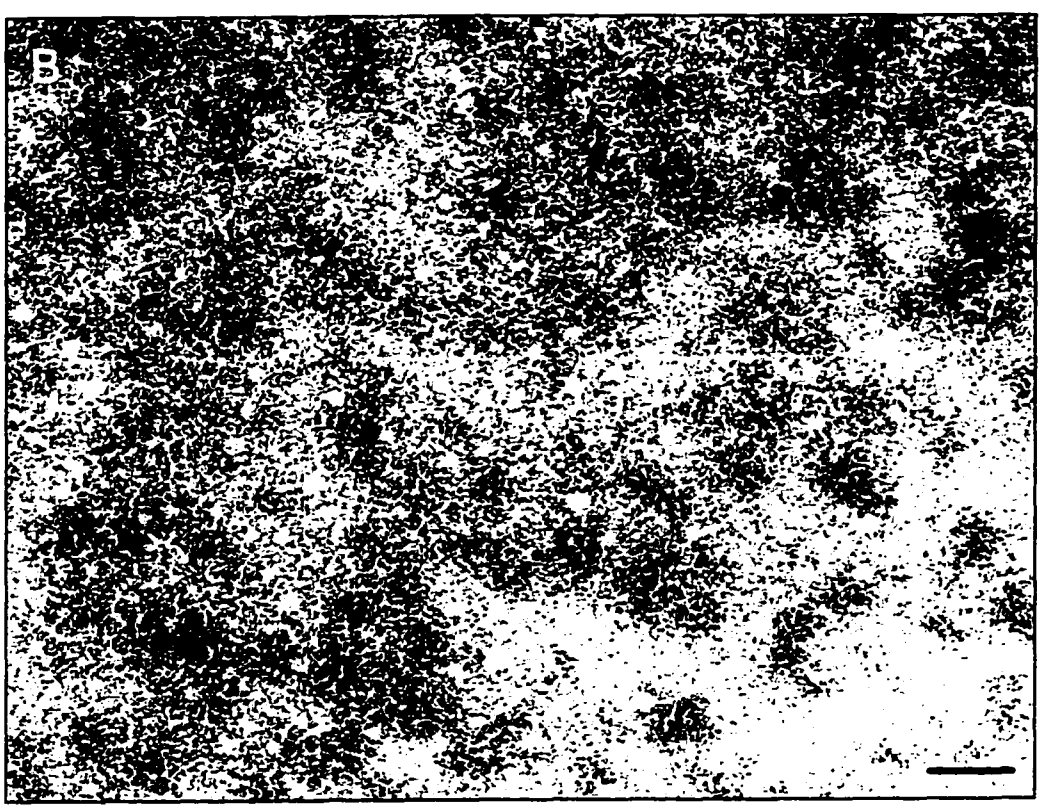

B

Figure 5. Brain clathrin (1.0 mg/ml) dialysed against buffer B without the addition of AP or calcium. A, Sucrose gradient fractionation of dialysed brain clathrin. (■) absorbance at 280nm; (o) sucrose concentration (wt/wt). B, Electron microscopy of unassembled triskelia remaining at the top of the gradient after centrifugation. Uranyl acetate staining. Bar = 100 nm.

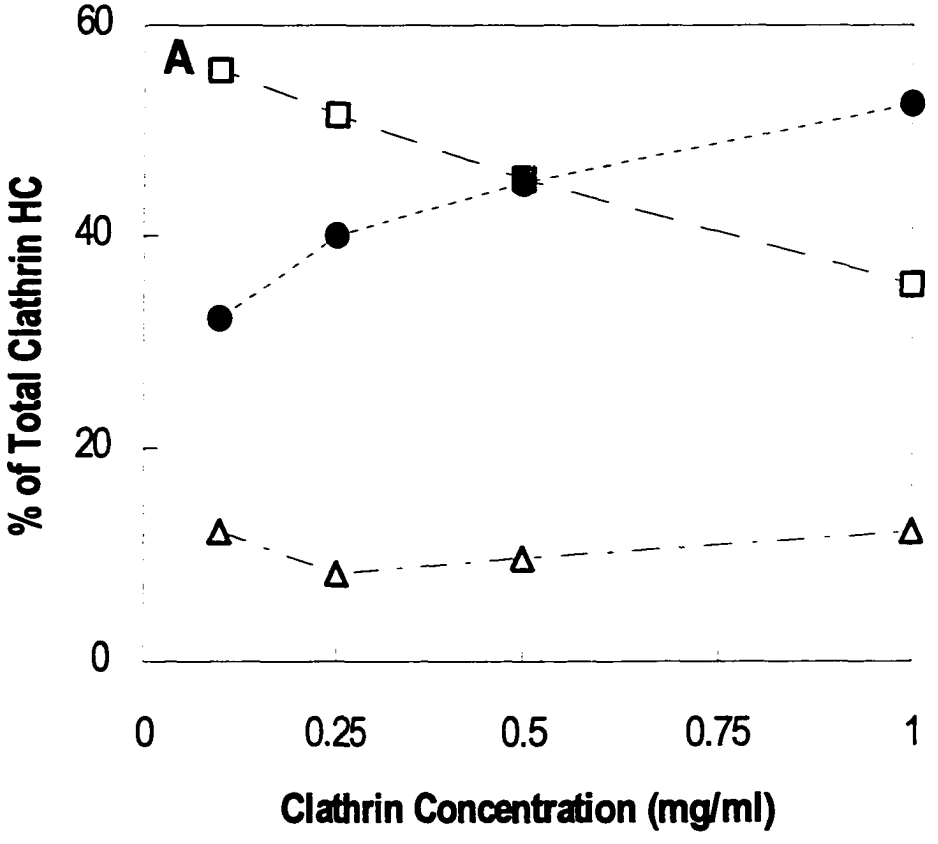

A
% Sucrose (wt/wt)
Abs 280 nm
Fraction Number
35
30
25
20
15
10
5
0
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25

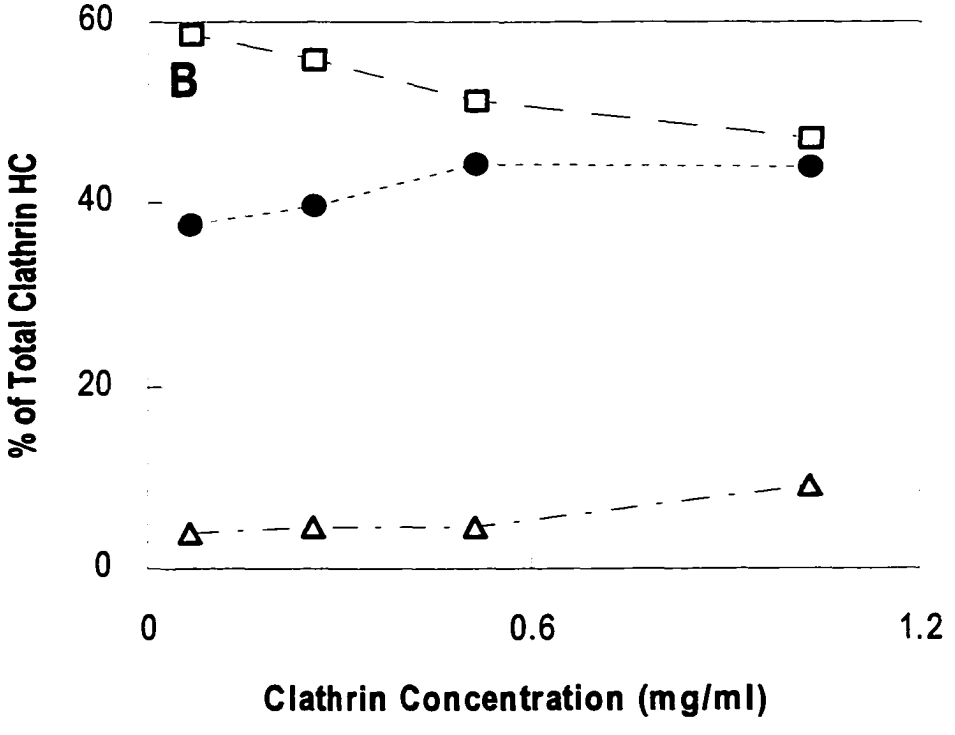


Ungewickell, 1992). We employed a modification of this assay to determine the effects of increasing concentration, pH and time on the extent and rate of self-assembly in pea. Non-commercial microdialysis cells constructed from Eppendorf tubes were fitted with dialysis tubing (see methods). A sample size of 0.1 ml was used for all experiments. Cells were placed in a common flotation device constructed of closed-cell Styrofoam sheet. After dialysis, two centrifugation steps allow separation of samples into aggregated material (low-speed pellet), and assembled (high-speed pellet) and unassembled (high-speed supernatant) fractions. Each sample (in its entirety) was subjected to SDS-PAGE. Instead of employing traditional densitometry, protein quantification was accomplished through a two step process in which Coomassie G stained gels (or black-and-white photographs of gels) were scanned using a flat bed scanner and protein bands quantified using image analysis software. Assembly is expressed in terms of percentage of the total clathrin HC in the high speed pellet over that present in the aggregated, unassembled and assembled fractions combined.

Over a ten fold increase in clathrin concentration from 0.1 to 1.0 mg/ml, the proportion of assembled clathrin increased from roughly 32 to 53% when dialyzed against buffer B at pH 6.5 (Fig. 6A). This amount compares roughly to that reported for AP or calcium mediated assembly in brain (Lindner and Ungewickell, 1992). In a similar experiment conducted at pH 7.2, a more "physiologically relevant" cytosolic pH for plant cells (Romani et al., 1996; Horn et al., 1992) the concentration effect was less marked, with a total increase of only 6%, from 38 to 44 % (Fig. 6B).

Figure 6. Effect of pH on concentration dependence of pea clathrin assembly. 0.1 ml samples containing 0.1 to 1.0 mg/ml pea clathrin were dialyzed against buffer B (pH 6.5) or E (pH 7.2) Percentages calculated as the amount of clathrin HC in the (•) assembled, (□) unassembled, or (Δ) aggregated fractions over the total HC present. A, Concentration dependence of pea clathrin at pH 6.5. B, Concentration dependence of pea clathrin at pH 7.2.


A
60
40
20
0
% of Total Clathrin HC
0
0.25
0.5
0.75
1
Clathrin Concentration (mg/ml)

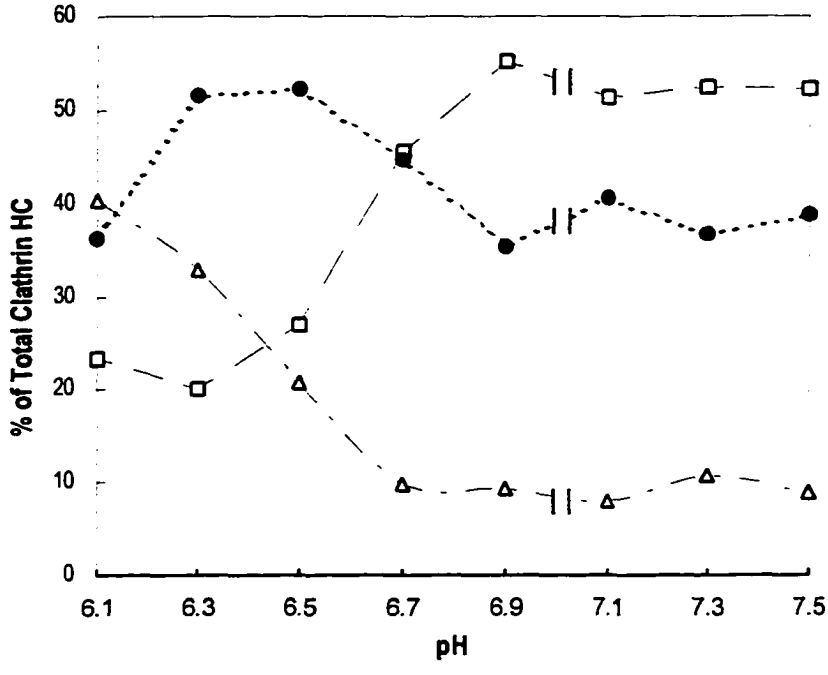

B
60
40
20
0
% of Total Clathrin HC
0
0.6
1.2
Clathrin Concentration (mg/ml)

Self-assembly of pea clathrin proceeds slowly in comparison to AP-mediated assembly in brain. While maximum AP-mediated assembly of brain coats occurs within four hours, pea clathrin self-assembly continues to at least 24 hours (data not shown). Inclusion of 2mM free Ca^{++} to this assay increased the percentage of assembled clathrin approximately 6% over that of pea clathrin self-assembly for each time point.

The small scale assay was used to determine whether the extent of self-assembly of pea clathrin into cages was pH dependent. Maximal self-assembly of pea clathrin occurs at pH 6.5 with roughly 52% of clathrin HC found in the high speed pellet. (Fig. 7). Maximum self-assembly of brain clathrin was also observed at pH 6.5, but less than 20% of clathrin assembled into cages (Zaremba and Keen, 1983). Higher levels of brain clathrin assembly, comparable to those seen in pea, occur only in low ionic strength buffers (Kirchhausen and Harrison, 1981; Van Jaarsveld et al., 1981) or in the presence of AP (Zaremba and Keen, 1983), divalent cations such as Ca^{++} or Mn^{++}, polybasic amines or exogenous basic protein such as lysozyme (Nandi et al., 1981).

Above the pH optima, the percentage of pea clathrin found assembled declines to approximately 75% of the highest level but then remains nearly constant between pH 6.9 and pH 7.5 (Fig. 7). In contrast, brain self-assembly declines to approximately 5% of total protein at pH 7.4 (25% of the maximum observed) (Zaremba and Keen, 1983).

Figure 7. Effect of pH on assembly of pea clathrin. 40 μg aliquots of pea cotyledon clathrin were dialyzed against buffer B (pH 6.1-6.9) or buffer E (pH 7.1-7.5) in microdialysis cups and processed as described in methods. Percentages calculated as the amount of clathrin HC in the (•) assembled, (□) unassembled, or (Δ) aggregated fractions over the total HC present.


60
50
40
30
20
10
0
% of Total Clathrin HC
6.1
6.3
6.5
6.7
6.9
7.1
7.3
7.5
pH

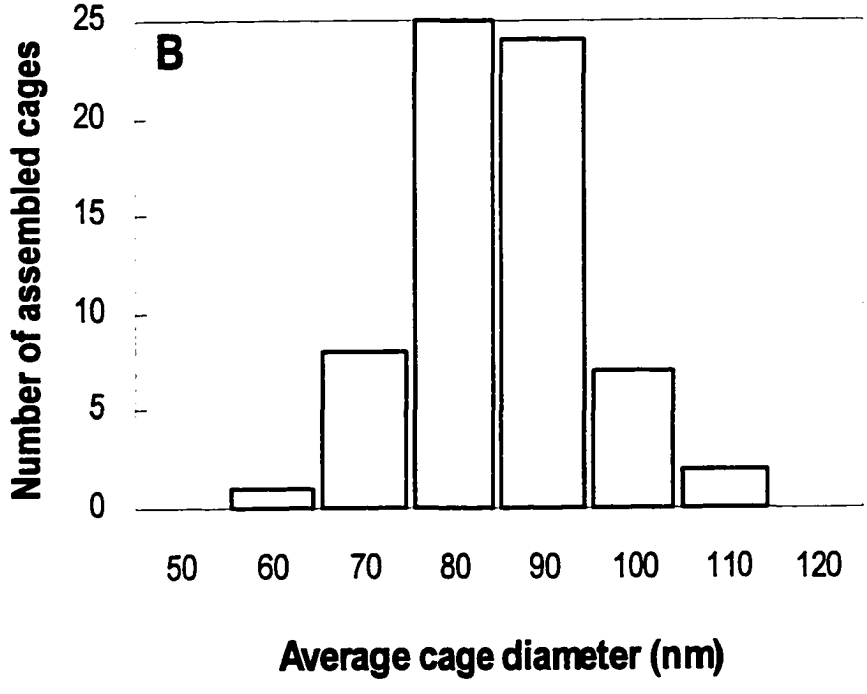
At the lowest pHs tested a high proportion of clathrin HC is found in the aggregated protein fraction (low speed pellet). With increasing pH, the amount of aggregated protein steadily declines and at pHs approaching more physiologically relevant (pH 6.7 and beyond) represents only a minor percentage (10%) of the total (Fig.7).

Inclusion of 2mM $CaCl_2$ in the standard assembly buffer affected cage assembly in both pea and brain. Reassembled cages were purified on sucrose density gradient separated to allow determination of approximate sedimentation rates and quantification of cage sizes. A marked calcium stimulation of brain clathrin assembly occurred, as has been observed by many other laboratories (Keen et al., 1979; Kirchhausen and Harrison, 1981; Nandi et al., 1981; Lindner and Ungewickell, 1992). Roughly 70% of the clathrin was found in a broad peak corresponding to sucrose concentrations from 10.8% to 21.5%. Polygonal structures similar to intact coated vesicles were present in electron micrographs of the peak fraction but the number of lattice vertices present per structure appeared greater than for brain CCVs (Fig. 8A). By image analysis these structures were found to have a mean diameter of 80.42nm ± SD = 9.52 with a minimum of 55.90nm, a maximum diameter of 109.22nm and were of relatively uniform size (Fig. 8B).

Examination of the protein content of gradient fractions of pea clathrin reassembled in the presence of 2 mM free Ca^{++} showed that two major peaks were present, in contrast to the single peak shown for brain clathrin assembled under the same conditions. Absorbance maxima were found at fractions 8 and 18

Figure 8. Assembly of brain clathrin in the presence of 2 mM free Ca^{++}. Purified brain clathrin (1.0 mg/ml) was dialyzed against buffer B including 2mM $CaCl_2$. A, Electron microscopy of reassembled cages recovered at 17.2% sucrose after gradient purification and fractionation. Negative staining with uranyl acetate. Bar = 100nm. B, Histogram of diameters of cages recovered at 17.2% sucrose after reassembly (n=66). Markers on x-axis represent cage sizes below, but not including the data point.


A


B
Number of assembled cages
25
20
15
10
5
0
50
60
70
80
90
100
110
120
Average cage diameter (nm)

corresponding to sucrose concentrations of 24.2% (the major peak) and 17.7% (minor peak) respectively. Cages from these fractions exhibited strong differences in morphology, both numerically and visually. Polygonal structures present in fraction 8, like those of brain, appeared to have a larger number of lattice vertices than fraction 18 (Fig. 9A), but unlike those of brain were much less uniform in size. Diameters ranged from a minimum of 64.21nm to a maximum of 128.27 nm, with a mean of 97.94 ±12.87 SD, n = 88. Electron microscopic examination of fraction 18 (absorbance maxima of the minor peak) showed cages to have many fewer lattice vertices than those from fraction 8 (Fig. 9B). These were of more uniform diameter with a mean of 69.61 ± 8.87 SD, n = 96. The minimum cage diameter measured was 55.58 nm with the maximum diameter 93.51 nm. Forty-nine of the cages measured fell within the 60-70 nm range with the next largest size class numbering approximately half that of the largest class (Fig. 9C).

AP Mediated Assembly of Pea and Brain Coats

Reassembly of pea and brain clathrin in the presence of their respective AP-produced very different results. In contrast to the insignificant amount of brain clathrin which self-assembles at pH 6.5, the inclusion of a Superose-6 mixed AP pool in assembly assays, resulted in approximately 75% of brain clathrin being assembled into coat structures incorporating the AP proteins. After sucrose gradient purification, two broad, poorly separated peaks were observed in the regions of the gradient from 10 to 27% sucrose (Fig. 10A). Reassembled coats from the larger peak, with a mean

Figure 9. Assembly of pea clathrin in the presence of 2mM free Ca^{++}. Purified pea cotyledon clathrin (1.0 mg/ml) was dialyzed against buffer B including 2mM $CaCl_2$. A, Electron microscopy of reassembled cages recovered at 24.2% sucrose after gradient purification and fractionation. Negative staining with uranyl acetate. Bar = 100 nm. B, Electron microscopy of cages found at 17.7% sucrose from the same experiment. C, Histogram of diameters of cages recovered at 17.7% sucrose after reassembly (n=96). Markers on x-axis represent cage sizes below, but not including the data point.

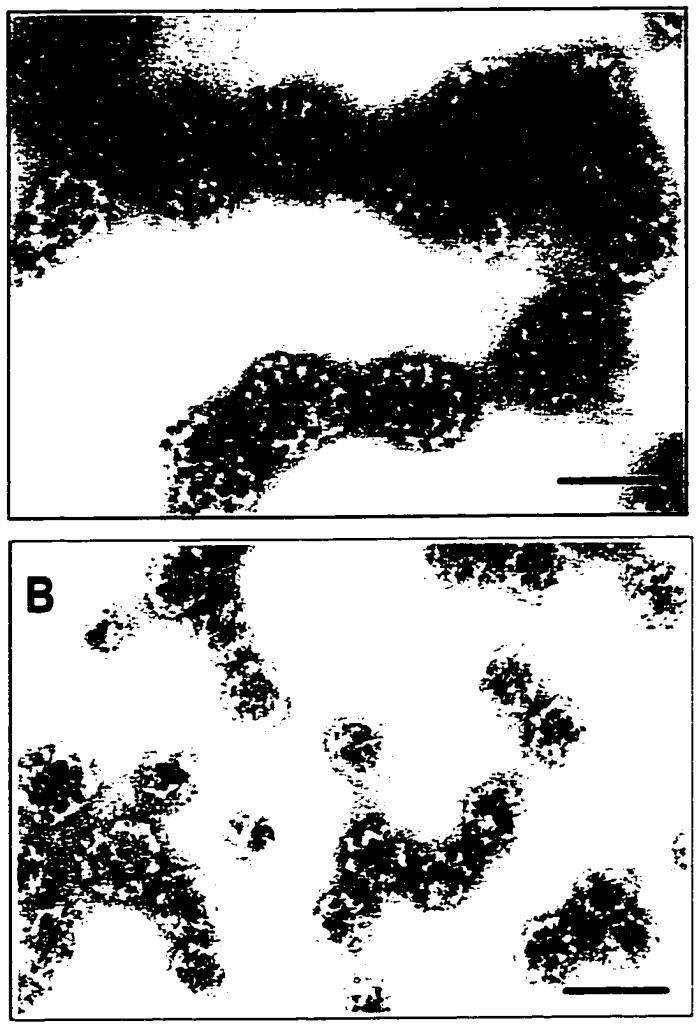
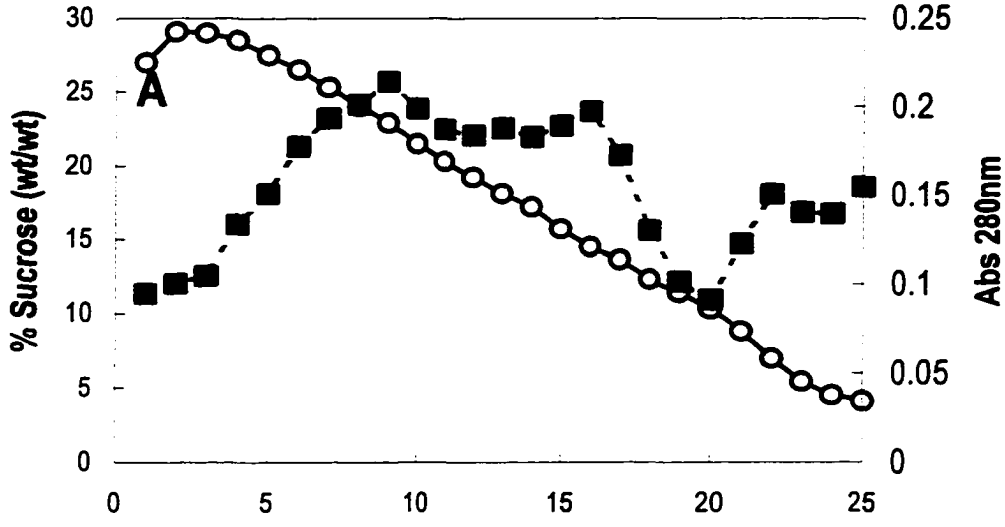
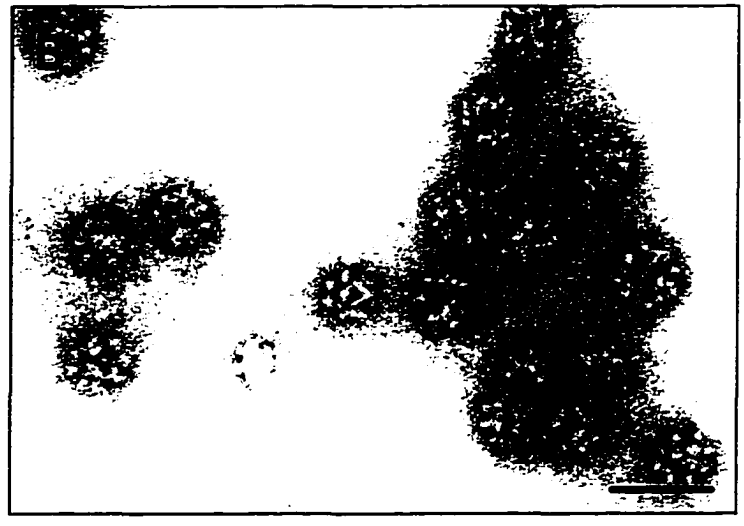
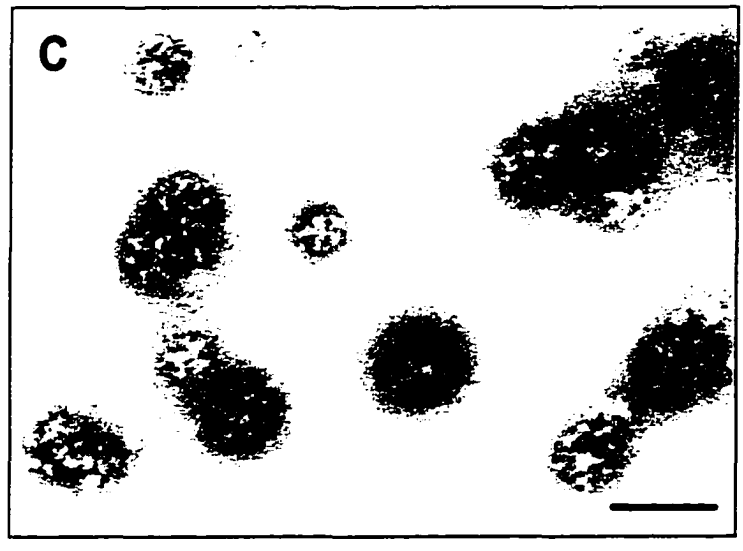

B
C
Number of assembled cages
0
10
20
30
40
50
50
60
70
80
90
100
110
Average cage diameter (nm)

Figure 10. Assembly of brain clathrin in the presence of brain AP. Purified brain clathrin (~1.0 mg/ml) was mixed with brain AP (0.5mg/ml) and dialyzed against buffer B. A, Sucrose gradient fractionation after dialysis. (■) absorbance at 280nm; (o) sucrose concentration (wt/wt). Polyhedric structures were found between 10-27% sucrose. B, Electron microscopy of reassembled coats recovered at 22.9% sucrose after gradient purification and fractionation. C, Electron microscopy of coats from the same experiment found at 14.5% sucrose. All negative staining with uranyl acetate. Bar = 100 nm.


A
% Sucrose (wt/wt)
Abs 280nm
Fraction Number
30
25
20
15
10
5
0
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25




C

of 65.72 ±5.04 SD (n = 90), were significantly smaller (at the 0.05 level) in diameter than brain clathrin cages formed with the inclusion of 2 mM free Ca^{++}. Coats from the second peak, centered at 14.5% sucrose were still smaller, with a mean of 58.94 nm ±7.44 SD (n = 61). While both classes of coats are morphologically quite distinct (Figs. 10B and 10C), AP proteins (as detected by immunoblotting) are incorporated into each equally. In addition, no difference in the relative proportion of adaptor β subunits (β1 versus β2) was detectable (Fig. 11).

Unlike brain, no AP stimulation of assembly occurs when isolated pea clathrin is dialyzed in the presence of a Superose-6 enriched pea adaptor fraction. In small scale experiments, with a clathrin concentration of 1.1 mg/ml, approximately 55% of the pea clathrin is found assembled from AP concentrations of 0-0.4 mg/ml (Fig. 12). No change is observed (past that expected due to the decreased clathrin concentration itself) if the clathrin concentration is reduced and AP concentration increased (see data for concentration dependence above). However, large scale assembly experiments with sucrose density purification demonstrate that, while pea AP fails to stimulate assembly, the coats assembled do incorporate AP (Fig. 13A). Coats containing AP were found in two incompletely resolved peaks between sucrose concentrations of 13-25% (Fig. 13B). This assembly profile resembles that observed for brain clathrin/brain AP (see Fig. 10A). While pea AP is incorporated into coats from both peaks, coat diameters of the 22.2% sucrose pea appear to incorporate more AP than those from the second peak at 15.9%. These coats (mean diameter = 76 nm ±14.36 SD n = 71) are significantly smaller than those of the major peak formed by

Figure 11. Electrophoretic and Western blot analysis of sucrose gradient fractions after assembly of brain clathrin in the presence of brain AP. 100 µl aliquots of fractions were subjected to SDS-PAGE on 8-18% acrylamide gels. A, Western blot using β-adaptin monoclonal antibody. ECL detection. B, Coomassie-G stained replicate gel. (*) immunoreactive polypeptides.

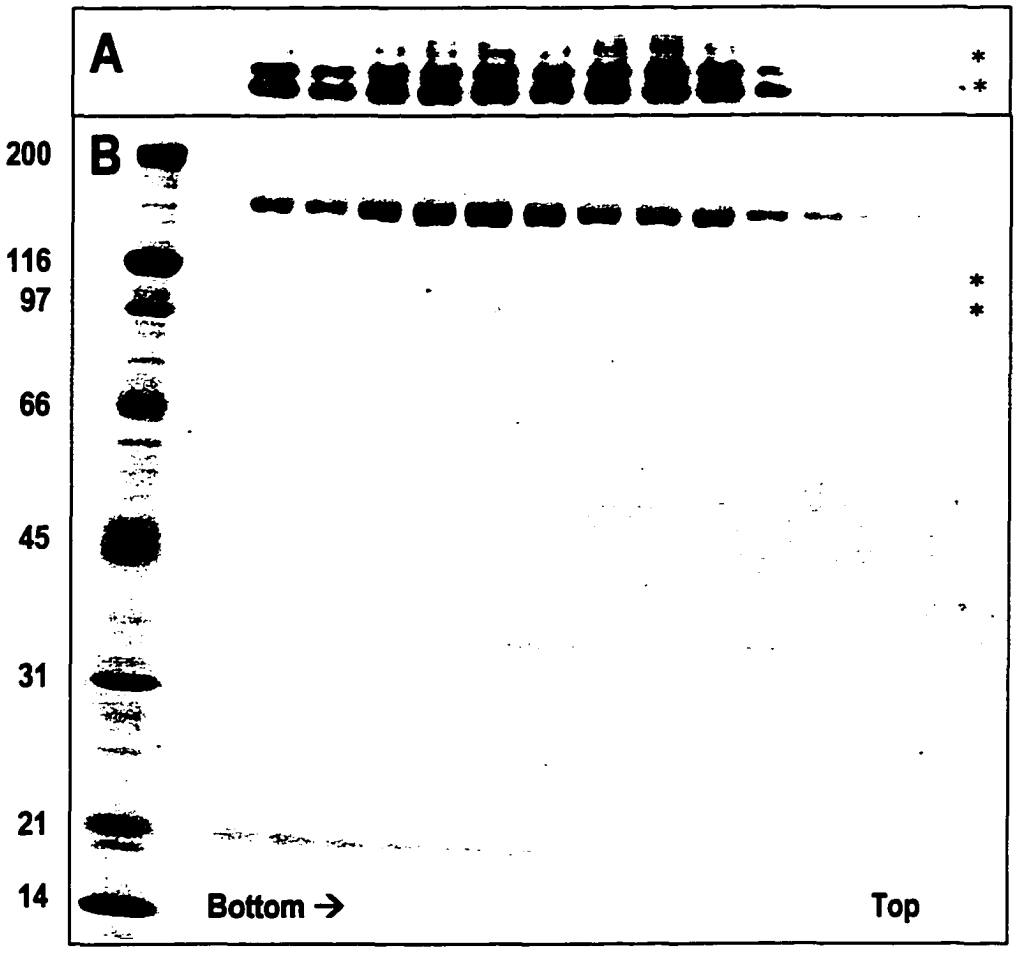

A
B
200
116
97
66
45
31
21
14
Bottom →
Top

Figure 12. Dependence of coat assembly on AP concentration. Pea cotyledon clathrin (1.1 mg/ml) was dialysed in microdialysis cups against buffer B in the presence of increasing amounts of pea cotyledon AP and processed as described in methods. Percentages calculated as the amount of clathrin HC in the (●) assembled, (□) unassembled, or (△) aggregated fractions over the total HC present.

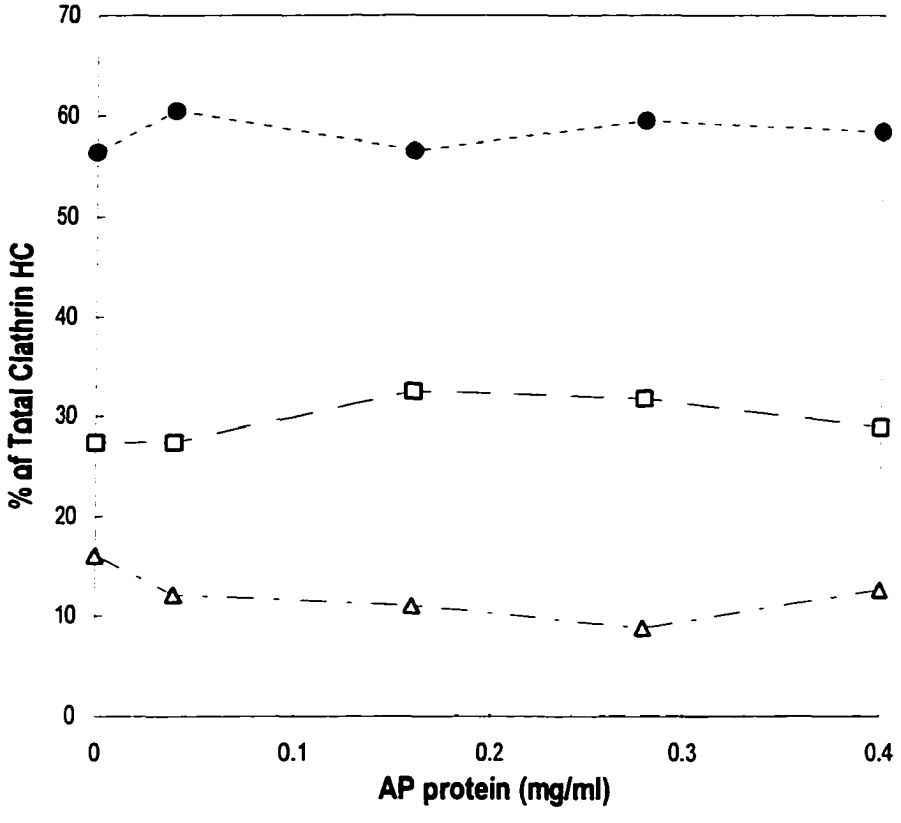
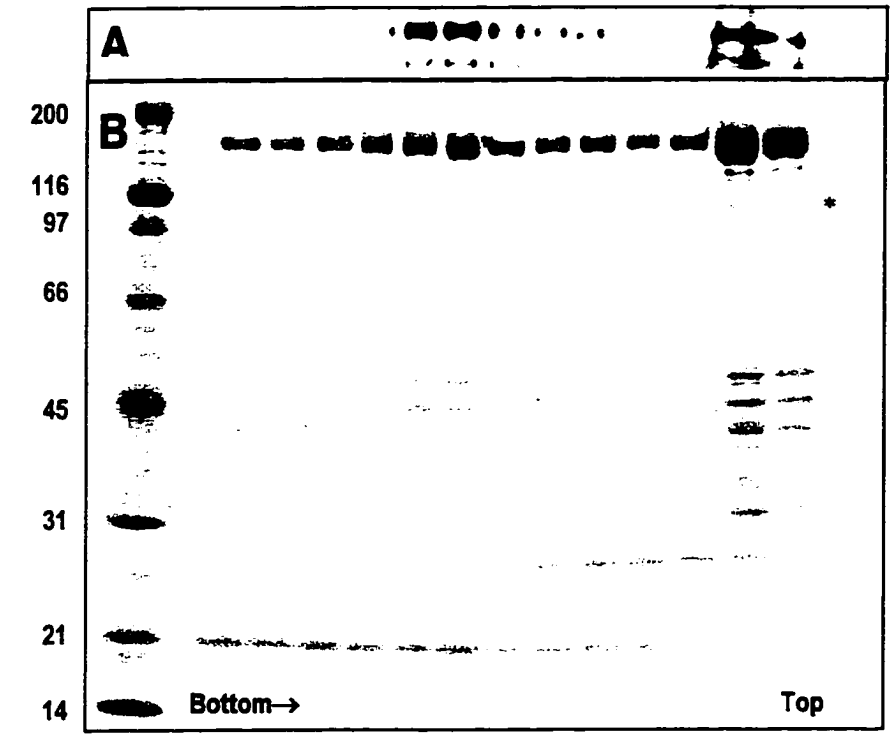

70
60
50
40
30
20
10
0
% of Total Clathrin HC
0
0.1
0.2
0.3
0.4
AP protein (mg/ml)

Figure 13. Assembly of pea cotyledon clathrin in the presence of pea cotyledon AP. Purified pea clathrin (~1.0 mg/ml) was mixed with pea AP (0.5mg/ml), dialyzed against buffer B and cages purified on sucrose density gradients. A, Western blot analysis of sucrose gradient fractions using β-adaptin monoclonal antibody. ECL detection. B, 100 µl aliquots of fractions were subjected to SDS-PAGE on an 8-18% acrylamide gel. (*) immunoreactive polypeptide. C, Sucrose gradient fractionation (■) absorbance at 280nm; (○) sucrose concentration (wt/wt).


A
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200
116
97
66
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14
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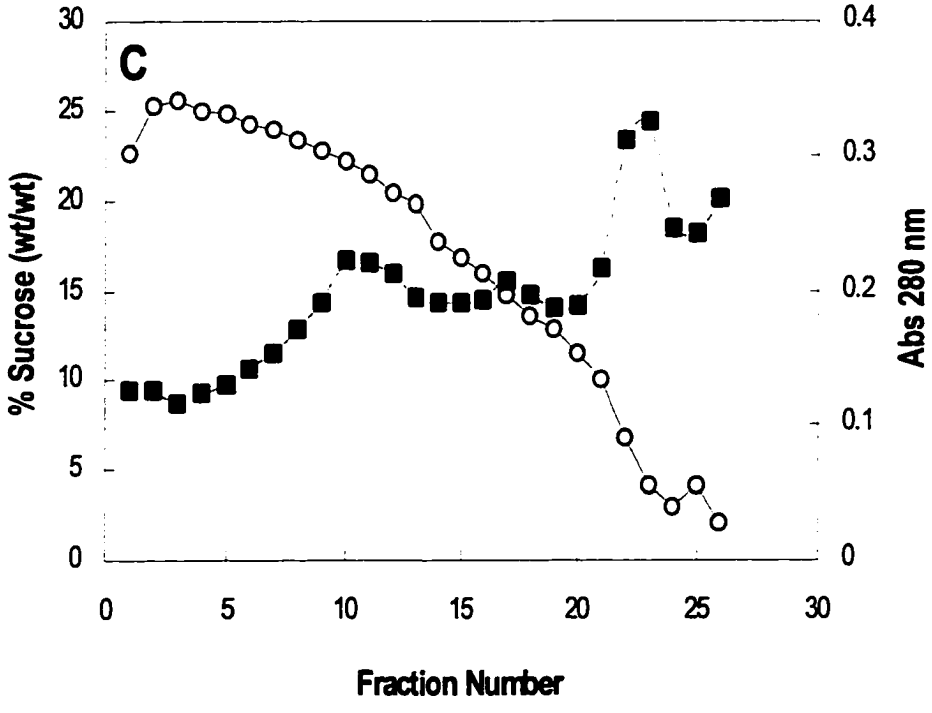
*


C
30
25
20
15
10
5
0
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
% Sucrose (wt/wt)
Abs 280 nm
Fraction Number

pea clathrin self-assembly or pea clathrin assembled in the presence of 2 mM free Ca^{++}. Coats from the smaller peak centered at fraction 17 (15.9% sucrose) had a mean of 69.55 nm (±8.58 SD).

Failing to find any stimulation of assembly by the Superose-6 enriched pea adaptor fraction, we searched for potential assembly stimulating proteins in other regions of the Superose-6 elution profile. Fractions between the clathrin peak and adaptor peak, and the fractions following the adaptor peak were pooled separately, concentrated and included in small scale assembly assays. Only a slight stimulation (6%) over the amount of pea clathrin self-assembly occurred with inclusion of the post-adaptor fraction at 0.28 mg/ml.

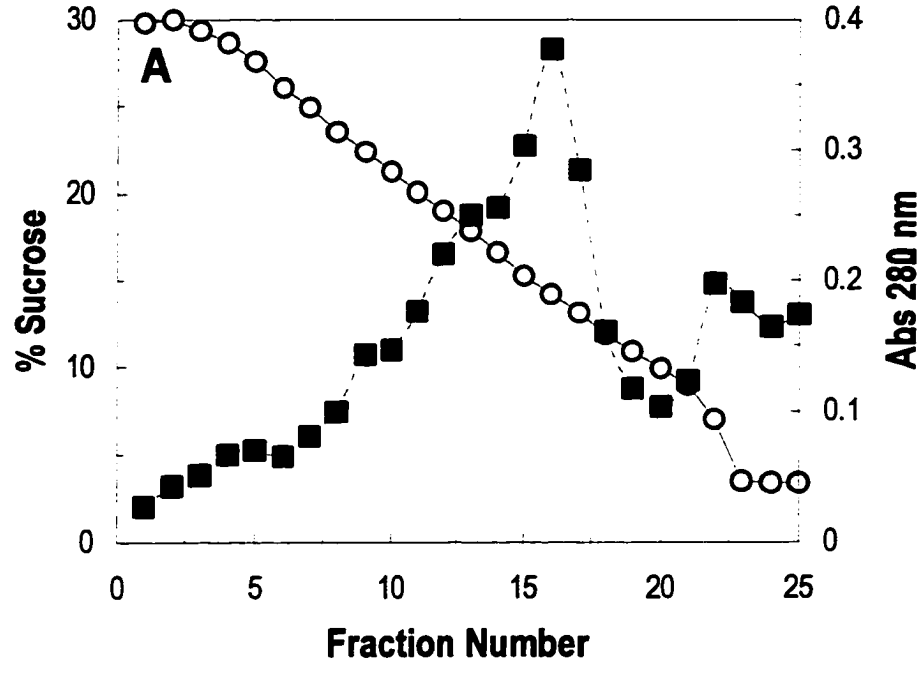
In brain, multiple GTPase appear to regulate assembly, invagination and the budding of coats (Schmid 1993) and a number of kinase activities are also known to be associated with isolated brain and pea CCVs (Lin et al., 1992). To determine whether nucleotide hydrolysis might be required for an AP-mediated stimulation of coat assembly in pea, GTP or ATP (to a final concentration of 2 mM) was added to clathrin dialysed in the presence of pea AP and incubated for 15 minutes at 25°C (Ungewickell et al., 1995). No change in the amount of AP-mediated assembly was observed under these conditions.

Formation of Hybrid Coats by Reciprocal Mixing of Pea Clathrin/Brain AP and Brain Clathrin/Pea AP

Dialysis of pea clathrin in the presence of brain AP, unlike dialysis in the presence of pea AP, produced a significant increase in assembly over that observed for pea clathrin self-assembly. While pea clathrin self-assembly at ~ 0.75 mg/ml and pH 6.5 averages 45%, with the inclusion of brain AP at 0.4 mg/ml, 67% of pea clathrin assembled into recognizable polygonal structures amounting to a 1.5 fold increase in assembly. Assembled material was found over a broad range of sucrose concentrations from 11-25% (Fig. 14). Immunodetection revealed that brain AP was incorporated into these coats across the entire range in which assembled structures were recovered. Electron microscopy and image analysis of peak fraction 16 at 14% sucrose showed these coats to be significantly smaller than those found in the major peak of self-assembled cages or those cages/coats formed in the presence of calcium or pea AP respectively. Mean diameters of coats was 70.19 nm (±5.95 SD, n=106). Coat morphology appeared strained; coats had fewer vertices than observed for either pea or brain coats formed in the presence of their respective AP's and leg angles appeared altered (Fig. 15).

The effect of pea AP on assembly of brain clathrin was strikingly different than for pea clathrin/brain AP. No appreciable assembly occurred; the protein absorbance profile of the fractionated sucrose density gradient highly resembled that of brain clathrin alone. In the presence of pea AP most of the brain clathrin remained at the top of the gradient. Electron microscopic examination of this material revealed

Figure 14. Assembly of pea cotyledon clathrin in the presence of brain AP. Purified pea clathrin (~1.0 mg/ml) was mixed with brain AP (0.5mg/ml), dialyzed against buffer B and coats purified on sucrose density gradients. A, sucrose gradient fractionation (■) absorbance at 280nm; (○) sucrose concentration (wt/wt). B-C. Electrophoretic and Western blot analysis of gradient fractionation. 100 µl aliquots of fractions were subjected to SDS-PAGE on replicate 8-18% acrylamide gels. (*) immunoreactive polypeptides, (□), pea clathrin LC. B, Western blot using β-adaptin monoclonal antibody. ECL detection. C, Coomassie-G stained gel.


A
30
20
10
0
0.4
0.3
0.2
0.1
0
% Sucrose
Abs 280 nm
0
5
10
15
20
25
Fraction Number

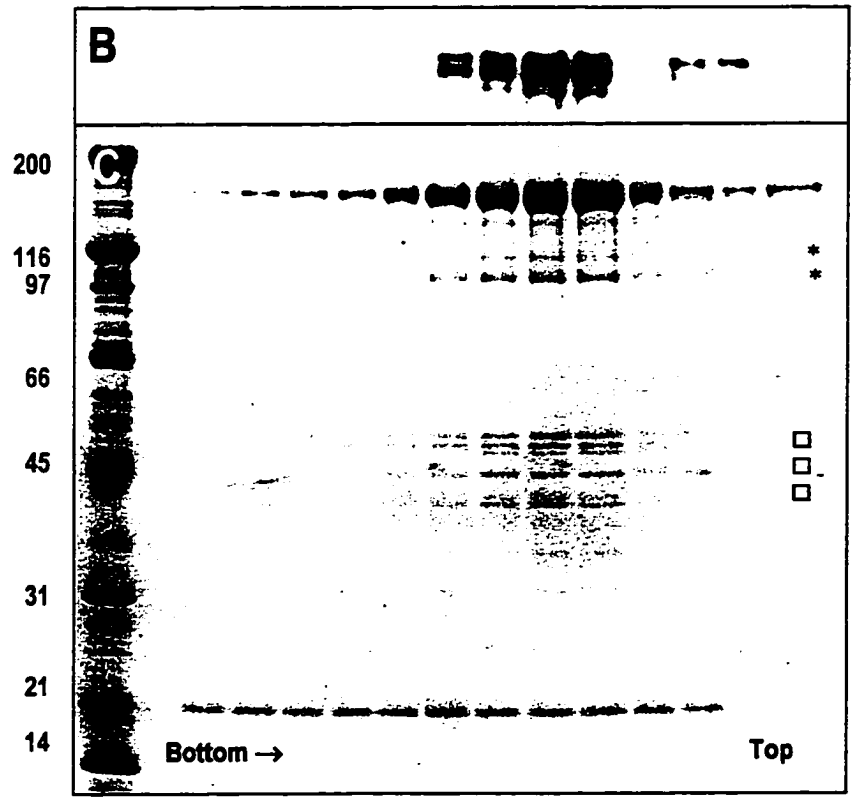
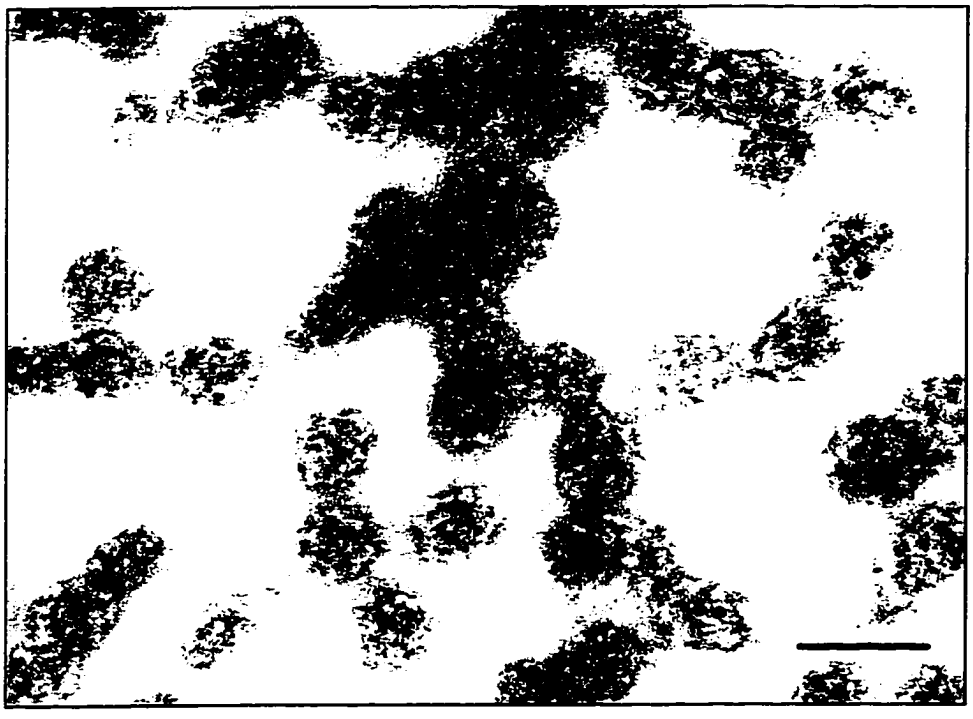

B
C
200
116
97
66
45
31
21
14
Bottom →
Top

Figure 15. Assembly of hybrid coats containing pea clathrin and brain AP. Electron microscopy of reassembled coats recovered at 14.2% sucrose after gradient purification and fractionation. Negative staining with uranyl acetate. Bar = 100 nm.



a jumble of unassembled triskelions. While no pea AP stimulation of assembly of brain clathrin into polygonal structures was observed in the large scale assembly assay, a small scale assembly assay demonstrated that pea AP is able to bind to pre-formed brain clathrin cages. When pea AP was dialyzed with brain clathrin in dialysis buffer containing 2 mM free Ca^{++}, no increase in assembly was observed beyond that attributable to calcium stimulation. However, when replicate samples were subjected to western blot analysis immunodetection revealed that a significant proportion of the pea AP was present in the high speed pellet. This is analogous to the observation that while AP1 does not appear to function as an assembly-stimulating protein in brain clathrin assembly *in vitro* (Lindner and Ungewickell, 1992) it will bind to pre-formed cages.

DISCUSSION

Under conditions of moderate ionic strength, assembly of clathrin triskelions of bovine brain into polyhedric structures *in vitro* is highly dependent on the addition of an "assembly promoting component" present in the dissociated coats of CCVs (Keen et al., 1979). The molecular composition of the dissociated assembly promoting proteins have been characterized, and three monomeric proteins (AP180, auxilin and p140) and two heterotetromeric protein complexes (AP1 and AP2) have been identified. The AP1 complex is composed of two ~100-110 kD polypeptides denoted as β1 and γ, along with single-copy 47 and 20 kD subunits. The AP2

complex is similarly composed of α and β2 (~100-110 kD) subunits, and 50 and 17 kD subunits. Of the dissociated proteins, AP2 and AP180 are the most active in promoting assembly of clathrin triskelions, accounting for 94% of the demonstrated assembly activity in Tris-solubilized coat proteins. Of the total, AP180 is responsible for 61% and AP2 contributes 33% of the observed assembly (Lindner and Ungewickell, 1992).

There are many more Tris-solubilized polypeptides in the coats of pea cotyledon CCVs than in brain CCV coats. This situation increases the difficulty in identifying potential assembly promoting polypeptides in plant CCVs. We reasoned that, as in brain, assembly promoting polypeptides would be found among those proteins which bind clathrin. A single cycle of assembly, in which Tris-solubilized proteins were dialyzed against moderate ionic strength buffer at pH 6.5, produced coats with a simplified polypeptide composition over that found in non-reassembled Tris-solubilized coat proteins. On Superose-6 fractionation of these reassembled coats Clathrin HC and LCs were found in the earlier eluting fractions along with a protein of 142 kD. Major co-eluting protein bands at ~110, ~80, 55 and 48 kD were present in fractions which elute after clathrin. In other experiments, we have demonstrated the ~110 kD protein reacts with a monoclonal antibody against the brain AP1/AP2 β-subunits. Thus, we consider it to be a likely candidate for pea β-adaptin. Identification of the ~80 kD protein, however, is problematical. In yeast, a putative homolog of β-adaptin with estimated molecular mass of ~80 kD has been identified (Kirchhausen, 1990). It is possible that the 80 kD protein may represent a β-adaptin of similar size

to that of yeast, however, it does not react with the monoclonal antibody to brain AP1/AP2 β-subunits. Other studies have shown that the β-adaptins of brain can be cleaved into 70-80 kD (containing the amino terminus) and 30-40 kD fragments by incubation with trypsin (Zaremba and Keen, 1985; Kirchhausen et al., 1989), thus the possibility exists that the 80 kD polypeptide is a proteolytic product of β-adaptin. While this possibility cannot be eliminated we consider it unlikely for the following reasons. The dialysis buffer contained protease inhibitors, and no 30 kD polypeptide is visible in coat proteins purified by reassembly. In other experiments where adequate precautions against proteolysis were not taken a band of ~67 kD cross reactive to β-adaptin is identified. An alternative possibility is that the prominent 80 kD polypeptide represents the plant equivalent of α or γ adaptins of brain. Unfortunately, antibodies to α or γ adaptins of brain do not identify comparable polypeptides in plants ((Holstein et al., 1994) and Chapter 2). It is unlikely that the 80 kD protein represents contaminating BP-80, a vacuolar targeting receptor previously shown to be present in CCVs (Kirsch et al., 1994). BP-80 is a transmembrane protein normally released from the membrane by treatment with CHAPS detergent. Proteins at 55 and 48 kD, which elute in the same fractions as the ~110 kD protein, as considered potential candidates for the adaptor μ-subunits. However, antibodies against brain μ-subunit candidates fail to detect any immunoreactive μ-subunit candidates in Tris-solubilized pea CCV coat proteins

(Chapter 2). While no candidates for the adaptor σ-subunits were apparent, this may be attributable to protein loading on SDS-PAGE.

Having identified a clathrin binding protein immunologically related to β-adaptin, and knowing that, following dialysis, Tris-dissociated pea clathrin coat proteins reassemble into polyhedral structures incorporating the potential assembly promoting polypeptide, we investigated the *in vitro* conditions under which assembly of pea clathrin would occur and compared the results with those of brain clathrin.

Little or no assembly of cages occurs when isolated brain clathrin triskelions are dialyzed against moderate ionic strength buffer at pH 6.5. However, roughly 70% of total clathrin in the assay was found to be assembled when isolated brain triskelions were dialyzed in the presence of a Superose pool containing brain AP, as has been demonstrated by many other studies (reviewed in (Pearse and Crowther, 1987)). Following sucrose gradient purification, assembled coats incorporating AP with mean diameters of ~66 and 59 nm were found in two poorly resolved peaks centered at 23 and ~15% sucrose. AP was incorporated equally across the peak fractions, with no changes in the relative amounts of β1/β2 adaptins observed, thus it seems unlikely that two distinct populations of coats exists; rather, the sedimentation range of coats suggest slight variability in the number of triskelions/coat.

In striking contrast to brain, reassembly of pea cotyledon triskelions purified from Tris-dissociated coat proteins does not require addition of any additional protein cofactor present in CCV coats. Instead, self-assembly of greater than 50% of total clathrin occurs. Sucrose density gradient purification of self-assembled pea clathrin

revealed two populations of cages at 20.4 and 15.2% with mean diameters of 91.22 nm (the larger of the peaks) and 71.60 nm. Inclusion of pooled Superose fractions containing the potential pea β-adaptin in assays conducted under the same conditions as for self-assembly, produced no increase in the total level observed in the absence of these proteins. We therefore conclude that pea AP proteins are not assembly promoting proteins.

Upon sucrose gradient purification, reassembled pea clathrin coats (formed of triskelions and APs) exhibited sedimentation behavior similar to that of brain coats. As in brain, two poorly resolved peaks were observed, centering at roughly 24 and 18% sucrose with mean diameters of 76 and 70nm respectively. The slightly greater molecular mass of pea clathrin HC (190 kD versus 180 kD for brain) may account for the slight increase in sedimentation in relation to brain coats. Pea β-adaptin, identified immunologically by western blot, was incorporated across the peaks, as was observed for the β1/β2-adaptins in brain assembly assays. This incorporation confirms the clathrin binding property of the ~110 kD polypeptide. While no stimulation of assembly occurred, the diameters of the assembled coats were smaller than the majority of self-assembled cages, suggesting that pea AP does, in fact, exert control over assembly of pea clathrin *in vitro* as does AP in brain.

Failure of pea AP to stimulate assembly may reflect the membrane origin of the CCVs isolated from pea cotyledon. Using immuno-affinity purified brain AP components, AP1, of Golgi origin, was shown to stimulate assembly by only 10% of the stimulation attributable to AP2 of plasma membrane origin (3% versus 33% of

total clathrin in the assay) (Lindner and Ungewickell, 1992). Since Pea AP, in similarity to AP1, also fails to stimulate assembly, the data suggests that it may be of the AP1 rather than the AP2 type. Thus, pea cotyledon CCVs may be of predominantly *trans*-Golgi origin. In brain, CCVs which bud from the Golgi (*trans*-Golgi) are involved in the intracellular transport of proteins to the lysosome. Vacuoles, similar functionally to lysosomes, are found in most plant cells. Since CCVs from pea cotyledon have been shown to transport unprocessed vacuolar hydrolases (Harley and Beevers, 1989b), a similar *trans*-Golgi origin for CCVs of pea cotyledon might be expected. Electron microscopic images of pea cotyledon showing many CCVs in close proximity to Golgi (but not plasma membrane) lend further support for the predominantly *trans*-Golgi origin (data not shown).

In brain, AP180, one of the monomeric polypeptides, is responsible for most of the AP-mediated clathrin assembly observed *in vitro* (Lindner and Ungewickell, 1992), but no polypeptide(s) equivalent to AP180 have yet been identified in pea CCV. The 142 kD protein present in the Superose-6 clathrin fraction of Tris-solubilized reassembled coat proteins may be a potential candidate for the plant equivalent of AP180. However, in contrast to brain AP180, the pea 142 kD polypeptide elutes with the clathrin fraction rather than leading the AP peak. If the 142 kD protein is an assembly-promoting polypeptide, it binds clathrin more tightly than does AP180. Assembly proteins tightly bound to clathrin triskelions might fail to dissociate under the conditions used since the coats of plant CCV have been shown more resistant to dissociation than those of brain (Chapter 2). No stimulation of

assembly was observed with the addition of the pea β-adaptin containing fractions, however, a slight stimulation of assembly over the level of self-assembly was observed when post AP Superose-6 fractions of Tris-solubilized coat proteins prepared without prior reassembly) were included in the assembly assay. This observed stimulation also suggests that unidentified assembly promoting polypeptides may be present in CCV coats.

Seeking an explanation for the striking difference in assembly behavior of animal and plant triskelions in the presence and absence of AP, we sought to determine whether other factors shown to affect brain clathrin assembly *in vitro* might explain the observed differences. With minor differences, pH, and clathrin concentration have similar effects on assembly of pea and brain clathrin cages *in vitro*. Maximal assembly for both occurs at pH 6.5, and assembly was found to be clathrin-concentration dependent.

The requirement for GTP-binding proteins in CCV formation at the cell surface (Carter et al., 1993) and Golgi (Stamnes and Rothman, 1993) and the observation that clathrin HC (Mooibroek et al., 1987), AP180 and the large subunits of both AP1 and AP2 (Morris et al., 1990), both μ subunits (Meresse et al., 1990) and LCb (in the presence of polylysine) of brain can be phosphorylated *in vitro* has led to speculation that protein phosphorylation may be involved in the regulation of CCV coating and/or uncoating processes *in vivo*. In similarity to brain, phosphorylation of pea cotyledon CCVs coat proteins have also been described (Lin et al., 1992). Since purified adaptors of bovine brain have been shown to be dephosphorylated during

purification (Morris et al., 1990), we considered the possibility that nucleotides might be required for AP-mediated stimulation of coat assembly in pea. When pea clathrin coats (pre-formed by dialysis without nucleotides) were incubated with either 2mM ATP or GTP, however, the extent of cage assembly was unaffected. The failure of ATP or GTP to stimulate assembly in our assay can be interpreted in the light of a recent study which demonstrated that upon phosphorylation, clathrin adaptors from bovine brain no longer bind clathrin (Wilde and Brodsky, 1996). If pea AP is also dephosphorylated upon purification, nucleotide mediated phosphorylation of adaptors would arrest, rather than stimulate, coat assembly.

Inclusion of 2 mM free Ca^{++} in the assembly assay stimulates assembly of both pea and brain triskelions into larger diameter polygonal structures than those resulting from AP-mediated coat assembly. Because clathrin light-chains (LCs) of brain have been shown to be calcium binding proteins (Mooibroek et al., 1987), it has been suggested that the site of influence for calcium may be clathrin LCs. The calcium binding site of mammalian LC has been localized to a 12 residue amino-terminal segment which appears to be a modified EF hand-loop similar to that of calmodulin and related proteins. An α-helix sequence involved in clathrin heavy chain binding and triskelion assembly is found adjacent to this EF hand-loop (Näthke et al., 1990). High concentrations of calcium (2 to 5mM) may stabilize this α-helix sequence and produce the stimulation of assembly of purified clathrin triskelions observed *in vitro* (Brodsky et al., 1991). The LC-like polypeptides of pea cotyledon CCVs have been shown to bind calcium (Lin et al., 1992). Thus the similarity of

response of pea and brain triskelions to the inclusion of free Ca^{++} in the assembly assay is indicative that pea clathrin LC may have sequence similarities to brain clathrin LC and serve a similar function *in vivo*.

The similarity in assembly response to pH, clathrin concentration, calcium and nucleotide additions indicates a considerable degree of homology in triskelions prepared from bovine brain and pea clathrin coated vesicles, and thus it is difficult to explain the ready self-assembly of the pea triskelion. Plant clathrin is considerably more resistant to dissociation than brain and it is possible that the self-assembly of cages from pea triskelions is mediated by unknown assembly promoting polypeptides incompletely dissociated from the triskelions. However negative staining of the triskelion fraction showed no evidence of partial cage assembly or polypeptides binding to clathrin as had been demonstrated for brain (Heuser and Keen, 1988). To maximize extraction of coat proteins from pea CCVs and to precipitate contaminating ferritin, near molar $MgCl_2$ is included in the dissociation buffer (Hyde et al., 1963; Woodward and Roth, 1978; Sczekan and Joshi, 1987). Ferritin, due to its density, co-migrates with CCVs on Ficoll/D_2O gradients. Without the addition of $MgCl_2$, large quantities of ferritin co-elute with AP upon gel filtration of dissociated pea coat proteins, as has been shown for placental APs (Pearse, 1982), thus making adaptor fractions unusable. It is conceivable that $MgCl_2$, sufficient to stimulate assembly (Woodward and Roth, 1978), remains in the triskelion and β-adaptin fractions. This seems highly unlikely however, since $MgCl_2$ would elute much further downstream than any protein (the Superose-6 column acting effectively as a large-scale desalting

column). In addition, assembly assays included dialysis against large volumes of buffer without high concentrations of $MgCl_2$, further decreasing the concentration of $MgCl_2$ and the possibility of $MgCl_2$ stimulated assembly.

The similarity in coat components between brain and pea CCVs is further demonstrated by the reciprocal mixing experiments. Dialyzing pea clathrin in the presence of brain AP resulted in assembly of recognizable polygonal structures which incorporate brain AP. In fact, more brain AP-mediated assembly of pea clathrin occurred than for pea self-assembly (or assembly in the presence of pea AP). This stimulation indicates that pea clathrin assembly *in vitro* is influence by AP (although not actually stimulated by the β-adaptin found in pea clathrin coats) and that similar binding sites for assembly proteins must be present on brain and pea cotyledon triskelions. Addition of pea AP to brain clathrin did not increase the amount of clathrin triskelions found assembled further suggesting the pea β-adaptin is of the AP1 type. While not stimulating the assembly of brain triskelions in cages, pea AP does bind to brain cages pre-formed in the presence of calcium. This suggests that not only are binding sequence conserved across the animal and plant kingdoms but that pea β-adaptin, in similarity to brain AP1, interacts with clathrin triskelia.

It is possible that the self assembly observed for pea triskelions, rather than the AP-mediated assembly demonstrated for brain, is the more "typical" situation. *In vitro* assembly of clathrin cages from mammalian sources has only been examined using clathrin purified from brain CCVs. AP180, responsible for most of the observed assembly in brain, is specific to neuronal cells or tissues (Ahle and

Ungewickell, 1986; Morris et al., 1993). Is brain atypical? AP1 (which does not promote assembly), rather than AP2 predominates in bovine adrenal gland CCVs (Ungewickell et al., 1994). What then is responsible for assembly of clathrin coats in adrenal gland and other tissues?

In summary, it appears that while pH, clathrin concentration and the addition of free Ca^{++} have similar effects on the assembly of pea cotyledon and brain clathrin *in vitro*, assembly of clathrin coats of developing pea cotyledons and brain is markedly different. The ability of pea clathrin to self-assemble and the failure of the pea β-adaptin Superose-6 fraction AP to stimulate additional assembly under our assembly conditions suggest that assembly of CCVs in plants may involve different components than in brain.

REFERENCES

Ahle S, Mann A, Eichelsbacher U, Ungewickell E (1988) Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. EMBO J **7:** 919-929

Ahle S, Ungewickell E (1986) Purification and properties of a new clathrin assembly protein. EMBO J **5:** 3143-3149

Balusek K, Depta H, Robinson DG (1988) Two polypepetides (30 and 38 kDa) in plant coated vesicles with clathrin light chain properties. Protoplasma **146:** 174-176

Blackbourn HD, Jackson AP (1996) Plant clathrin heavy chain: sequence analysis and restricted localization in growing pollen tubes. J Cell Sci **109:** 777-787

Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant - proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8:** 93-99

Brodsky FM, Hill BL, Acton SL, Näthke I, Wong DH, Ponnambalam S, Parham P (1991) Clathrin light chains: arrays of protein motifs that regulate coated-vesicle dynamics. Trends Biochem Sci **16:** 208-213

Butler JM, Beevers L (1993) Putative adaptor proteins of clathrin coated vesicles from developing pea. In DJ Morré, KE Howard, JJM Bergeron, eds. Molecular Mechanisms of Membrane Traffic, Vol **H74**. Springer-Verlag, Berlin-Heidelberg, pp 331-332.

Campbell C, Squicciarini J, Shia M, Pilch PF, Fine RE (1984) Identification of a protein kinase as an intrinsic component of rat liver coated vesicles. Biochemistry **23:** 4420-4426

Carter LL, Redelmeier TE, Woollenweber LA, Schmid SL (1993) Multiple GTP-binding proteins participate in clathrin-coated vesicle-mediated endocytosis. J Cell Biol **120:** 37-45

Cole L, Coleman JOD, Hawes CR, Horsley D (1987) Antibodies to brain clathrin recognise plant coated vesicles. Plant Cell Rep **6:** 227-230

Coleman J, Evans D, Hawes C, Horsley D, Cole L (1987) Structure and molecular organization of higher plant coated vesicles. J Cell Sci **88:** 35-45

Depta H, Robinson DG (1986) The isolation and enrichment of coated vesicles from suspension-cultured carrot cells. Protoplasma **130:** pp162-170

Fowke LC, Griffing LR, Mersey BG, Van der Valk P (1983) Protoplasts for studies of plasma membrane and associated cell organelles. Experientia **Suppl. 46:** 101-110

Glickman JN, Conibear E, Pearse BMF (1989) Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth factor II receptor. EMBO J **8:** 1041-1047

Harley SM, Beevers L (1989a) Isolation and partial characterization of clathrin coated vesicles for pea (*Pisum sativum* L.) cotyledons. Protoplasma **150:** 103-109

Harley SM, Beevers L (1989b) Coated vesicles are involved in the transport of storage proteins during seed development in *Pisum sativum* L. Plant Physiol **91:** 674-678

Heuser JE, Keen J (1988) Deep-etch visualization of proteins involved in clathrin assembly. J Cell Biol **107:** 877-886

Hoh B, Schauermann G, Robinson DG (1991) Storage protein polypeptides in clathrin coated vesicle fractions are not due to endomembrane contamination. J Plant Physiol **138:** 309-316

Hohl I, Robinson DG, Chrispeels MJ, Hinz G (1996) Transport of storage proteins to the vacuole is mediated by vesicle without a clathrin coat. J Cell Sci **109:** 2539-2550

Holstein SEH, Drucker M, Robinson DG (1994) Identification of a β-type adaptin in plant clathrin-coated vesicles. J Cell Sci **107:** 945-953

Horn MA, Heinstein PF, Low PS (1989) Receptor-mediated endocytosis in plant Cells. Plant Cell **1:** 1003-1009

Horn MA, Meadows RP, Apostol I, Jones CR, Gorenstein DG, Heinstein PF, Low PS (1992) Effect of elicitation and changes in extracellular pH on the cytoplasmic and vacuolar pH of suspension-cultured soybean cells. Plant Physiol **98:** 680-686

Hyde BB, Hodge AJ, Kahn A, Birnsteil ML (1963) Studies on phytoferritin .1. identification and localization. Ultrastructural Res **9:** 248-258

Joachim S, Robinson DG (1884) Endocytosis of cationic ferritin by bean leaf protoplasts. Eur J Cell Biol **34:** 212-216

Keen JH (1987) Clathrin assembly proteins: affinity purification and a model for coat assembly. J Cell Biol **105:** 1989-1998

Keen JH, Chestnut MH, Beck KA (1987) The clathrin coat assembly polypeptide complex. J Biol Chem **262:** 3864-3871

Keen JH, Black MM (1986) The phosphorylation of coated membrane proteins in intact neurons. J Cell Biol **102:** 1325-1333

Keen JH, Willingham MC, Pastan IH (1979) Clathrin-coated vesicles: isolation, dissociation and factor-dependent reassociation of clathrin baskets. Cell **16:** 303-312

Kirchhausen T, Nathanson KL, Matsui W, Vaisberg A, Chow EP, Burne C, Keen JH, Davis AE (1989) Structural and functional division into two domains of the large (110- to 115-kDa) chains of the clathrin-associated protein complex AP-2. Proc Natl Acad Sci USA **86:** 2612-2616

Kirchhausen T (1990) Identification of a putative yeast homolog of the mammalian β chains of the clathrin associated protein complexes. MolCell Biol **10:** 6089-6090

Kirchhausen T, Harrison SC (1981) Protein organization in clathrin trimers. Cell **23:** 755-761

Kirsch T, Beevers L (1993) Uncoating of clathrin coated vesicles by uncoating ATPase from developing peas. Plant Physiol **103:** 205-212

Kirsch T, Paris N, Butler JM, Beevers L, Rogers JC (1994) Purification and initial characterization of a plant vacuolar targeting receptor. Proc Nat Acad Sci USA **91:** 3403-3407

Kirsch T, Saalbach G, Raikel N, Beevers L (1996) Interaction of a potential vacuolar targeting receptor with amino- and carboxyl-terminal targeting determinants. Plant Physiol **111:** 469-474

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:** 680-685

Lin H, Harley SM, Butler JM, Beevers L (1992) Multiplicity of clathrin light-chain-like polypeptides from developing pea (*Pisum sativum* L.) cotyledons. J Cell Sci **103:** 1127-1137

Lindner R, Ungewickell E (1992) Clathrin-associated proteins of bovine brain coated vesicles. An analysis of their number and assembly-promoting activity. J Biol Chem **267:** 16567-16573

Low PS, Chandra S (1994) Endocytosis in plants. Annu Rev Plant Physiol Plant Mol. Biol **45:** 609-631

Maizel JV (1971) Polyacrylamide gel electrophoresis of viral proteins. Methods Virol **5:** 179-246

Meresse S, Ludwig T, Frank R, Hofflack B. (1990) Phosphorylation of the cytoplasmic domain of the bovine cation-independent mannose 6-phosphate receptor. J Biol Chem **31:** 18833-18842

Mersey BG, Griffing LR, Rennie PJ, Fowke LC (1985) The isolation of coated vesicles from protoplasts of soybean. Planta **163:** 317-327

Mooibroek M, Michiel DF, Wang HH (1987) Clathrin light chains are calcium-binding proteins. J Biol Chem **262:** 25-28

Morris SA, Schroder S, Plessmann U, Weber K, Ungewickell E (1993) Clathrin assembly protein AP180: primary structure, domain organization and identification of a clathrin binding site. EMBO J **12:** 667-765

Morris SA, Mann A, Ungewickell E (1990) Analysis of 100-180-kDa phosphoproteins in clathrin-coated vesicles from bovine brain. J Biol Chem **265:** 3354-3357

Näthke I, Hill BL, Parham P, Brodsky FM (1990) The calcium-binding site of clathrin light chains. J Biol Chem **265:** 18621-18627

Nandi PK, Van Jaarsveld PP, Lippoldt RE, Edelhoch H (1981) Effect of basic compounds on the polymerization of clathrin. Biochemistry **20:** 6706-6710

Nandi PK, Pretorius HT, Lippoldt RE, Johnson ML, Edelhoch H (1980) Molecular properties of the reassembled coat protein of coated vesicles. Biochemistry **19:** 5917-5921

Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis **9:** 255-262

Paris N, Stanley CM, Jones RL, Rogers JC (1996) Plant cells contain two functionally distinct vacuolar compartments. Cell **85:** 563-572

Pearse BMF (1975) Coated vesicles from pig brain: purification and biochemical characterization. J Mol Biol **97:** 93-98

Pearse BMF, Crowther RA (1987) Structure and assembly of coated vesicles. Annu Rev Biophys Biophys Chem **16:** 49-68

Pearse BMF (1982) Coated vesicles from human placenta carry ferritin, transferrin, and immunoglobulin G. Proc Nat Acad Sci USA **79:** 451-455

Pearse BMF (1988) Receptors compete for adaptors found in plasma membrane coated pits. EMBO J **7:** 3331-3336

Robinson, DG, Balusek K, Depta H, Hoh B, Holstein SEH (1991). Isolation and characterization of plant coated vesicles. *In* Endocytosis, Exocytosis and Vesicle Traffic in Plant Cells, Cambridge University Press, Cambridge, pp65-79

Robinson DG, Balusek K, Freundt H (1989) Legumin antibodies recognize polypeptides in coated vesicles from developing pea. Protoplasma **150:** 79-82

Romani G, Beffagna N, Meraviglia G (1996) Role for the vacuolar H^+-ATPase in regulating the cytoplasmic pH; an *in vivo* study carried out in *chl1* and *Arabidopsis thaliana* mutant impaired in NO_3^- transport. Plant Cell Physiol **37:** 285-291

Rosenbluth J, Wissig SL (1964) The distribution of exogenous ferritin in toad spinal ganglia and the mechanism of its uptake by neurons. J Cell Biol **23:** 307-325

Rosenbluth J, Wissig SL (1963) The uptake of ferritin by toad spinal ganglion cells. J Cell Biol **19:** 91A

Roth TF, Porter KR (1964) Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti* L. J Cell Biol **20:** 313-332

Schmid SL (1993) Biochemical requirements for the formation of clathrin-and COP-coated transport vesicles. Curr Op Cell Biol **5:** 621-627

Sczekan SR, Joshi JG (1987) Isolation and characterization of ferritin from soyabeans (*Glycine max*)*. J Biol Chem **262:** 13780-13788

Staehelin LA, Moore I (1995) The plant Golgi apparatus: Structure, functional organization and trafficking mechanisms. Annu Rev Plant Physiol Plant Mol Biol **46:** 261-288

Stamnes MA, Rothman JE (1993) The binding of AP-1 clathrin adaptor particles to golgi membranes requires ADP-ribosylation factor, a small GTP-binding protein. Cell **73:** 999-1005

Tanchak MA, Griffing LR, Mersey BG, Fowke LC (1884) Endocytosis of cationized ferritin by coated vesicles of soybean protoplasts. Planta **162:** 481-486

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA **76:** 4350-4354

Ungewickell E, Plessmann U, Weber K (1994) Purification of Golgi adaptor protein 1 from bovine adrenal gland and characterization of its β(β') subunit by microsequencing. Eur J Biochem **222:** 33-40

Ungewickell E, Ungewickell H, Holstein SEH, Lindner R, Prasad K, Barouch W, Martin B, Greene LE, Eisenberg E (1995) Role of auxilin in uncoating clathrin-coated vesicles. Nature **378:** 632-635

Van Jaarsveld PP, Nandi PK, Lippoldt RE, Saroff H, Edelhoch H (1981) Polymerization of clathrin protomers into basket structures. Biochemistry **20:** 4129-4135

Vigers GPA, Crowther RA, Pearse BMF (1986) Location of the 100 kD-50 kD accessory proteins in clathrin coats. EMBO J **5:** 2079-2085

Wilde A, Brodsky FM (1996) *In vivo* phosphorylation of adaptors regulates their interaction with clathrin. J Cell Biol **135:** 635-645

Woodward MP, Roth TF (1978) Coated vesicles: Characterization, selective dissociation, and reassembly. Proc Natl Acad Sci USA **75:** 4394-4398

Zaremba S, Keen JH (1985) Limited proteolytic digestion of coated vesicle assembly polypeptides abolishes reassembly activity. J Cellular Biochem **28:** 47-58

Zaremba S, Keen JH (1983) Assembly polypeptides from coated vesicles mediate reassembly of unique clathrin coats. J Cell Biol **97:** 1339-1347

CHAPTER IV

Interaction of Plant Clathrin Adaptors with a Putative Plant Vacuolar Receptor

ABSTRACT

We have previously isolated a complex of proteins containing a β-type adaptin from clathrin coated vesicles (CCVs) of developing pea cotyledon. In mammalian systems, clathrin adaptors are known to mediate protein sorting in both the endocytic and secretory pathways through their interaction with the cytosolic domains of transmembrane receptors present at the plasma membrane (AP2) and the *trans*-Golgi network (AP1). Coat proteins released by Tris-HCl treatment of CCVs from developing pea cotyledon interact *in vitro* with a 80 kD vacuolar targeting receptor (BP-80) present in CCVs and a less dense membrane fraction (LDM). BP-80, isolated by CHAPS treatment of pea cotyledon CCVs and a less dense membrane fraction (LDM) and affinity purified, specifically binds Superose-6 purified pea cotyledon adaptor proteins. We have further constructed an Affigel affinity matrix containing the 37 amino acid residues present in the *Arabidopsis* homolog of the BP-80 C-terminal domain and demonstrated that it specifically binds purified adaptor proteins from bovine brain, wheat germ and pea cotyledons. This binding is largely eliminated when adaptor proteins are incubated with a truncated peptide containing

the portion of the C-terminal domain of BP-80 corresponding to the canonical tyrosine binding sequence identified as necessary for receptor targeting to endosomal compartments in mammalian systems. The binding of plant clathrin APs to the C-termini of the 80 kD receptor provides a selective mechanism for recruitment of receptor and ligand from the trans-Golgi for assembly into CCVs and confirms both the identification of BP-80 as a vacuolar targeting receptor and an "adaptor" function for AP proteins of plant CCVs.

INTRODUCTION

Each of the specialized compartments of the endomembrane system is dependent for its function on high fidelity sorting and transport of organellar specific proteins from their site of synthesis on ER-bound ribosomes. Both transport and sorting are mediated by a variety of small membrane-bound vesicles bearing protein coats. In addition, coated vesicles also mediate endocytosis from the cell surface to the endosome and lysosome. Although several types of coated vesicles are known (for recent reviews, see (Robinson, 1997; Rothman and Wieland, 1996)), clathrin-coated vesicles (CCVs), remain the best characterized. Among the coat proteins of CCVs are specialized polypeptides termed adaptors which interact with Tyr-based sorting signals present in the cytoplasmic domains of transmembrane proteins and protein receptors. In mammalian systems these adaptors are components of two heterotetromeric complexes: AP2, which mediates association of clathrin with the

plasma membrane, and AP1, present in CCVs associated with the *trans*- Golgi network. The AP2 complex consists of two ~100 kD large subunits (α- and β2 or β1-adaptins) one 50 kD medium chain (μ2) and a 17 kD small subunit (σ2). The AP1 complex is similarly composed of four subunits (γ-adaptin, β1-adaptin, μ1 and σ1). Within the AP complexes the homologous μ-subunits appear to interact directly with Tyr-based sorting motifs present on receptors (Boll et al., 1995; Ohno et al., 1995; Boll et al., 1996).

In mammalian cells, endocytosis is mediated by the interaction of AP2 with Tyr-based receptors containing either NPXY-type sorting signals (such as the LDL receptor) or YXXØ-type sorting signals (such as the transferrin receptor or the asialoglycoprotein receptor). Sorting of lysosomal hydrolases in mammalian cells, however, is dependent on recognition of mannose-6 phosphate residues (added to Asn-linked oligosaccharide chains of lysosomal proteins in the Golgi) by receptors, present in the *trans*-Golgi network, which contain, in addition to the luminal mannose-6 phosphate binding sequences, Tyr-based sorting signals of the YXXØ-type in their cytoplasmic C-terminal domains. After binding, receptor/cargo complexes are then packaged into AP1-type CCVs for transport to the lysosome (Pfeffer and Rothman, 1987).

While our knowledge of clathrin mediated-sorting in plants is still limited, CCVs present in plant cells, like those of mammalian cells appear to function at both the plasma membrane and at the plant TGN. Early studies of endocytosis

using isolated soybean protoplasts clearly demonstrated the internalization of exogenous ferritin via coated vesicles (Tanchak et al., 1884), and more recent studies demonstrate the involvement of CCVs in plasma membrane recycling during cell-plate formation (Samuels et al., 1995) and pollen tube growth in lily (Blackbourn and Jackson, 1996). In addition, CCVs appear to have a role in the intracellular transport of newly synthesized proteins in plants as well as in mammalian cells. CCVs isolated from developing pea cotyledons are known to contain precursors of lectin and hydrolytic enzymes (Harley and Beevers, 1989). In legumes, hydrolytic enzymes appear to be stored in specialized lytic vacuoles shown, by the presence of TIP-Ma27 (also known as γ-TIP) in the tonoplast membrane, to be distinct from vacuoles containing the storage proteins utilized during seedling germination and development (Marty-Mazars et al., 1995; Paris et al., 1996).

In plants, however, sorting of proteolytic enzymes to lytic vacuoles (thought to be equivalent to the mammalian lysosome due to high concentrations of proteolytic enzymes and an acidic pH), appears not to be dependent on the mannose-6 phosphate system since no mannose-6 posphate containing proteins or receptors have been identified in plant systems (Gaudreault and Beevers, 1984). Instead, sorting to pre-vacuolar and vacuolar compartments in plants is dependent on signals present in the amino-terminal (Holwerda and Rogers, 1992; Matsuoka and Nakamura, 1991) and carboxy-terminal propeptides (Saalbach et al., 1996), and in the mature proteins

themselves (Nakamura and Matsuoka, 1993; Chrispeels and Raikel, 1992; Gal and Raikhel, 1993).

Recently, our laboratory has isolated from pea cotyledon CCVs and a less dense membrane (LDM) present in the Ficoll/D_2O gradients used for final purification of CCVs, a plant vacuolar-targeting receptor (BP-80) (Kirsch et al., 1994) which has been shown to interact with sorting signals present in both amino-terminal propeptides (barley aleurain and prosporamin from sweet potato) and the carboxy-terminal propeptide of 2S albumin from Brazil nut (Kirsch et al., 1996). In similarity to many mammalian receptor proteins shown to interact with clathrin adaptors, BP-80 contains a Tyr-based sorting signal of the YXXØ type in its cytoplasmically oriented carboxy-terminus (Paris et al., 1997). In addition to the isolation of BP-80, we have also isolated clathrin adaptor proteins from both pea cotyledon ((Butler and Beevers, 1993)and Chapter 2) and wheat germ CCVs (unpublished results).

In the present study, we have demonstrated the *in vitro* interaction of a total coat protein fraction containing adaptors obtained from pea cotyledon CCV with purified LDM containing BP-80, and protein-protein interaction between purified pea cotyledon AP and BP-80. We have further demonstrated the binding of AP protein from pea cotyledon, wheat germ, and bovine brain to an *Arabidopsis* homolog of BP-80 using an immobilized peptide corresponding to the entire cytoplasmic domain of this homolog (the wt peptide) and confirmed, by electrophoretic analysis, the subunit composition of pea cotyledon AP obtained by immuno-affinity chromatography

(Chapter 2). In addition, we have demonstrated that binding of plant APs to the wt peptide is largely eliminated when adaptors are incubated with a truncated peptide containing the portion of the carboxy-terminal domain corresponding to the canonical Tyr-based binding sequence of BP-80. Finally, we have shown that degree of binding of pea cotyledon AP to a peptide modified by an substitution of Ala for the Tyr in the binding motif of the truncated peptide is essentially unchanged from that of the binding of pea cotyledon AP to the truncated peptide, suggesting that, as has been shown for the cation dependent 46 kD mannose-6 phosphate receptor (Höning et al., 1997), that multiple AP binding sites may be present in the cytoplasmic tail of BP-80 and that *in vivo* binding of AP to the BP-80 receptor may be the result of multiple binding events.

MATERIALS AND METHODS

Materials

Clathrin coated vesicles (CCVs) were isolated from developing pea, bovine brain and wheat germ. A less dense membrane fraction (LDM) enriched in BP-80 (Kirsch et al., 1994; Kirsch et al., 1996; Paris et al., 1997) was isolated from developing pea. Garden peas, *Pisum sativum* L. cv. Burpeeana, were locally grown, hand-harvested at 24-27 days post anthesis, mechanically shelled, quick frozen in LN_2, and frozen at -80°C. Bovine brains, stripped of meninges and frozen in LN_2 within 30 minutes of slaughter, were purchased from Pel-Freez Biologicals, Rogers,

AR. Organically grown wheat germ, *Triticum aestivum* L., milled and packaged by Arrowhead Mills, Hereford, TX, was obtained locally or as a gift from Arrowhead Mills. Wheat germ used in this study was judged to be approximately 95% viable by calculating the weight percentage of flotation of a sample layered on a 1:3 (vol/vol) mixture of cyclohexane:carbon tetrachloride. Prior to homogenization, wheat germ was imbibed with tap water overnight.

Clathrin Coated Vesicle (CCV) Isolation

The following buffers were used. Buffer A: 0.1 M NaMes (2-(N-morpholinomethane sulfonic acid), 0.1 M EGTA, 0.5 mM $MgCl_2$, 0.02% sodium azide pH 6.5; Buffer B: Buffer A containing 0.025 mg/ml trypsin inhibitor, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM dithiolthreitol (DTT); Buffer C: Buffer B plus 0.3 M sorbitol. All buffers were adjusted to final pH at RT.

Clathrin coated vesicles were isolated using a protocol which combined the methods of (Lin et al., 1992) and (Campbell et al., 1984) utilizing sequential Ficoll/Sucrose and Ficoll/D_2O gradient centrifugations (Chapters 2-3). All procedures were carried out at 4°C. Frozen pea cotyledon and bovine brain were quick thawed and homogenized for 1-2 minutes in a Waring blender (pea:buffer C, 1:1 wt/vol; brain:buffer B, 1:1 wt/vol). To reduce ribosomal contamination, imbibed wheat germ was homogenized in buffer C (1:1 wt/vol) which had been modified to eliminate $MgCl_2$ and include 5mM EDTA. This strategy has been successfully employed for the

isolation of CCVs from zucchini hypocotyl and pea cotyledon (Demmer et al., 1993). PMSF, DTT, and trypsin inhibitor were added to buffers immediately prior to centrifugation. Plant homogenates were first squeezed through cheesecloth, and all homogenates were centrifuged at 200 x g (all values calculated as r^{avg}), for 5 minutes, followed by 30 minutes at 17,000 x g in a Sorvall RC5B centrifuge using a GSA rotor. Supernatants were collected by aspiration and centrifuged at 120,000 x g for 40-50 minutes in either a Beckman L2 or L8-80 ultracentrifuge operated at 4°C using Beckman 45Ti and/or Beckman 42.1 rotors. Postmicrosomal pellets were resuspended in buffer B (for wheat germ, modified as buffer C above) using a ground glass hand homogenizer to a final volume of 10mls/150 g tissue. Pea and wheat germ homogenates were incubated with pancreatic ribonuclease A (1mg/ml of homogenate) for 1 hr at 4°C with gentle shaking. Homogenates were diluted 1:1 with 12% Sucrose/12% Ficoll in buffer B (modified as above for wheat germ) and centrifuged at 20,000 x g (30,000 x g for wheat germ) in a Beckman 42.1 rotor for 30 minutes. Supernatants were collected by Pasteur pipette, diluted with at least 3 volumes of buffer B, and centrifuged at 120,000 x g for 50 minutes as above. Pelleted CCVs were resuspended in buffer A (modified as above for wheat germ) to a final volume of 25g initial weight/ml buffer. In some cases resuspended pellets were frozen at -80°C without apparent loss of CCV yield. Finally, the resuspended pellets were layered (5-10 mls/gradient) on 20 ml linear gradients consisting of 5% Ficoll/10% D_2O to 25% Ficoll/90% D_2O in buffer A (modified for wheat germ) and centrifuged at 55,000 x g

for 16 hrs in a Beckman SW28 swinging bucket rotor. Gradient regions containing CCVs (determined by SDS-PAGE analysis of fractionated gradients to be highly reproducible) with Ficoll concentration equivalent in refractive index to 18-22% sucrose were collected by Pasteur pipette, diluted with buffer A (all tissues) and pelleted as above. Pellets containing CCVs were resuspended in a small amount of buffer and frozen at -80°C.

Gel Filtration Chromatography of Brain and Plant CCV Coat Proteins

Brain coat proteins were dissociated from CCVs by addition of 0.33 vol of 2 M Tris-HCl, pH 7.0, 8mM EDTA, 0.4 mM PMSF incubated for 15 min on ice (Lindner and Ungewickell 1992) followed by ultracentrifugation at 170,000 x g for 50 min in a T65 rotor. Extractions were repeated at least once and supernatants combined. Coat proteins (15-30 mg in 3-6 ml) obtained from 1-2 kg brain were loaded on a 2.5 x 66 cm Superose-6 gel filtration column, equilibrated in Buffer D (a 1:1 mixture of buffer A and 1 M Tris-HCl, pH 7.0), and eluted downward at ~ 0.6 ml/min at 10°C. Four ml fractions were collected. After SDS-PAGE and immunoblotting (see below) to locate APs within the elution profile, fractions 37-42 containing dissociated clathrin triskelions and fractions 50-58 containing APs were pooled separately and concentrated using Amicon Centri-Prep10™ concentrators. Only AP fractions were used in this study.

To obtain pea coat proteins CCVs from 3 kg cotyledons were pelleted, resuspended in 1.75 ml dissociation buffer containing 0.05 M Mes, 0.5 M Tris, 0.75 M

$MgCl_2$ (Holstein et al., 1994) augmented with 0.02% Sodium azide, 0.025 mg/ml trypsin inhibitor, 0.05 mM PMSF and 1 mM DTT, incubated on ice for 60 min with gentle shaking and ultracentrifuged at 170,000 x g in T65 rotor for 50 min. Pellets containing partially uncoated CCVs were resuspended in 1.0 ml dissociation buffer, extracted as above for 30 min, spun as above and the first and second supernatants were combined. Coat proteins (6-8 mg in 2.5 ml) were loaded on a 1x94 cm Superose-6 column equilibrated in Buffer D and eluted downward at 0.2 ml/min at 10°C. One ml fractions were collected. After SDS-PAGE and immunoblotting (see below) to locate APs within the elution profile fractions 36-44 containing clathrin triskelions and fractions 50-55 containing APs were pooled separately and concentrated using Amicon Centri-Prep10™ concentrators. Wheat germ coat proteins were obtained and separated on Superose-6 using essentially the same protocol for pea cotyledon coat proteins.

BP-80 Isolation and Purification

In addition to CCVs, a less-dense membrane fraction (LDM), equivalent in refractive index to 9-12% sucrose was recovered from the Ficoll/D_2O gradients of pea cotyledon CCV preparations. This fraction and CCVs were used to prepare purified BP-80 by its affinity for the N-terminal targeting determinant of the vacuolar thiol protease proaleurain. A synthetic peptide containing this targeting determinant was synthesized at the Washington University Medical School Protein Chemistry Laboratory and chemically coupled to Sulfolink™ agarose beads (Pierce) according to

the manufacturer's protocol. Surface proteins from LDM and CCVs were dissociated by incubation with 1 M Tris-HCl (pH 8.3, room temperature, 30 min) and centrifuged at 165,000 x g for 40 min using a Beckman T65 rotor. Pellets were resuspended and incubated for 30 min at RT in a 20 mM Hepes-NaOH buffer, pH 7.1, containing 150 mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 1% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate. Membranes were then pelleted (as above) and the supernatant containing ~ 1mg/ml protein was applied to the proaleurain column. The column was incubated for 30 minutes with shaking, and unbound protein drained. After washing with 2 and 10 ml aliquots of the CHAPS incubation buffer, bound protein, highly enriched in BP-80, was eluted with 25mM sodium acetate, pH 4.0 containing 150 mM NaCl, 1 mM EGTA and 1 % CHAPS (Kirsch et al., 1994).

Reassembly of LDM and CCV Coat Proteins

LDM (from a 1500 g CCV preparation) was further purified on a gradient constructed of a 3 ml 52% sucrose cushion overlaid with a 24 ml 15-40% linear sucrose gradient. Sucrose solutions were made in buffer A. Gradients were centrifuged for 16 hrs at 90,000 x g in a Beckman SW27 rotor. LDM containing fractions (25-31% sucrose) were diluted, pelleted, and finally resuspended in 0.5 ml of buffer A.

CCVs from 1500 g pea cotyledon, suspended in 1.0 ml buffer A were incubated for 30 min at RT with an equal volume of 1M Tris-HCl, pH 7.0. After incubation, dissociated coats were separated from vesicle membranes by centrifugation at 200,000 x g for 40 min using a Beckman T65 rotor. Pea cotyledon cytosol was prepared by homogenizing 50 g of cotyledon in 100 mls of buffer A, followed by centrifugation at 200,000 x g as above.

One ml of coat protein were combined with 200 µl of cytosol, 200 µl of LDM, and adjusted to 1mM GTP and 1 mM ATP in the presence of 1 mM PMSF and 0.25mg/ml trypsin inhibitor. To exchange the buffer, the mixture was applied to a 15 ml Sephadex G-25 column equilibrated with 0.1M Mes pH 6.5 containing 5mM magnesium acetate. All proteins eluting in the void volume were collected and applied to a sucrose gradient constructed as above. After isopycnic centrifugation (see above) the gradient was fractionated into 1.2 ml aliquots. A mixture containing 1 ml coat proteins and 200 µl of cytosol (containing identical concentrations of nucleotides and protease inhibitors), but without LDM was processed as above. LDM plus protease inhibitors (without nucleotides) was applied to a third gradient, centrifuged and fractionated as above. Fractions from each gradient were analyzed by SDS-PAGE.

Reassembly of Pea Adaptor and Affinity Purified BP-80

Using individual microdialysis cups constructed from microcentrifuge tubes ((Lindner and Ungewickell, 1992) and Chapter 3), Superose-6 fractionated pea cotyledon AP (final concentration 0.067 mg/ml) and affinity purified BP-80 (final concentration 0.060mg/ml) were dialyzed overnight against 2 x 600 ml of buffer B alone and in combination in a final volume of 0.1 ml. Following high speed centrifugation (150,000 x g for 40 min using a Beckman T65 rotor) the distribution of BP-80 in pellets (assembled protein) and supernatants (unassembled) was analyzed by SDS-PAGE.

Affinity Columns and Chromatography

For construction of the affinity columns used in the AP-BP-80 interaction, a synthetic peptide corresponding to the C-terminal portion of the *Arabidopsis* homolog of BP-80, EST cDNA Z38123, (NH_2-Lys-Tyr-Arg-Ile-Arg-Ser-Tyr-Met-Asp-Ala-Glu-Ile-Arg-Gly-Ile-Met-Ala-Gln-Tyr-Met-Pro-Leu-Glu-Ser-Gln-Pro-Pro-Asn-Thr-Ser-Gly-His-His-Met-Asp-Ile-COOH) and two truncated peptides, one containing the Tyr-based sorting motif (NH_2-Arg-Gly-Ile-Met-Ala-Gln-Tyr-Met-Pro-Leu-COOH) and a second with Ala substituted for Tyr, were synthesized. The *Arabidopsis* sequence was used because the full-length sequence of BP-80 from pea was unavailable at the time of synthesis. When the full length sequence of BP-80 became available, sequence comparison showed that, in the truncated peptide, only a single

amino acid difference between the sequences of *Pisum* (pea cDNA NP471) and *Arabidopsis* (EST cDNA Z38123) existed, a substitution of Ala (*Pisum*) to Gly (*Arabidopsis*) in position 601. All peptides were synthesized by the Molecular Biology Resource Facility of the William K. Warren Medical Research Institute of the University of Oklahoma Health Sciences Center. Peptides were coupled to either Sulfolink™ (Pierce) or Affigel-10 (Biorad) according to the manufacturers' protocols. For Affigel-10 affinity chromatography, APs were dialyzed overnight, bound and eluted according to (Sosa et al., 1993) with the following modifications: no CHAPS was included in the dialysis/storage/wash buffer, and trypsin inhibitor was substituted for BSA in the column equilibration buffer in some experiments. Bound proteins were eluted by boiling the agarose beads in SDS-PAGE sample buffer (Singh, 1994) and loaded directly onto 12% agarose gels. Flow through and wash samples were precipitated with 12% trichloroacetic acid before solublization in the same buffer. Sulfolink™ columns were washed with 25mM Hepes, pH 7.0 containing 250 mM NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. Instead of dialysis, AP containing Superose-6 fractions were desalted on mini-G-25 columns constructed of microcentrifuge tubes and trypsin inhibitor (0.2 mg/ml) was added to samples before loading. Bound proteins were eluted sequentially with 0.5 M Tris-HCl, pH 7.0 containing 750 mM $MgCl_2$ followed by 0.1 M sodium carbonate, and flow through and bound proteins subjected to SDS-PAGE. Gels were either stained with Coomassie-G or subjected to western blot analysis. Column were stored in buffer A at 4°C.

Electrophoresis and Immunoblotting

All SDS-PAGE was conducted using 8-18% or 12% standard gels with an acrylamide:bis-acrylamide ratio of 37.5:1 (Laemmli 1970) and stained either with Coomassie Brilliant Blue-G (Neuhoff et al., 1988) or silver (Blum et al., 1987).

For immunoprobing, proteins were first electroblotted onto Millipore Immobilon-P transfer membrane at 100 mA overnight using buffer containing 25mM Tris, 192mM glycine and 20% methanol, pH 8.3 (Towbin et al., 1979). Immunoreactive bands were subsequently detected using the Amersham ECL system. For adaptor immunodetection the primary antibody used was the monoclonal antibody against the brain β1/β2 subunit (clone 100/1, (Ahle et al., 1988)) obtained from Sigma Immunochemicals (A-4450). Polyclonal antibodies against the μ- and σ-adaptor subunits of brain were the generous gift of Dr. M.S. Robinson (Page and Robinson, 1995). Since BP-80 was known to be a glycoprotein (Kirsch et al., 1994), antibodies specific for plant Asn-linked oligosaccharides were purified from rabbit anti-pea β-*N*-acetylhexosaminidase antiserum (Harley and Beevers, 1987) by chromatography on a pineapple stem bromelain-agarose affinity column (S. M. Harley and L. Beevers, unpublished data). Because antibodies against the polypeptide portions of these two enzymes do not crossreact, only antibodies specific for plant oligosaccharides were retained on the column and then eluted for use in these experiments.

RESULTS

Binding of Clathrin Coat Proteins to LDM

When coat proteins from pea cotyledon CCVs, containing primarily clathrin heavy chain (HC), light chains (LC) and adaptor proteins, were mixed with cytosol and the nucleotides GTP and ATP, separated on sucrose density gradients and analyzed by SDS-PAGE, a small amount of BP-80, identified by immunoblot of a replicate gel, can be found associated with the assembled clathrin and 110 kD adaptor protein in fractions between 42-51% sucrose (Fig. 1). In contrast, when purified LDM was separated on a parallel gradient without the addition of coat proteins or nucleotides, all the BP-80 was found banded between 25-30% sucrose (Fig. 2). The addition of coat proteins, cytosol and nucleotides to the same quantity of LDM prior to separation on a parallel gradient, however, resulted in a marked change in the location of some BP-80 within the gradient. While the majority of BP-80 is still found banded between 25-50% sucrose, quantities of BP-80, far in excess of that seen to associate with clathrin coat proteins centrifuged alone (see Fig. 1), were found associated with the coat proteins banded between 42-51% sucrose (Fig. 3). This migration of BP-80 to a higher concentration of sucrose suggests that BP-80 was incorporated into the reassembled clathrin coats.

Figure 1. Sucrose gradient fractionation of LDM from developing pea cotyledon. A, Electrophoretic analysis of gradient fractions on an 8-18% SDS-PAGE gel. (★), contaminating clathrin HC; (⊕), position of BP-80. B, Immunoblot of replicate gel probed with antibody against plant Asn-linked oligosaccharides (see Methods). ECL detection.

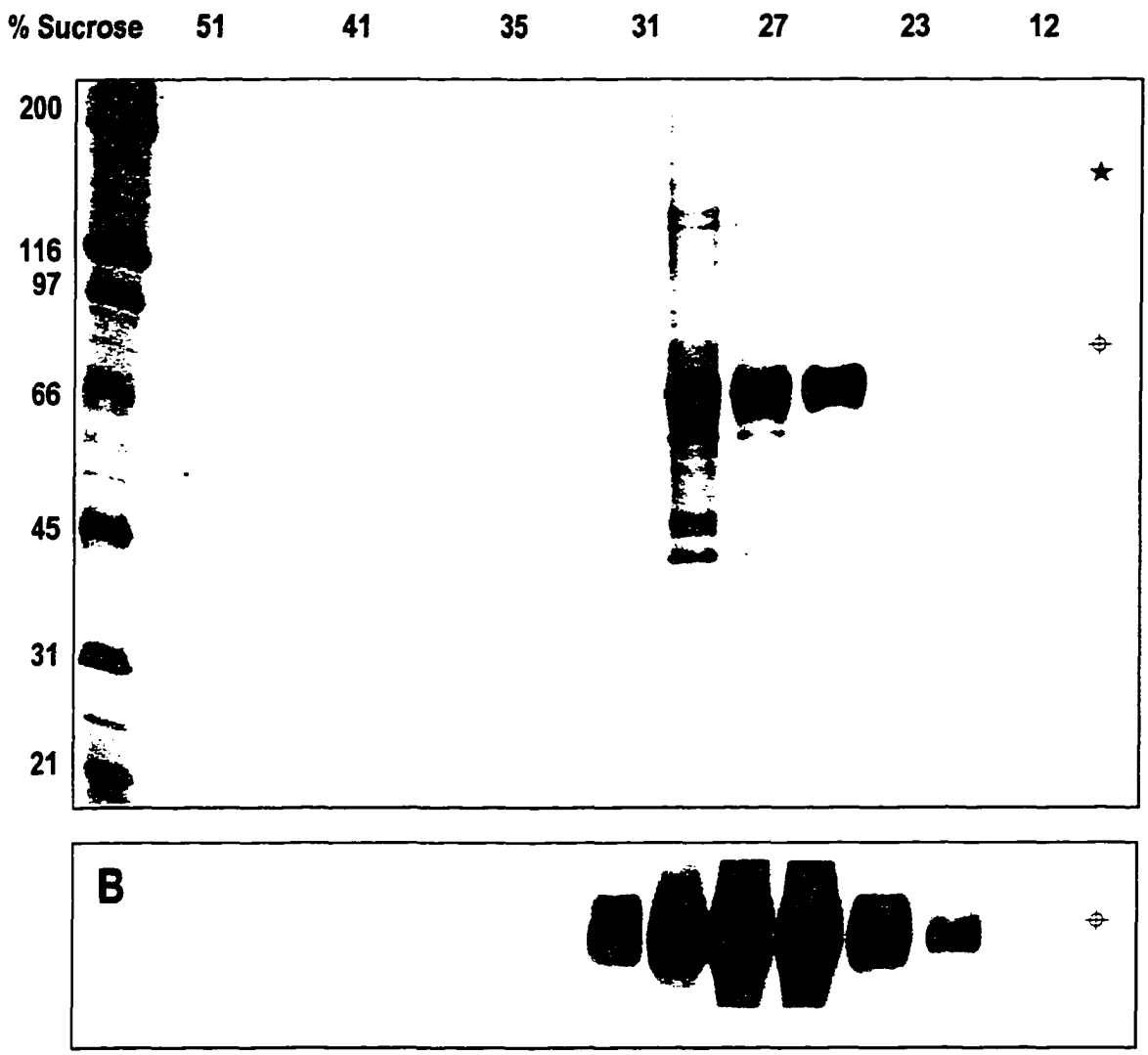
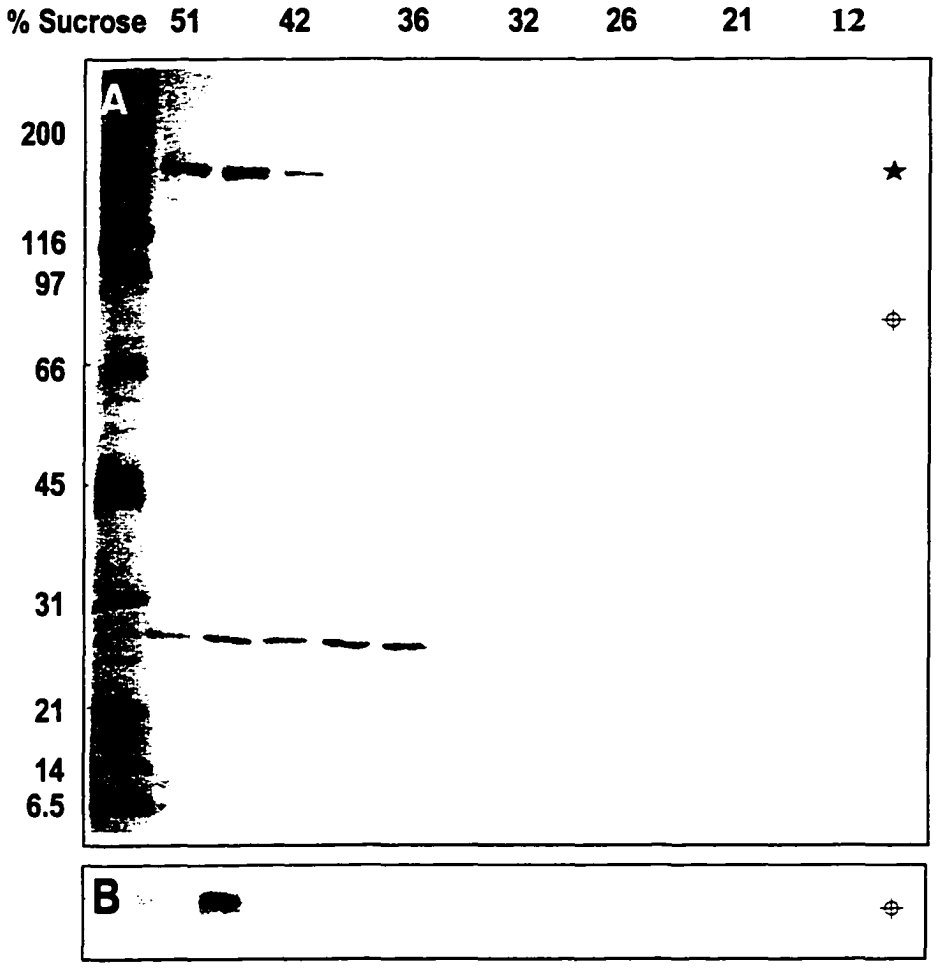

% Sucrose
51
41
35
31
27
23
12
200
116
97
66
45
31
21
B

Figure 2. Sucrose gradient fractionation of coat proteins dissociated from pea cotyledon CCVs with Tris-HCl after buffer exchange to assembly conditions. A, Electrophoretic analysis of gradient fractions on an 8-18% SDS-PAGE gel. (★), clathrin HC; (⊕), position of BP-80. B, Immunoblot of replicate gel probed with antibody against plant Asn-linked oligosaccharides (see methods). ECL detection.


% Sucrose 51 42 36 32 26 21 12
A
200
116
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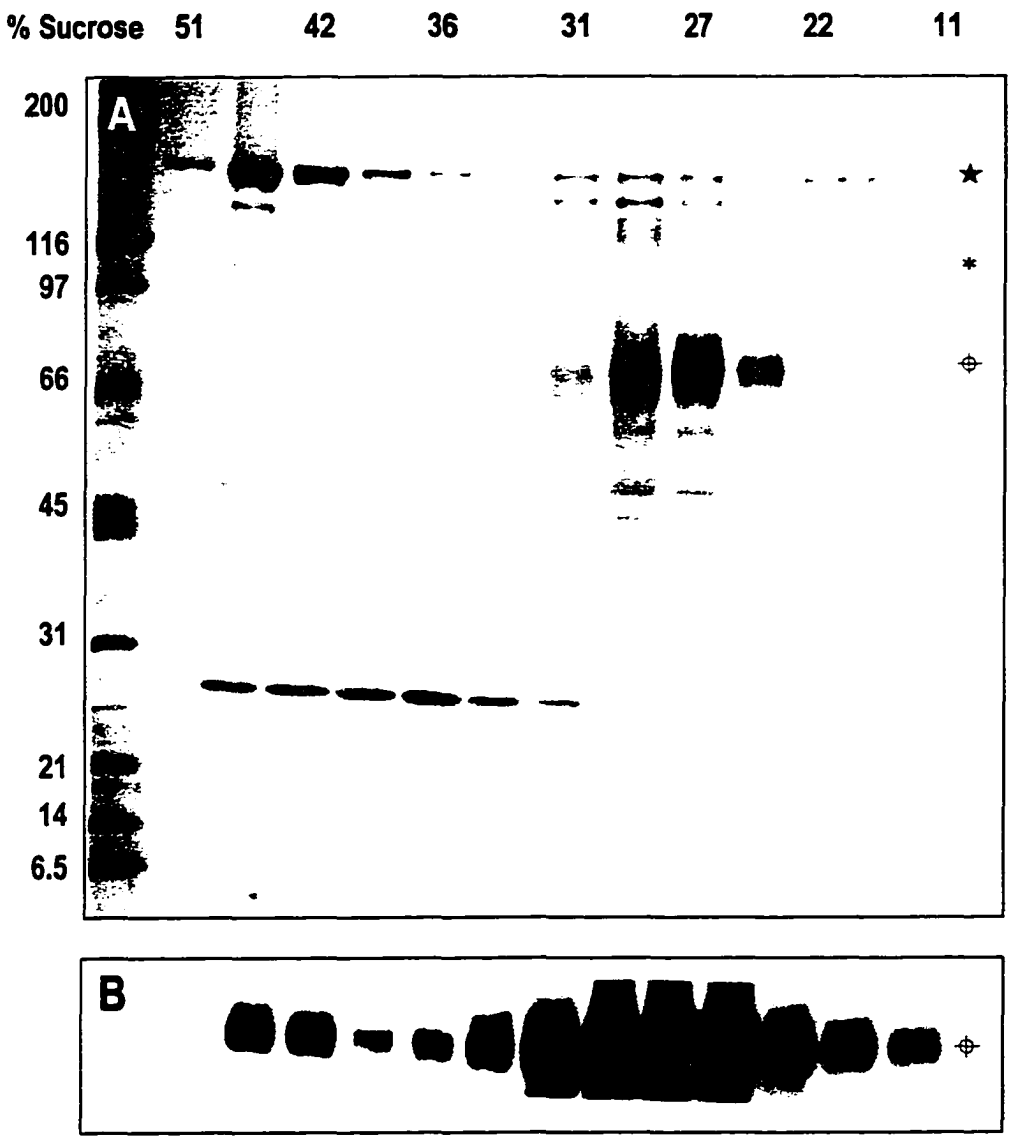
66
45
31
21
14
6.5
B

Figure 3. Sucrose gradient fractionation of LDM and Tris-HCl dissociated coat proteins from pea cotyledon CCVs associated after buffer exchange to assembly conditions. A, Electrophoretic analysis of gradient fractions on an 8-18% SDS-PAGE gel. (★), clathrin HC; (⊕), BP-80; (*), β-adaptin. B, Immunoblot of replicate gel probed with antibody against plant Asn-linked oligosaccharides (see methods). ECL detection.


% Sucrose 51 42 36 31 27 22 11
200
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45
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21
14
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A
B

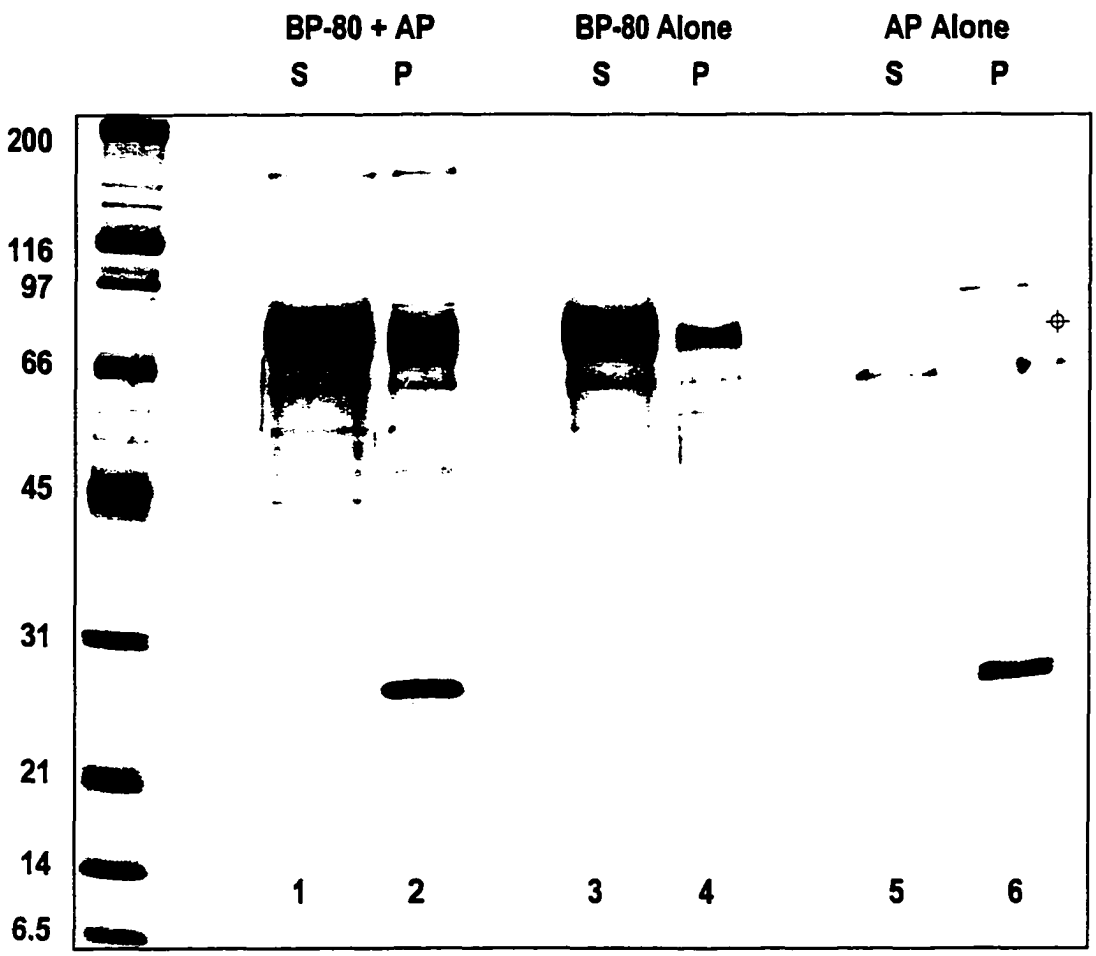
Assembly of BP-80 with Pea AP

We further investigated the interaction of pea cotyledon AP with BP-80 (purified as in methods) by overnight dialysis of purified proteins against the moderate ionic strength (buffer B) used in clathrin assembly experiments (Chapter 3). Dialysis was followed by ultracentrifugation to separate associated protein found in the pellet from the non-associated protein present in the supernatant. When BP-80 was dialyzed alone, most of the protein remained in the supernatant, while only a small amount of protein was observed in the pellet. In contrast, in the presence of Superose-6 purified pea adaptor protein, much more BP-80 protein was found in the pellet, suggesting that interaction between the two proteins had occurred (Fig. 4).

Binding of Plant Adaptor to the Carboxy-Terminus of BP-80

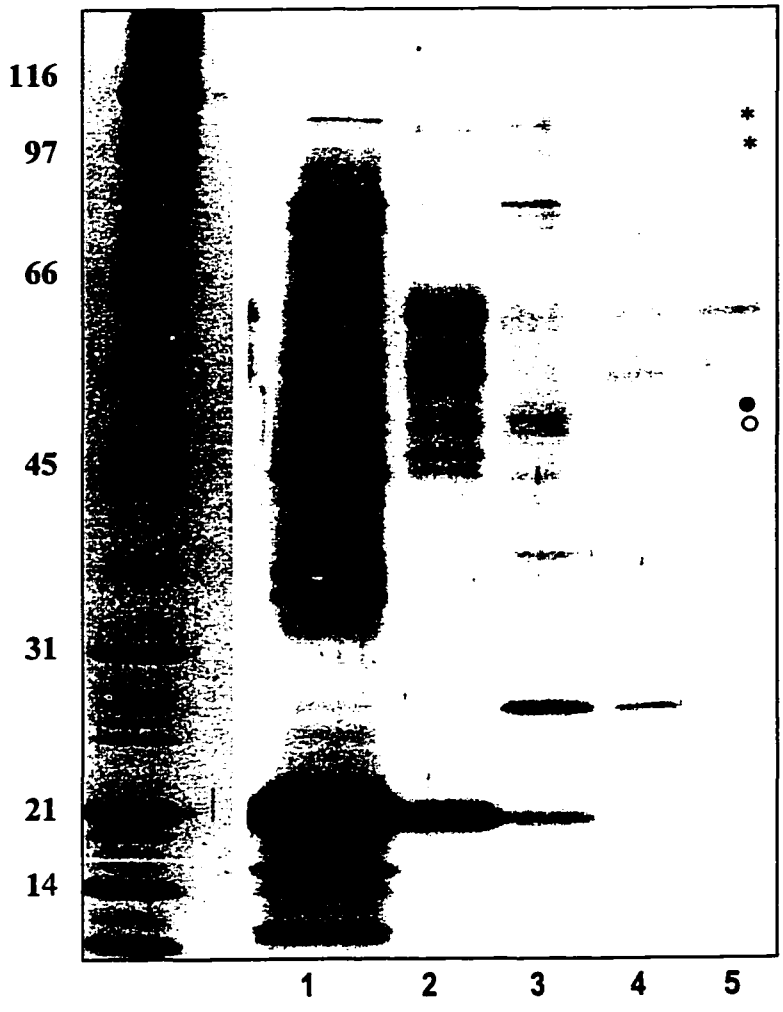
Having shown that LDM containing the vacuolar targeting receptor BP-80 associated with clathrin coat proteins from pea cotyledon, and that purified BP-80 associated with the adaptor protein component of these coat proteins, we sought to determine which portion of BP-80 was involved in the binding with the pea cotyledon adaptor. From analogy to mammalian receptors, and from N-terminal sequencing of BP-80 from control and proteolytically treated CCVs which established the transmembrane orientation of BP-80 (Kirsch et al., 1994), we reasoned that the cytoplasmic carboxy-terminus of the protein should contain the binding site for clathrin adaptor protein. To test this hypothesis, a synthetic peptide consisting of a

Figure 4. Interaction of purified pea cotyledon AP with BP-80. Purified proteins were dialyzed overnight against Buffer B, and subjected to high-speed ultracentrifugation to separate associated protein found in the pellet (P) from non-associated proteins present in the supernatant (S). Fifty μl of Superose-6 separated pea cotyledon AP (0.067 mg/ml) was dialyzed overnight with or without 50 μl of affinity purified BP-80 (0.06 mg/ml). All samples were adjusted to a final volume of 0.1 ml with buffer B prior to dialysis. Lanes 1-2, pea cotyledon AP with BP-80. Lanes 3-4, BP-80 alone. Lanes 6-7, Pea cotyledon AP alone. Silver staining.


BP-80 + AP
BP-80 Alone
AP Alone
S
P
S
P
S
P
200
116
97
66
45
31
21
14
6.5
1
2
3
4
5
6

an N-terminal Cys residue followed by the carboxy-terminal 37 amino acids of the *Arabidopsis* homolog of BP-80 was linked to Sulfolink™ affinity resin according to the manufacturer's protocol. This column was then used to assess the binding of Superose-6 purified pea cotyledon AP. When an aliquot of Superose-6 separated pea cotyledon AP, pooled and concentrated as in Methods, was applied to the C-terminal column and eluates subjected to SDS-PAGE, many polypeptides ranging in molecular mass from 110 to 7 kD were found in the flow through and wash fractions upon SDS-PAGE electrophoresis (Fig. 5, lanes 1-2). Upon elution of the column under conditions which dissociate adaptors from CCV membranes and clathrin (0.5M Tris-HCl, pH 7.0 containing 0.75M $MgCl_2$), prominent polypeptides of 110, 100, 80, 50, 47, 38 and 28 kD were found to have bound specifically (Fig 5, lane 3). The 110, 100, 50, and 47 kD polypeptides correspond in molecular mass to those of the large and medium subunits of the mammalian clathrin adaptors, and exactly with the molecular masses of polypeptides purified by affinity chromatography of pea cotyledon APs on Sulfolink™ resin coupled to a monoclonal antibody preparation against the mammalian β1/β2 large subunits (Chapter 2). Stripping the BP-80 C-terminal column with 0.1M sodium carbonate eluted only small residual amounts of the 80, 60, and 28 kD polypeptides (Fig. 5, lanes 4-5). Superose-6 separated AP proteins from CCVs isolated from wheat germ were also found to specifically bind to, and elute from the same column under identical conditions (data not presented).

Figure 5. SDS-PAGE analysis of the interaction of pea cotyledon AP with the wt peptide corresponding to the C-terminal tail of the *Arabidopsis* homolog of BP-80. 12% acrylamide gel, Coomassie-G staining. Wt peptide was bound to Sulfolink™ resin according to the manufacturers' protocol. 80 μg desalted AP, protected with trypsin inhibitor (0.2 mg/ml final concentration) was loaded on the column in a final volume of 1.0 ml and the column washed extensively, followed by sequential elution with Tris-HCl and sodium carbonate. On the left, molecular mass standards. Lane 1, Flow through/ first wash; lane 2, second wash; lane 3, Tris-HCl/$MgCl_2$ elution; lane 4-5, sodium carbonate elution. Positions of the ~100 kD adaptins (*); and the putative 50 kD (●) and 47 kD (○) adaptor μ-subunits are marked.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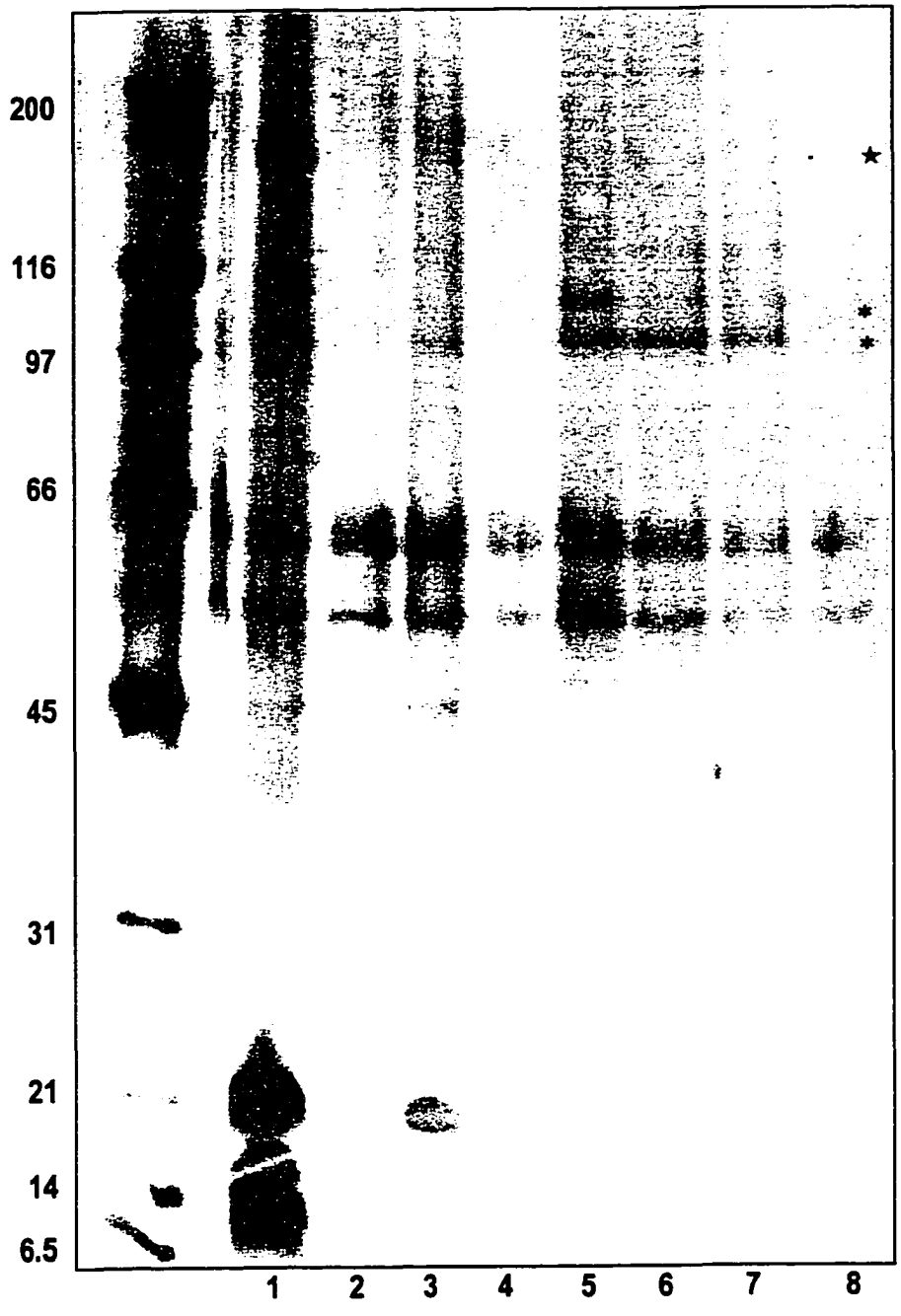
Thus, it appears that the C-terminal peptide of the Arabidopsis homolog of BP-80 does contain the adaptor binding sequence.

Binding of Brain AP to the C-Terminal Peptide of BP-80

Since it has been shown that both the AP1 and AP2 brain adaptor complexes bind to Tyr-based sorting motifs (Pearse, 1988; Glickman et al., 1989; Sosa et al., 1993; Ohno et al., 1995; Boll et al., 1996; Ohno et al., 1996) we sought to determine whether the Tyr-binding motif present in the BP-80 cytoplasmic domain would also interact with clathrin adaptor proteins from brain as it had with those from pea cotyledon and wheat germ. Two different affinity columns were used to assess the binding of a pooled brain adaptor fraction (similarly separated on Superose-6) to the peptide corresponding to the cytoplasmic portion of the *Arabidopsis* homolog of BP-80. SDS-PAGE analysis of column flow through and eluates showed that, like pea cotyledon and wheat germ AP, bovine brain AP bound specifically to, and was eluted from the Sulfolink™ column using Tris-HCL/$MgCl_2$ elution buffer (Fig. 6). To confirm the results of this experiment, a second peptide also corresponding to the C-terminal 37 amino acids of the *Arabidopsis* homolog of BP-80, minus the Cys-residue required for Sulfolink™ linkage, was constructed. This full-length, wild-type (wt) peptide was coupled to Affigel-10 (Bio-Rad) according to the manufacturer's protocol. The coupling procedure for Affigel-10 produces covalently-linked monomeric peptides with exposed carboxyl termini connected to the activated

Figure 6. SDS-PAGE analysis of the interaction of brain AP with the wt peptide corresponding to the C-terminal tail of the *Arabidopsis* homolog of BP-80. 12% acrylamide gel, Coomassie-G staining. Wt peptide was bound to Sulfolink™ resin according to the manufacturers' protocol. 80 μg desalted AP, protected with trypsin inhibitor (0.2 mg/ml final concentration) was loaded on the column in a final volume of 1.0 ml and the column washed extensively, followed by sequential elution with Tris-HCl and sodium carbonate. On the left, molecular mass standards. Lane 1, Flow through/ first wash; lane 2, blank lane; lane 3, second wash; lane 4, blank lane; lane 5, Tris-HCl/$MgCl_2$ elution; lane 6-7, sodium carbonate elution; lane 8, blank lane. Positions of ~100 kD adaptins (*); and residual clathrin HC (★) present in AP sample. Two artifactual bands are present in all lanes between 45 and 66 kD.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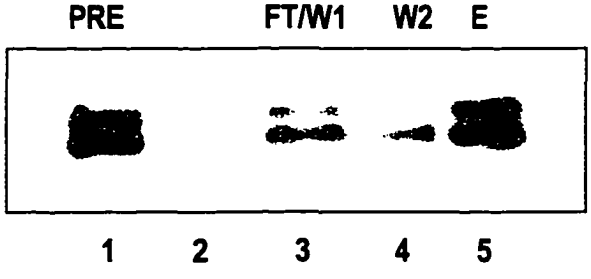
agarose matrix through a neutral 10-atom spacer arm. Brain AP was applied to this Affigel affinity column, eluates subjected to SDS-PAGE, western blots prepared and probed with the monoclonal antibody against the β1/β2 adaptor subunits. In similarity to the binding of brain AP to the Sulfolink™ C-terminal column, both the β-adaptins were found to bind specifically to the Affigel-10 affinity column, with smaller amounts found in the flow through and wash fractions (Fig. 7). Results of reprobing the blot with polyclonal antibodies against the brain medium subunits (μ1 and μ2) and small subunits (σ1 and σ2) were positive for all subunits, further confirming that both AP1 and AP2 complexes were retained on the column (data not presented). However, brain AP appears to bind more strongly to the C-terminal peptide immobilized on Affigel-10; after extensive washing, sucessful recovery of the bound proteins required heating the resin in electrophoresis sample buffer (Asai et al., 1996; Okabayashi et al., 1996).

Peptide Binding Specifity of Plant AP

Specificity of pea cotyledon and wheat germ adaptor binding to the cytoplasmic tail of BP-80 was investigated by competition with a modified peptide constructed of the Tyr-binding sequence flanked by the immediately N-terminal six amino acids found in the full-length, wild-type peptide. Adaptors were preincubated for a total of 2 hours (1.5 hours at 4°C and 30 min at 30°C) with and without a 20-fold excess (in comparison with the immobilized wt peptide) of the free peptide

Figure 7. Immunoprobe analysis of the interaction of brain AP with the wt peptide corresponding to the C-terminal tail of the *Arabidopsis* homolog of BP-80. The wt peptide was coupled to Affigel-10 according to the manufacturers' protocol. 50 μg Superose-6 separated AP from bovine brain APs, suitably protected against proteolysis, was dialyzed overnight, loaded on a 0.2 ml column containing the bound peptide. The column was washed extensively and eluted (see methods) followed by SDS-PAGE and blotting of column fractions. Immunoprobed with the antibody against the β1/β2 adaptin large subunits. ECL detection. Only that portion of the blot containing the immunoreactive bands is shown. Lane 1, 10 μg column load (Pre); lane 2, blank; lane 3, flow through and first wash; lane 4, second wash; lane 5, immunoreactive polypeptides eluted from column by boiling the resin in sample buffer (see methods).


PRE
FT/W1
W2
E
1
2
3
4
5

before being loaded to the wt column. For pea cotyledon AP, binding to the wt column was reduced by approximately half when the adaptor fraction was pre-incubated with the modified peptide. When wheat germ AP, also preincubated with the modified peptide, was loaded on the wt column the effect was similar to that observed for pea cotyledon AP. However, while some pea cotyledon AP was still retained on the column, preincubation of the wheat germ AP with the modified peptide resulted in no AP being bound (Fig. 8).

In addition to the competition experiments we sought to further investigate the specificity of binding of pea cotyledon AP to the Tyr-binding motif of BP-80 using modified peptides. The truncated peptide, and another peptide in which Ala was substituted for the Tyr residue, were immobilized on Affigel-10. For each column, most of the applied AP eluted in either the flow through and wash fractions (Fig. 9, lanes 2-3 and 6-7). Both peptides bound pea cotyledon AP, however, the amount of bound peptide was somewhat more on the Ala substituted column than on the column constructed with the shortened peptide (compare lanes 5 and 9).

DISSCUSSION

The affinity of BP-80, purified from CCVs and LDM of pea cotyledon, for the amino-terminal targeting sequence of barley (*Hordeum vulgare* L.) proaleurain and sweet potato (*Ipomoea batatas* (L.) Lam.) sporamin led to the identification of BP-80 as a probable vacuolar targeting receptor which is specifically transported in CCVs

Figure 8. Immunoprobe analysis of the interaction of pea and wheat germ AP with the wt peptide corresponding to the C-terminal tail of the *Arabidopsis* homolog of BP-80 in the absence (lanes 2-5) or presence (lanes 6-9) of free peptide corresponding to the Tyr-binding motif present in the C-terminal portion of BP-80 and its *Arabidopsis* homolog. The wt peptide was coupled to Affigel-10 according to the manufacturers' protocol. APs were incubated in the presence or absence of an excess of the truncated peptide prior to loading. 50 μg aliquots of Superose-6 separated AP from pea cotyledon and wheat germ APs, suitably protected against proteolysis, were dialyzed overnight and loaded on 0.2 ml columns. Columns were washed extensively, eluted with boiling sample buffer and column fractions subjected to SDS-PAGE and blotting. Immunoprobed with the antibody against the β1/β2 adaptin large subunits. ECL detection. Only that portion of the blot containing the immunoreactive bands is shown. Analysis of binding of pea cotyledon, upper portion; wheat germ lower portion. Lane 1, column load; lanes 2 and 6, flow through; lanes 3 and 7, first wash ; lanes 4 and 8, second wash; lanes 5 and 9, elution.

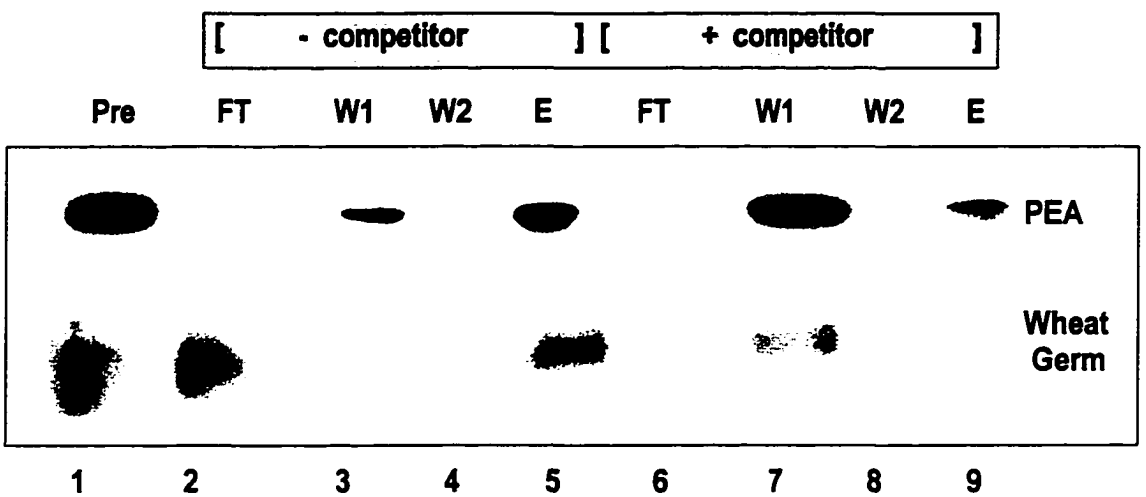
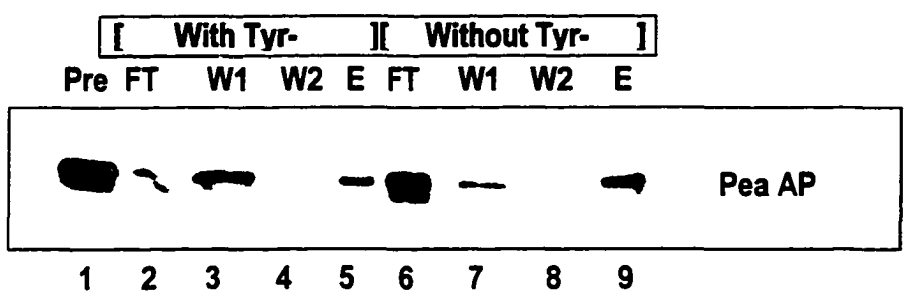

[- competitor] [+ competitor]
Pre
FT
W1
W2
E
FT
W1
W2
E
PEA
Wheat Germ
1
2
3
4
5
6
7
8
9

Figure 9. Immunoprobe analysis of the interaction of pea cotyledon AP with the truncated peptide corresponding to the Tyr-binding motif present in the C-terminal portion of BP-80 and its *Arabidopsis* homolog and another peptide with an Ala substitution for the Tyr in the binding motif. Lanes 2-5, fractions from peptide column containing Tyr; lanes 6-9, fractions from peptide column containing the Ala substitution. Both peptides were coupled to Affigel-10 according to the manufacturers' protocol. 50 μg aliquots of Superose-6 separated AP from pea cotyledon APs, suitably protected against proteolysis, were dialyzed overnight and loaded on 0.2 ml columns. Columns were washed extensively, eluted with boiling sample buffer and column fractions subjected to SDS-PAGE and blotting. Immunoprobed with the antibody against the β1/β2 adaptin large subunits. ECL detection. Only that portion of the blot containing the immunoreactive bands is shown. Lane 1, column load; lanes 2 and 6, flow through; lanes 3 and 7, first wash; lanes 4 and 8, second wash; lanes 5 and 9, elution.


[With Tyr-][Without Tyr-]
Pre FT W1 W2 E FT W1 W2 E
Pea AP
1 2 3 4 5 6 7 8 9

(Kirsch et al., 1994; Kirsch et al., 1996; Paris et al., 1997). Purification of adaptor proteins from both pea cotyledon and wheat germ AP has provided additional tools to investigate the role of BP-80 in CCV-mediated protein targeting in plants.

Clathrin coat proteins of developing pea, removed from the vesicle membrane by chaotropic agents such as Tris-HCL reassemble into recognizable coats when dialyzed or exchanged into low or moderate ionic strength buffer (Chapter 3, and references within). Using an assay similar to that which first demonstrated the interaction between the mannose-6 phosphate receptor and clathrin adaptors in mammals (Pearse, 1985), BP-80, present in LDM, has been shown to be incorporated into newly assembled clathrin coats when exchanged into a moderate ionic strength buffer in the presence of cytosol, GTP and ATP. In addition, we have shown that purified pea cotyledon AP binds purified BP-80. These results suggest that the incorporation of BP-80 into newly formed clathrin coated vesicles is a result of binding between APs and receptor. This provides additional support for the role of BP-80 as a vacuolar targeting receptor and the first demonstration of an "adaptor" function for the AP proteins of plant CCVs. It appears, comparing the degree of BP-80/AP interaction observed between the purified proteins to the degree of LDM-coat protein interaction in the assembly experiments, that the inclusion of cytosol and nucleotides did not stimulate additional assembly of AP onto LDM (compare the amount of BP-80 found in Fig.3 between 42 and 51% sucrose, and Fig. 4, lane 2). This is consistent with earlier studies of AP binding to Golgi membranes in

mammalian cells which demonstrated only a slight increase in binding of γ-adaptin to rat liver Golgi membranes on addition of ATP, GTP and an ATP-regeneration system (Stamnes and Rothman, 1993). In that study, significant stimulation of binding required the addition of purified ARF (ADP-Ribosylation Factor) and GTPγS. It is possible that our crude cytosol contained insufficient as-yet unidentified cellular factors to stimulate significant binding. However, because the amount of BP-80 in these experiments exceeded the amount of AP provided, it is also possible that our results may reflect the saturation of available adaptor binding sites rather than a failure of ATP, GTP and cytosol to stimulate assembly. From our data, however, we cannot exclude the possibility that ATP, GTP or cellular factors present in cytosol are required for regulated adaptor-receptor assembly *in vivo*. While not providing quantifiable evidence, these experiments did serve to demonstrate interaction between BP-80 and plant adaptors on a protein-to-protein basis and showed that both components were purified in a binding competent form.

Having localized BP-80 to newly formed coats, and having demonstrated interaction between the purified adaptor and receptor, it was appropriate to identify the location of the sequence(s) responsible for adaptor binding within BP-80. We have determined that Superose-6 separated adaptor proteins from pea cotyledon and wheat germ bind specifically to, and are eluted from an affinity matrix containing immmobilized peptides corresponding to the entire 37 amino acid C-terminus of the *Arabidopsis* homolog of BP-80 (the wt peptide). These experiments suggest that

interaction of adaptor and receptor *in vitro* is dependent on signals present in the cytoplasmic tail of BP-80 and its homologs.

Strikingly, a mixed adaptor preparation from bovine brain, in addition to those of pea and wheat, bound the immobilized C-terminal tail of the *Arabidopsis* homolog of BP-80. SDS-PAGE analysis of flow through and bound Sulfolink™ affinity column fractions showed that both β1 and β2 adaptin subunits were present in the eluate. Affigel-10 affinity chromatography and immunoblotting confirmed not only the binding of the β1 and β2 adaptins, but further demonstrated that all the brain adaptor medium and small subunits were present, suggesting that intact mammalian clathrin adaptor complexes were involved in the interaction. In similarity to our results, peptides corresponding the the targeting motif of TGN38, lamp-1, CD68, the transferrin receptor and CD3-γ (Ohno et al., 1995) and the cation-independent mannose-6 phosphate receptor (Glickman et al., 1989) have also been shown to bind both AP1 and AP2. The binding of AP1 and AP2 to both mammalian and plant receptors suggest a universality in the mechanisms for protein sorting and targeting in the endomembrane systems of all eukaryotes.

Pioneering studies demonstrated the binding of clathrin adaptors to transmembrane receptors bearing Tyr-based sorting signals in their cytoplasmic tails (Pierce, 1985; Pearse, 1988; Glickman et al., 1989 and others) and recent studies have implicated the μ-subunits of clathrin adaptors in this interaction. Using the yeast two-hybrid approach, a triple repeat of the Tyr-based sorting signal of the *trans*-Golgi

integral membrane protein (TGN38) was used to screen a mouse spleen cDNA library for interactive clones (Ohno et al., 1995). From this screening only two reactive clones (out of ~2.5 x 10^6) were identified, and found to correspond to the μ2 subunit of AP2. In addition, two different combinatorial selection methods were used to show that a tyrosine-containing tetrapeptide sequence of the Y-polar-polar-hydrophobic type (YppØ) was both necessary and sufficient for interaction with μ2 (Boll et al., 1996). Interestingly, for highest affinity binding of μ2 the preferred hydrophobic residue in the Y + 3 position was shown to be Lys (Boll et al., 1996). The binding of brain AP to the wt C-terminal column used in our study of plant adaptor/receptor interaction may reflect this preference for Lys, since the Tyr-based sorting signal of BP-80 and its *Arabidopsis* homolog also contain Lys in the Y + 3 position (Paris et al., 1996).

Because the recognition of Tyr-based sorting signals in mammals appears to involve the adaptor μ-subunits, and both AP1 and AP2 of bovine brain bind to the C-terminal peptide of the *Arabidopsis* homolog of BP-80, μ-subunits may also be involved in the recognition of Tyr-based sorting signals in plants. Support for this hypothesis comes from our data demonstrating that polypeptides of 47 and 50 kD, as well as of 110 and 100 kD, approximately the same molecular mass as the mammalian adaptins and μ-subunits, present in Superose-6 separated pea cotyledon AP preparations, bind to and elute from the C-terminal wt column. The binding of polypeptides of 47 and 50 kD (as well as 100 and 110 kD) present in Superose-6

separated pea cotyledon AP preparations to a Sulfolink affinity matrix containing immobilized anti- bovine brain β1/β2-subunit antibodies (Chapter 2), further supports the identification of these polypeptides as potential μ-subunits.

Preincubation of APs with an excess of free peptide corresponding to the tyrosine-binding motif present BP-80 and its *Arabidopsis* homolog (the truncated peptide) inhibited binding of pea and wheat germ APs to the wt peptide affinity column, suggesting that AP/receptor interaction *in vitro* is dependent on the Tyr-sorting motif as was shown for lysosomal acid phosphatase (LAP)(Sosa et al., 1993). Substitution of Ala for the Tyr in the binding motif of LAP reduced the amount of HA-2 adaptors bound from 98 to 25% (Sosa et al., 1993), and a complete elmination of binding of μ2 was observed with an Ala substitution for Tyr in the binding motif of TGN38 (Ohno et al. 1995). In contrast, the substitution of Ala for Tyr in the truncated peptide did not result in a reduction of the amount of AP bound. While surprising, our results are potentially interpretable in light of two recent studies. In addition to actual sequence specifics, the degree of interaction of Tyr-based sorting motifs with the adaptor μ-subunits has been shown in mammalian systems to be dependent on the position of the motif in the cytoplasmic tail of the receptor (Ohno et al., 1996). Signals presented at the absolute carboxy-terminus (as both our truncated and Ala modified peptide) were shown to bind with higher avidity than signals presented in the midst of a longer sequence. Furthermore, for internally presenting sorting motifs the number of residues between the C-terminus and the motif also

affect the avidity of binding. (Ohno et al., 1995). Since the Tyr-based sorting motif in *Arabidopsis* and pea are both internal to the C-terminus by 14 and 16 residues respectively, the increased binding of pea cotyledon AP to the modified peptide may primarily reflect the terminal position of the binding motif in this peptide. Most recently, the 46 kD mannose-6 phosphate receptor has been shown to contain not one, but multiple high-affinity binding sites for both AP1 and AP2 (Höning et al., 1997). Interestingly, none of the binding sites include the Tyr-binding motif, suggesting that uncharacterized binding motifs not dependent on Tyr may be present in other receptors. While to date no other AP-binding motifs have been identified in BP-80 and its homologs, we have observed Pea cotyledon AP binding to peptide sequences lacking Tyr-binding motifs (data not presented). Additional studies will be required to determine if non Tyr-based signals, shown to be present in the 46 kD mannose-6 phosphate receptor, are also present in plant targeting receptors. If present, their role in vacuolar targeting will need to be established.

While preliminary, this study represents the first demonstration of interaction between clathrin assembly proteins and targeting receptors in plants. Many more questions, however, remain to be addressed. Primary among these is the need to further examine the interaction using more quantifiable techniques. Recently, the interaction of mammalian APs with receptors has been examined using surface plasmon resonance (SPR) spectroscopy (Heilker et al., 1996; Höning et al., 1996; Höning et al., 1997). Since SPR allows both the binding and dissociation kinetics of

interactions to be readily quantified in real time, evaluation of short term binding is simplified. Furthermore, the stoichiometry of binding can be easily determined because the technique allows quantification of the density of peptide binding to the sensor. Use of SPR spectroscopy would allow a much more thorough examination of the intraction of between plant adaptors and targeting receptors. Now that a full length sequence of BP-80 is available, additional peptides can be generated from the BP-80 (rather than the *Arabidopsis)* sequence, allowing a more complete understanding of the adaptor/receptor interactions in pea cotyledon CCVs.

Although early ultrastructural studies demonstrated the occurrence of CCVs in plant cells, their function(s) are not well established (Beevers, 1996; Robinson, 1996). This report of the interaction of plant clathrin adaptor proteins with the vacuolar targeting receptor BP-80 and its homologs provides a mechanistic model for the selective retrieval of vacuolar proteins or their precursors from the *trans*-Golgi and their sequestration into CCVs. These finding are consistent with the proposed involvement of CCVs in transport of vacuolar proteins in the plant secretory system (Harley and Beevers, 1989). This mechanism of retrieval is similar to that observed in the recruitment of soluble lysosomal proteins in mammalian systems. It is noteworthy the the Tyr-based sorting motifs involved in receptor/adaptor interaction in plants are similar to those encountered in the better characterized mammalian system. Thus, the chemical basis for the mechanistic model appears to have been conserved during the evolutionary divergence of plants and mammals.

REFERENCES

Ahle S, Mann A, Eichelsbacher U, Ungewickell E (1988) Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. EMBO J **7:** 919-929

Asai N, Murakami H, Iwashita T, Takahashi M (1996) A mutation at tyrosine 1062 in MEN2A-Ret and MEN2B-Ret impairs their transforming activity and association with Shc adaptor proteins. J Biol Chem **271:** 17644-17649

Beevers L (1996) Clathrin-coated vesicles in plants. Int Rev Cytol **167:** 1-35

Blackbourn HD, Jackson AP (1996) Plant clathrin heavy chain: sequence analysis and restricted localization in growing pollen tubes. J Cell Sci **109:** 777-787

Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant - proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8:** 93-99

Boll W, Gallusser A, Kirchhausen T (1995) Role of the regulatory domain of the EGF-receptor cytoplasmic tail in selective binding of the clathrin-associated complex AP-2. Curr Biol **5:** 1168-1178

Boll W, Ohno H, Songyang Z, Rapoport I, Cantley LC, Bonificino JS, Kirchhausen T (1996) Sequence requirements for the recognition of tyrosine-based endocytic signal by clathrin AP-2 complexes. EMBO J **15:** 5789-5795

Butler JM, Beevers L (1993) Putative adaptor proteins of clathrin coated vesicles from developing pea. *In* DJ Morré, KE Howell, JJM Bergeron, eds. Molecular Mechanisms of Membrane Traffic, Vol. **H 74**. Springer-Verlag, Berlin Heidelberg, pp331-332

Campbell C, Squicciarini J, Shia M, Pilch PF, Fine RE (1984) Identification of a protein kinase as an intrinsic component of rat liver coated vesicles. Biochemistry **23:** 4420-4426

Chrispeels MJ, Raikel NV (1992) Short peptide domains target proteins to plant vacuoles. Cell **68:** 613-616

Demmer A, Holstein SEH, Hinz G, Schauermann G (1993) Improved coated vesicle isolation allows better characterization of clathrin polypeptides. J Exp Bot **44:** 23-33

Gal S, Raikhel NV (1993) Protein sorting in the endomembrane system of plant cells. Curr Op Cell Biol **5:** 636-640

Gaudreault P-R, Beevers L (1984) Protein bodies and vacuoles as lysosomes. Investigations into the role of mannose-6-phosphate in intracellular transport of glycosidases in pea cotyledons. Plant Physiol **76:** 228-232

Glickman JN, Conibear E, Pearse BMF (1989) Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth factor II receptor. EMBO J **8:** 1041-1047

Harley SM, Beevers L (1987) Isozymes of β-N-acetylhexosaminidase from pea seeds (*Pisum sativum* L.) Plant Physiol **85:** 1118-1122

Harley SM, Beevers L (1989) Coated vesicles are involved in the transport of storage proteins during seed development in *Pisum sativum* L. Plant Physiol **91:** 674-678

Heilker R, Manning-Krief U, Zuber J-F, Spiess M (1996) *In vitro* binding of clathrin adaptors to sorting signals corelates with endocytosis and basolateral sorting. EMBO J **15:** 2893

Holstein SEH, Drucker M, Robinson DG (1994) Identification of a β-type adaptin in plant clathrin-coated vesicles. J Cell Sci **107:** 945-953

Holwerda BC, Rogers JC (1992) Purification and characterization of aleurain: a plant thiol protease functionally homologous to mammalian cathepsin H. Plant Physiol **99:** 848-855

Höning S, Griffith J, Geuze HJ, Hunziker W (1996) The tyrosine-based lysosomal targeting signal in lamp-1 mediates sorting into Golgi-derived clathrin-coated vesicles. EMBO J **15:** 5230-5239

Höning S, Sosa M, Hille-Rehfeld A, von Figura K (1997) The 46-kDa mannose 6-phosphate receptor contains multiple binding site for clathrin adaptors. J Biol Chem **272:** 19884-19890

Kirsch T, Paris N, Butler JM, Beevers L, Rogers JC (1994) Purification and initial characterization of a plant vacuolar targeting receptor. Proc Nat Acad Sci USA **91:** 3403-3407

Kirsch T, Saalbach G, Raikel N, Beevers L (1996) Interaction of a potential vacuolar targeting receptor with amino- and carboxyl-terminal targeting determinants. Plant Physiol **111:** 469-474

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:** 680-685

Lin H, Harley SM, Butler JM, Beevers L (1992) Multiplicity of clathrin light-chain-like polypeptides from developing pea (Pisum sativum L.) cotyledons. J Cell Sci **103:** 1127-1137

Lindner R, Ungewickell E (1992) Clathrin-associated proteins of bovine brain coated vesicles. An analysis of their number and assembly-promoting activity. J Biol Chem **267:** 16567-16573

Maldonado-Mendoza IE, Nessler CL (1996) Cloning and expression of a plant homologue of the small subunit of the Golgi-associated clathrin assembly protein AP19 from *Camptotheca acuminata.* Plant Mol Biol **32:** 1149-1153

Marty-Mazars D, Clémencet M-C, Cozolme P, Marty F (1995) Antibodies to the tonoplast form the storage parenchyma cells of beetroot recognize a major intrinsic protein related to TIPs. Eur J Cell Biol **66:** 106-118

Matsuoka K, Nakamura K (1991) Propeptide of a precursor to a plant vacuolar protein required for vacuolar targeting. Proc Nat Acad Sci USA **88:** 834-838

Nakamura K, Matsuoka K (1993) Protein targeting to the vacuole in plant cells. Plant Physiol **101:** 1-6

Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis **9:** 255-262

Ohno H, Fournier M-C, Poy G, Bonifacino JS (1996) Structural determinants of interaction of tyrosine-based sorting signals with the adaptor medium chain. J Biol Chem **271:** 29009-29015

Ohno H, Stewart J, Fournier M-C, Bosshart H, Rhee I, Miyatake S, Saito T, Gallusser A, Kirchhausen T, Bonifacino JS (1995) Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science **269:** 1872-1875

Okabayashi Y, Sugimoto Y, Totty NF, Hsuon J, Kido Y, Sakaguchi K, Gout L, Waterfield MD, Kasuga M (1996) Interaction of Shc with adaptor protein adaptins. J Biol Chem **271:** 5265-5269

Page LJ, Robinson MS (1995) Targeting signals and subunit interactions in coated vesicle adaptor complexes. J Cell Biol **131:** 619-630

Paris N, Rogers SW, Jiang L, Kirsch T, Beevers L, Phillips TE, Rogers JC (1997) Molecular cloning and further characterization of a probable plant vacuolar sorting receptor. Plant Physiol **115:** 29-39

Paris N, Stanley CM, Jones RL, Rogers JC (1996) Plant cells contain two functionally distinct vacuolar compartments. Cell **85:** 563-572

Pearse BMF (1985) Assembly of the mannose-6-phosphate receptor into reconstituted clathrin coats. EMBO J **4:** 2457-2460

Pearse BMF (1988) Receptors compete for adaptors found in plasma membrane coated pits. EMBO J **7:** 3331-3336

Pfeffer RS, Rothman JE (1987) Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. Annu Rev Biochem **56:** 829-852

Robinson DG (1996) Clathrin-mediated trafficking. Trends Plant Sci **1:**349-355

Robinson MS (1997) Coats and vesicle budding. Trends Cell Biol **7:** 99-102

Rothman JE, Wieland FT (1996) Protein sorting by transport vesicles. Science **272:** 227-234

Saalbach G, Rosso M, Schumann U (1996) The vacuolar targeting signal of the 2S albumin from Brazil nut resides at the C terminus and involves the C-terminal propeptide as an essential element. Plant Physiol **112:** 975-985

Samuels AL, Giddings TH Jr, Staehelin LA (1995) Cytokinesis in tobacco BY-2 and root tip cells: a new model of cell plate formation in higher plants. J Cell Biol **130:** 1345-1357

Schmid SL (1997) Clathrin-coated vesicle formation and protein sorting: an integrated process. Annu Rev Biochem **66:** 511-548

Singh R (1994) Odorless SDS-PAGE of proteins using sodium 2-mercapto-ethanesulfonate. BioTechniques **17:** 263-265

Sosa M, Schmidt B, von Figura K, Hille-Rehfeld A (1993) In vitro binding of plasma membrane-coated vesicles adaptors to the cytoplasmic domain of lysosomal acid phosphatase. J Biol Chem **268:** 12537-12543

Stamnes MA, Rothman JE (1993) The binding of AP-1 clathrin adaptor particles to golgi membranes requires ADP-ribosylation factor, a small GTP-binding protein. Cell **73:** 999-1005

Tanchak MA, Griffing LR, Mersey BG, Fowke LC (1884) Endocytosis of cationized ferritin by coated vesicles of soybean protoplasts. Planta **162:** 481-486

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA **76:** 4350-4354

Chapter V

SUMMARY

1. Based on presence among dissociated coat proteins, molecular sizing chromatography, immunoreactivity and probable subunit composition, we have identified adaptor proteins from CCVs isolated from developing pea cotyledons.

2. Polypeptides of 110, 100, 50 and 47 kD, similar in molecular mass to adaptor proteins of mammals, co-elute on molecular sizing chromatography at ~295 kD, suggesting that plant adaptors, like mammalian adaptors, exist as multimeric complexes.

3. In similarity to the adaptor complexes of bovine brain, plant adaptor complexes visualized by freeze-etch electron microscopy appear as large globular proteins of ~13 nm in diameter.

4. The 110 kD polypeptide is identified as a β-type adaptin by immunoreaction against two different monoclonal antibodies against the mammalian β1/β2 adaptin.

5. The 100 kD polypeptide, based on its coelution with the 110 kD adaptin on molecular sizing chromatography, hydroxylapatite chromatography and immunoaffinity chroma-tography, is considered a candidate for α/γ adaptin subunit.

6. Another polypeptide of similar size to a yeast adaptor protein, which coelutes with the 110 and 100 kD polypeptides on molecular sizing chromatography, and slightly before on hydroxylapatite chromatography, is considered a candidate for another adaptor large subunit.

7. μ-adaptin candidates, polypeptides of 47 and 50 kD, are bound to and elute from a Sulfolink™ affinity matrix coupled to antibodies against the mammalian β1/β2 adaptins along with peptides of 110 and 110 kD. The existence of these two μ-adaptin candidates, plus the identification of the 80 kD polypeptide, provide evidence for two adaptor complexes.

8. Identification of pea cotyledon APs as the AP1 (*trans*-Golgi) type is supported by their failure, in similarity to brain AP1, to bind CNBR-activated clathrin Sepharose and by the knowledge that pea cotyledon CCVs carry vacuolar hydrolases (as brain AP1 complexes carry lysosomal hydrolases).

9. Tris-HCl dissociated total pea CCV coat proteins reassemble into polyhedral structures incorporating the immunoreactive β-type adaptin.

10. Pea clathrin reassembles into polyhedric coats without the addition of pea AP, in sharp contrast to brain clathrin which exhibits little or no self assembly.

11. Pea cotyledon APs are incorporated into, and exert control over the size of reassembled pea clathrin coats. However, pea APs do not stimulate the degree of reassembly over that observed for pea clathrin alone. We conclude, therefore, that pea cotyledon APs are not assembly proteins.

12. Further evidence for identification of pea cotyledon AP as of the AP1 (*trans*-Golgi) type is found in the failure of pea cotyledon AP to stimulate coat reassembly of either pea or brain clathrin.

13. Ca^{++}, pH, and clathrin concentration have similar effects on reassembly of both pea and brain clathrin.

14. AP from bovine brain markedly stimulates assembly of pea clathrin coats. The brain β-type adaptins are incorporated into these coats.

15. While no stimulation of brain clathrin assembly by pea APs is observed, pea AP does bind to brain clathrin cages preformed by Ca^{++}, indicating some degree of sequence conservation between pea and brain APs.

16. BP-80, present in LDM, is incorporated into newly assembled clathrin coats, and purified pea AP binds purified BP-80, suggesting that binding occurs between pea cotyledon adaptors and receptors on a protein-to-protein basis.

17. Binding of pea cotyledon AP and receptor demonstrates an "adaptor" function for pea AP and confirms the identification of BP-80 as a vacuolar targeting receptor.

18. APs of pea cotyledon and wheat germ bind specifically to an immobilized peptide corresponding to the entire 37 amino acid C-terminus of the *Arabidopsis* homolog of BP-80 suggesting that the interaction between adaptor and receptor is dependent on signal(s) present in the C-terminal tails of BP-80 and its homologs.

19. A universality in the mechanisms of protein sorting and targeting in the endomembrane systems of plants and mammals is suggested by the binding of both the brain β1/β2 adaptins to the C-terminus of the *Arabidopsis* homolog of BP-80. This binding may reflect the preference of the mammalian AP2 μ2-subunit for the presence of Lys in the Y + 3 position in Tyr-based sorting signals present in mammals and in BP-80 and its homologs.

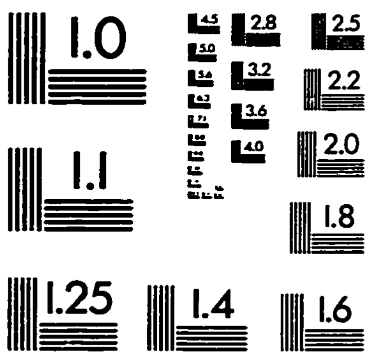
20. The binding of brain APs (known to involve μ-subunit interaction with Tyr-based sorting signals in mammals) to the C-terminus of the *Arabidopsis* homolog of BP-80

suggests that plant APs μ-subunits are also involved in receptor binding. This is supported by the binding of pea APs, shown, by electrophoretic analysis, to include μ-subunits, to the peptide corresponding to the C-terminus of the *Arabidopsis* homolog of BP-80.

21. Competition experiments using a truncated peptide containing the Tyr-based sorting signal of BP-80 suggest that AP/receptor interaction is dependent on the Tyr-based sorting motif present in the C-terminus of the *Arabidopsis* homolog of BP-80.

22. Substitution of Ala for Tyr in the truncated peptide did not result in a reduction in the amount of pea AP bound, indicating that binding of pea AP may be sensitive to the position of the sorting signal, and/or that other non-Tyr binding sites are also responsible for adaptor/receptor interactions in plants as is known for the mammalian 46 kD mannose-6 phosphate receptor.

IMAGE EVALUATION TEST TARGET (QA-3)



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