CHARACTERISATION OF THE AP-3 ADAPTOR-LIKE COMPLEX

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Declaration

I hereby declare that this thesis, entitled "Characterisation of the AP-3 adaptor-like complex" is the result of my own work, and no part of this thesis has been submitted for any other degree or diploma at the University of Cambridge.

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Abbreviations

ARF	ADP ribosylation factor
ATP	Adenosine 5'-triphosphate
BFA	Brefeldin A
BSA	Bovine serum albumin
CPY	Carboxypeptidase Y
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediamine-tetraacetic acid
EGTA	Ethyleneglycol-bis(β-aminoethylether)N,N,N',N'tetraacetic acid
ER	Endoplasmic reticulum
EST	Expressed sequence tag
GDP	Guanosine 5'-diphosphate
GST	Glutathione-S-transferase
GTP	Guanosine 5'-triphosphate
GTPγS	Guanosine 5'-O-(3-thiotriphosphate)
IgG	Immunoglobulin class G
LDL	Low density lipoprotein
M6P	Mannose-6-phosphate
MDBK	Madin-darby bovine kidney cell
NRK	Normal rat kidney
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
SDS	Sodium dodecylsulphate
SNAP	Soluble NSF attachment protein
SNARE	SNAP receptor
TGN	Trans-Golgi Network
TPA	Teradecanoyl phorbol acetate
Tris	Tris[Hydroxymethyl]aminomethane
Xgal	5-Bromo-4-chloro-3-indoly-β-D-galactopyranoside

Summary

Clathrin coated vesicles were the first type of coated vesicle to be characterised. The coat consists of two components, clathrin and adaptor (or AP) complexes, the AP-1 complex is associated with the clathrin coated vesicles that bud from the TGN and the AP-2 complex is associated with the clathrin coated vesicles that bud from the plasma membrane.

A new type of adaptor-like complex was discovered in our laboratory and was published in 1996. The complex has been shown to consist of two known proteins, β3B and μ3B, and two unknown proteins of 160kD and 22kD. Unlike the conventional adaptor complexes this complex is not associated with clathrin.

The aim of this thesis was to complete the characterisation of the adaptor-like complex and to establish its function. My studies have shown that, the adaptor-like complex consist of a α/γ like subunit, δ, a β subunit (β3A/B), a μ subunit (μ3A/B) and a σ subunit (σ3A/B). We named the adaptor-like complex AP-3, by analogy with the AP-1 and AP-2 complexes. The AP-3 complex is localised to perinuclear and more peripheral membranes in non-neuronal cells, with little overlap with endocytic markers. The β subunit of the AP-3 complex is the major target for phosphorylation. Analysis of mice with mutations in the β3A subunit, and in the δ subunit of the AP-3 complex, have revealed that the β subunit is required for the stability of the μ subunit and that the δ subunit is essential for the stability of the whole complex. Further analysis of the mutant mice indicated that the mice lack significant levels of functional AP-3 complex. Studies on fibroblasts generated from these mice revealed that the AP-3 complex plays a role in the trafficking of LAMPI to lysosomes.

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Chapter 1

Introduction

A characteristic feature of any eukaryotic cell is the organisation of its cytoplasm into a complex set of membrane bound organelles. This compartmentalisation allows the cell to perform many different, and sometimes competing, biochemical reactions. These compartments have a defined lipid and protein composition that must be maintained if the organelle is to remain functional. The organisation of the cytoplasm raises several questions including, how are proteins and lipids transported between these organelles and how is the composition of these defined structures maintained? There have been many studies investigating these questions, however three key studies outline the basic principals of protein and lipid transport in the endocytic and secretory pathways: vesicle budding, vesicle targeting and vesicle fusion.

Biochemical and electron microscopy studies by G. Palade on exocrine pancreas tissue indicated that proteins could move in a vectorial fashion between distinct organelles of the secretory pathway via vesicular intermediates (Palade, 1975). Morphological studies by Roth and Porter on the endocytic pathway of mosquito oocytes showed that the formation of endocytic carrier vesicles was driven by the formation of a bristle coat on the membrane, and raised the possibility that the coat could both enable the vesicle to form and select the vesicle cargo (Roth and Porter, 1964). Biochemical *in vitro* reconstitution studies by Rothman have indicated that there are mechanisms for regulating the fusion of transport vesicles to the correct acceptor membrane, by the pairing of specific vesicle and membrane associated transmembrane proteins (SNARE hypothesis) (Sollner et al., 1993). A combination of these mechanisms (cargo selection and targeted fusion) would provide selectivity in vesicular transport and thus help to maintain the integrity of these distinct organelles.

1.1 Overview of the Secretory and Endocytic Pathways

Secretory pathway

Proteins destined for the secretory pathway are co-translationally inserted into the endoplasmic reticulm (ER) where they are proteolytically processed, folded and acquire covalent modifications (O and N-linked glycosylation, palmitoylation, and disulphide formation) (Ng and Walter, 1994). Proteins that have not folded properly are either retained in the ER until they have correctly folded or are exported into the cytoplasm and degraded (Hebert et al., 1996; Pilon et al., 1997). Proteins that are not ER resident are exported from the ER to the Golgi complex via the intermediate compartment. Escaped ER resident proteins are retrieved from this compartment and directed back to the ER (Barlowe, 1998; Lowe and Kreis, 1998). Other proteins are transported to the Golgi stack, where they undergo sequential processing as they transit through the Golgi cisternae (carbohydrate modification and tyrosine sulphation).

The Golgi complex structurally consists of several flattened stacked membranes termed the cis, medial and trans cisternae, each cisternum is defined by the presence of a specific set of glycosyltransferases. The cis-Golgi contains N-acetylglucosamine (GlcNAc)-1-phosphodiester α-N-acetylglucosaminidase that is required for the addition of mannose-6-phosphate residues to the oligosaccharide chains of lysosomal hydrolases. The medial cisternum contains GlcNAc-transferase I and the trans cisternae contains the majority of sialyltransferase, a terminal sugar processing enzyme (Fullekrug and Nilsson, 1998). However each enzyme is found in more than one cisternum, indicating that there is a gradient of processing enzymes throughout the Golgi stack (Nilsson et al., 1993). Proteins are then transported to the trans-Golgi network (TGN) where they are sorted and transported via vesicular intermediates to various destinations within the cell including the plasma membrane, endosomes and/or lysosomes .

Endocytic pathway

Cells internalise material from the extracellular environment via many methods (receptor mediated endocytosis, macropinocytosis, phagocytosis and caveolae). Receptor mediated endocytosis is the best characterised endocytic process and involves the efficient internalisation of molecules bound to cell surface receptors.

Molecules such as LDL (low density lipoprotein), EGF (epidermal growth factor) and transferrin are bound by cell surface receptors and internalised via clathrin-coated vesicles. These vesicles pinch off from the cell surface, uncoat and fuse with early endosomes. The receptors and ligands have several possible fates. The receptor and ligand can be recycled back to the cell surface (e.g., the transferrin receptor). The ligand can dissociate from its receptor in the acidic environment of the endosome, with the receptor being recycled back to the cell surface (e.g., the LDL receptor). The receptor and ligand can be targeted further into the endocytic pathway for degradation in lysosomes (e.g., the EGF receptor) (Schmid, 1997).

The morphology of the endocytic pathway is very complex consisting of interconnected tubules and vesicles, however three main classes of organelles have been defined biochemically and morphologically. Early endosomes are found in the cell periphery and are the first structures to be labelled with internalised tracers. Early endosomes can be further subdivided into "sorting" and "recycling" endosomes (sorting endosomes contain both transferrin and LDL, however, recycling endosomes only contain transferrin). It is thought that Late endosome (pre-lysosomes) are found closer to the nucleus and label strongly for M6P receptor but not the transferrin receptor and often contain internal membranes. Lysosomes are the most acidic endocytic structures, they do not contain M6P receptor, are very rich in acid hydrolases and often have electron dense cores (Gruenberg and Maxfield, 1995) (Griffiths and Gruenberg, 1991).

The secretory and endocytic pathways can not be considered separate entities as newly synthesised endosomal and lysosomal proteins are targeted into the endocytic pathway and certain proteins also cycle between the endocytic and secretory pathways (furin, TGN38 and M6P receptor) (Le Borgne and Hoflack, 1998).

A common feature shared between the endocytic and exocytic pathways is the use of coated carrier vesicles to shuttle membranes and proteins between distinct compartments. Several distinct types of coated carrier vesicle have been identified and characterised, including COPI, COPII and clathrin-coated. Each coat consists of a distinct set of proteins; however, many of the mechanisms regulating the processes of vesicle formation and cargo selection are conserved between these coat complexes. I have mainly focused my introduction on the mechanisms involved in the biogenesis of clathrin-coated vesicles although many of the points discussed also apply to the generation of other types of coated vesicles.

1.2 Clathrin-coated vesicles

Clathrin-coated vesicles were first observed by Roth and Porter in the early 1960s. Roth and Porter were studying yolk protein uptake in mosquito oocytes. They observed that during yolk uptake the surface of the oocytes became invaginated and numerous vesicles appeared just under the cell surface. Both the invaginations and vesicles were covered with a bristle like coat. They speculated that the formation of the invaginations might be driven by the formation of the coat on the membrane, and the coat might also be playing a role in the selection of the content of the vesicle (Roth and Porter, 1964). The true nature of the bristle coat only became apparent when coated vesicles were isolated from guinea pig brains and negatively stained, revealing that the coat consisted of a polyhedral lattice (Kanaseki and Kadota, 1969). In 1975 the bristle coated vesicles were purified to near homogeneity and identification of the protein composition of the coat became possible (Pearse, 1975).

Clathrin heavy and light chains

The major protein in purified coated vesicles was a protein of 180kD which was named clathrin (Pearse, 1975). Two other proteins of 33kD and 36kD also consistently co-purified with clathrin and were named (LCa and LCb) (Pearse, 1978). Crosslinking studies indicated that soluble clathrin consists of a trimer of 3 heavy chains and 3 light chains (Kirchhausen and Harrison, 1981). Negative staining indicated that the trimer forms a three-legged structure joined at the centre (or vertex) called a triskelion (Ungewickell and Branton, 1981). Morphologically three regions in each leg are distinguishable; there is a bend approximately midway which subdivides the leg into a 17nm long proximal segment (close to the vertex) and a 25nm long distal segment. A globular domain is joined to the distal segment via a flexible linker (Kirchhausen et al., 1986) (Fig. 1.1a). Interestingly, purified clathrin triskelions under suitable conditions can reassemble *in vitro* into polyhedral cages resembling the lattice structures observed on coated vesicles, suggesting clathrin is the major structural component of the coat (Crowther and Pearse, 1981).

Clathrin heavy chain is ubiquitously expressed in all eukaryotes and sequence comparisons between species show that it is highly conserved (Kirchhausen et al., 1987; Pley and Parham, 1993). *Saccharomyces cerevisiae* clathrin heavy chain is ~50% identical to rat heavy chain. A second clathrin heavy chain has been identified, CLH2, which is ~85% identical to CLH1. CHL2 is also ubiquitously expressed but is most highly expressed in skeletal muscle (Kedra et al., 1996). The high degree of homology shared between the two isoforms would suggest that they are performing similar functions.

Higher eukaryotes ubiquitously express two types of clathrin light chain, LCa and LCb, that are ~60% identical to each other. In neurons there are two additional forms LCa and one additional form of LCb created by differential splicing (Ponnambalam et al., 1994). *S. cerevisiae* only express one form of clathrin light chain that is 18% identical to LCb (Brodsky et al., 1991).

Function of the clathrin heavy and light chains

The ability of the clathrin heavy chains to self-assemble *in vitro* into a regular polygonal lattice indicates that the heavy chains act as a structural scaffold for the budding vesicle. The role of the light chains is still unclear. It is known that clathrin heavy chains can self-assemble in the absence of light chains (Winkler and Stanley, 1983), and the absence of the light chains does not effect the lattice's stability. However under these conditions the assembly of lattices is no longer calcium dependent *in vitro* (Ungewickell and Ungewickell, 1991). Clathrin light chains contain a calcium binding domain so they have been proposed to play a role in regulating clathrin assembly (Mooibroek et al., 1987). Interestingly yeast lacking clathrin light chain have the same phenotype as yeast lacking clathrin heavy chain, indicating that clathrin light chains are required *in vivo* for vesicle formation (Silveira et al., 1990).

Clathrin structure

As mentioned earlier the basic building block of the polyhedral lattice is the triskelion. The structure of the lattice has been determined by cryo-electron microscopy on clathrin cages embedded in vitreous ice. The simplest cage consists of 12 pentagons and 6 hexagons (36 triskelions) (Crowther and Pearse, 1981; Smith et al., 1998) (Fig. 1.1b). A study by Heuser (1980), gave an indication of how clathrin-coated vesicles might be formed *in vivo*. The study revealed that the plasma membrane is coated with clathrin-coated structures that range from being flat sheets consisting of only hexagons to curved structures that contained both hexagons and pentagons. He proposed that the curvature of the lattice can be changed by altering the ratio of hexagons and pentagons (Heuser, 1980). It is still unclear what mechanisms exist to facilitate this process.

Two papers have recently been published that increase our understanding of clathrin heavy chain structure and function. The N-terminal globular domain (terminal domain) and a region of the proximal domain of the clathrin heavy chain have recently been crystallised and their structures solved (ter Haar et al., 1998; Ybe et al., 1999). The N-terminal globular domain forms a seven

bladed beta propeller similar to that found in the beta subunits of heterotrimeric G proteins. Several prominent grooves are formed by this structure that potentially could be sights of protein binding. From the cro-electron microscopy studies it is known that the propeller region is orientated so it is facing into the inside of the vesicle (Crowther and Pearse, 1981; Smith et al., 1998). This orientation would allow the propeller domain to interact with proteins that are involved in cargo selection. Interestingly, the binding of beta arrestin (Goodman et al., 1997), and β adaptin (personal communication, Dr T. Kirchhausen) to clathrin has been mapped to one of these groves (beta arrestin is thought to play a role in the internalisation of heterotrimeric G proteins (Kirchhausen et al., 1997)).

A section of linker that connects the propeller to the distal leg was also resolved in the ter Haar et al. study. The linker region is made up of alternating short alpha helices connected by loops that form a zigzag structure. The structure formed is thought to provide a flexible linker between the propeller and the distal leg. The structure of the proximal domain also has a zigzag type motif formed by alternating alpha helices. However the structure of the proximal domain is more organised than the distal linker domain, thus suggesting the proximal domain is not as flexible. The proximal domain plays a role in clathrin light chain binding and clathrin heavy chain-heavy chain interactions, so the crystal structure should help define residues that are required for these processes.

Clathrin adaptor interactions

The second major component of clathrin-coated vesicles are adaptor complexes. Adaptor complexes were initially isolated from coated vesicles by Pearse and Robinson (1984). Cryo-electron microscopy studies revealed that the adaptor complexes form a layer between the vesicle membrane and the clathrin lattice (Vigers et al., 1986a; Vigers et al., 1986b). Clathrin cage assembly in vitro only occurs under non-physiological conditions such as low pH~6.5 and low ionic strength buffers. The clathrin cages that are formed under these non-physiological conditions are of a non-uniform size. However

the addition of adaptor complexes allows the clathrin cages to assemble under more physiological conditions and the cages that are formed are of a more uniform size (Zaremba and Keen, 1983).

1.3 Adaptor Complexes (AP-1 and AP-2)

Adaptor composition and structure

Two types of adaptor (AP-1 and AP-2) were purified from clathrin-coated vesicles and separated by their different elution profiles on hydroxyapatite (Pearse and Robinson, 1984) (Fig. 1.2). Crosslinking studies indicated that adaptors are heterotetrameric complexes consisting of two ~100kD polypeptides named adaptins, and two smaller proteins of ~50kD and ~20kD (Ahle et al., 1988; Keen, 1987b; Virshup and Bennett, 1988). The AP-1 complex consists of γ-adaptin (~97kD), β1-adaptin (~105kD), μ1 (47kD) and σ1 (~19kD). The AP-2 complex consists of α-adaptin (~105kD), β2-adaptin (~105kD), μ2 (~50kD) and σ2 (~17kD) (reviewed by Robinson 1992)(Robinson, 1992).

Rotary shadowing studies on purified AP-2 complexes indicated that the adaptor complexes form a brick-like core "head" attached to two smaller appendages "ears" via flexible linkers (Heuser and Keen, 1988). Treatment of purified AP-2 with elastase results in removal of the "ears", as only the adaptor core was visualised by rotary shadowing. Analysis of the digested AP-2 complexes by SDS-PAGE revealed that the two adaptins had been cleaved. Sequencing of the digested adaptins revealed that the ears correspond to the C-terminal portions of β and α adaptins (Kirchhausen et al., 1989).

Adaptor genes

The AP-1 and AP-2 subunits have been cloned and are highly conserved between mammalian species. Sequence comparison between α/γ-adaptin and β-adaptin reveal that there is homology between these proteins indicating they are derived from a common ancestor. There is also homology between the

small subunits and the medium subunits. Adaptors subunits also share limited homology with coatomer subunits, β COP shares homology to β adaptin, γ COP with γ adaptin, δ COP with μ, ζ COP with α (Lowe and Kreis, 1998).

β1 and β2 are ubiquitously expressed and are highly related to each other (~84% identical). There is also a alternatively spliced form of β2 expressed only in neurons that has a 14 amino acid insert in its hinge region (Ponnambalam et al., 1990).

In mammalian cells there are two ubiquitously expressed isoforms of α-adaptin, α_A and α_C, which are 80% identical and encoded by two genes (Robinson, 1989). It is not known if α_A and α_C are functionally equivalent but both isoforms have been shown to be present in the in the same coated pits. α_A is also alternatively spliced in brain tissue with an extra 22 amino acids in its hinge domain (Ball et al., 1995).

There are also two ubiquitously expressed isoforms of γ-adaptin, γ and γ2, which are ~60% identical to each other (Robinson, 1990) (Lewin et al., 1998; Takatsu et al., 1998). γ2 has recently been described and it can assemble into an AP-1 complex's. However, it is not clear if γ and γ2 have distinct localisations within the cell. However γ2 can bind γ-adaptin's binding partner γ-synergin, indicating the two isoforms may have similar functions (W. Lui, personal communication).

The medium subunits μ1 and μ2 are ubiquitously expressed and are 39% identical to each other. Recently a new μ isoform has been described, μ1B, which is ~79% identical to μ1 and is only expressed in epithelial tissue. The restricted expression of μ1B might indicate that μ1B is involved in polarised trafficking (Ohno et al., 1999).

The small subunits σ1 and σ2 are ubiquitously expressed and are ~42% identical to each other. Recently a new σ isoform has been described, σ1B, which is ubiquitously expressed and is ~87% identical to σ1. Yeast two hybrid

studies have indicated that σ1B can interact with γ-adaptin and may therefor co-assemble into classical AP-1 complexes (Page et al., 1999; Takatsu et al., 1998).

Homologues of the various adaptor subunits have been identified and cloned in many species. The other main organism where research into adaptor complexes has been performed is *Saccharomyces cerevisiae.* Genetic and biochemical studies have indicated that the yeast genes *APL4*, *APL2*, *APM1* and *APS1* encode proteins that are equivalent to γ, β1, μ1 and σ1 of the AP-1 complex and that this complex might associate with clathrin (Phan et al., 1994; Rad et al., 1995; Stepp et al., 1995). It has also been proposed that the products of the genes *APL3*, *APL1*, *APM4* and *APS2* may encode a complex equivalent of the AP-2 complex in yeast but there is no functional data to support this (Cowles et al., 1997a). Recently a third adaptor complex has been identified and characterised in yeast, AP-3. Unlike the yeast AP-1 complex it does not interact with clathrin (discussed later) (Cowles et al., 1997a; Panek et al., 1997; Stepp et al., 1997).

Subunit interactions

Crosslinking studies on AP-2 adaptor complexes, using a reversible crosslinker, indicated that there were close associations between the various subunits of the complex (Virshup and Bennett, 1988). A fully crosslinked complex ran with a molecular weight of 260kD on SDS-PAGE consistent with a composition of all four subunits. Partially crosslinking revealed intermediates species of 200kD, 150kD and ~110kD. The 200kD species is probably composed of the two adaptins, the 150kD species is μ2 plus one of the adaptins and the 110kD species is probably one of the adaptins associated with σ2. The group were unable to determine which of the adaptin subunits were associated with the smaller subunits. The above findings were supported by a study performed by Page and Robinson 1995. Using the yeast two hybrid system they were able to detect interactions between the two large adaptins subunits of the AP-1 and AP-2 complexes (β and α/γ,) interactions between the β and μ subunits and interactions between the α/γ and σ subunits

(Page and Robinson, 1995). Interestingly the coatomer subunit that share homology to the adaptor subunits also interact in the yeast two hybrid system in a similar manner as β and μ and α /γ and σ (i.e. γ and ζ COP interact, and β and δ COP interact) (Faulstich et al., 1996).

Adaptor localisation

Antibodies raised against α and γ-adaptins demonstrate that the AP-1 and AP-2 complexes are localised to distinct compartments. The anti-α antibody labels the plasma membrane and the anti-γ antibody mainly labels the perinuclear region of the cell (Ahle et al., 1988; Robinson, 1987). By immuno-electron microscopy AP-1 positive coated pits are localised predominantly to the trans-Golgi network and partially colocalise with the M6P receptor (Klumperman et al., 1993). AP-1 staining is also present on endosomal structures (Futter et al., 1998). In addition, in specialised secretory cells AP-1 staining is present on immature secretory granules (Dittie et al., 1996). These findings indicate that the AP-1 complex may have multiple functions.

1.4 Function of adaptor complexes and clathrin

Biochemical studies

Structural studies indicated that the adaptor complexes are positioned between the clathrin lattice and the vesicle membrane (Vigers et al., 1986b), so it was proposed that adaptors play a role in the binding of clathrin to the membrane. Several biochemical studies support this hypothesis. Membranes can be treated under conditions that strip clathrin from the membrane but leave the adaptors still bound (Unanue et al., 1981; Wiedenmann et al., 1985). If these stripped membranes are then treated with elastase (to cleave the adaptors) the clathrin is unable to rebind to the membranes. Further support for this hypothesis comes from experiments where the AP-2 complex is mistargeted on to an endosomal compartment by the addition of GTPγS. Clathrin was found to follow the mistargeted AP-2 on to the endosomes (Seaman et al., 1993).

The localisation and concentration of molecules such as the transferrin receptor and M6P receptor into clathrin-coated vesicles, led to the hypothesis that adaptor complexes might be playing a role in the selection of these molecules. Biochemical binding studies have indicated that adaptor complexes are able to bind to the cytosolic transmembrane tails of molecules such as the LDL receptor, M6P receptor and LAMPI (lysosomal associated membrane protein I) (Pearse, 1988) (Glickman et al., 1989) (Honing et al., 1996). These bindings studies indicate that adaptor complexes are potentially able to interact and possibly concentrate molecules into clathrin-coated vesicles.

Genetic studies

Genetic studies have given several insights into adaptor and clathrin function *in vivo*. The clathrin gene has been disrupted in two unicellular organisms, yeast and *Dictyostelium* (Lemmon and Jones, 1987; O'Halloran and Anderson, 1992; Payne et al., 1987; Payne and Schekman, 1985). Surprisingly both organisms are viable although growth is impaired. In clathrin knock out yeast, Kex2p (processing protease) and dipeptidyl aminopeptidase A (DPAP A) are mislocalised from the TGN to the cell surface, although the sorting of carboxy peptidase Y (CPY) to the vacuole is unimpaired. Interestingly, when a temperature sensitive mutant of clathrin heavy chain is initially shifted to the restricted temperature a defect in the sorting of CPY to the vacuole is observed, however after a few hours the CPY missorting defect is no longer detected.

Surprisingly, disrupting all four subunits of the AP-1 complex in yeast causes no obvious sorting defect. A study by Huang et al. (1999) has recently been published that indicates that disruption of yeast AP-1 and AP-2 complexes and the yeast AP180 homologues (clathrin assembly proteins) all in the same strain does not lead to a clathrin deletion-like phenotype, and that the clathrin-coated vesicles in these cells are indistinguishable from clathrin-coated vesicles isolated from a wild type strain (Huang et al., 1999). Their findings indicate that in yeast clathrin is able to function in the absence of adaptor

complexes and assembly proteins. However, it is not known whether there are other proteins in yeast that may function like adaptor complexes (e.g. β arrestin).

Clathrin heavy chain and several of the adaptor subunits have also been disrupted in multicellular organisms. The clathrin heavy chain has been disrupted in *Drosophilia melanogaster* and has been found to be essential (Bazinet et al., 1993). Disruption of the α-adaptin gene in *Drosophilia* also leads to embryonic death. μ1A and γ-adaptin have both been knocked out in mice and both are essential and lead to early embryonic death (Zizioli et al., 1999). Cells have been generated from the μ1A knock out embryos and the trafficking of several proteins investigated. M6P receptor traffics more via the cell surface in these cells compared to wild type cells, however that trafficking of LAMPI appears to be unaffected (Dr Peter Schu, personal communication). These findings indicate that AP-1 complex is required for the efficient trafficking of the M6P receptor *in vivo*. These findings indicate that adaptor complex's are not essential for unicellular function, however in mullticellular organisms they are essential.

1.5 Adaptor subunit function

Clathrin binding

The localisation of adaptor complexes between the vesicle membrane and the clathrin lattice, and the ability of adaptors to promote clathrin assembly *in vitro* indicates that adaptors can directly interact with clathrin. *In vitro* binding studies indicated that adaptor complexes are able to bind to the terminal globular domains of clathrin heavy chains and are also able to bind the hub regions of clathrin triskelions (Hanspal et al., 1984; Murphy and Keen, 1992). As the crystal structure of the terminal globular domain is now known potential adaptor binding sites can be investigated by mutagenesis studies.

The high degree of homology shared between β1 and β2 subunits made them likely candidates for directly interacting with clathrin. Initial *in vitro* binding

studies indicated that recombinant β2 could stimulate clathrin cage assembly and also could compete with AP-2 for the binding of these cages (Ahle and Ungewickell, 1989; Gallusser and Kirchhausen, 1993). Using recombinant β2 fusion proteins the clathrin binding domain of β2 was mapped to the hinge region (Shih et al., 1995). However there have been no *in vivo* studies to confirm these *in vitro* observations.

Cargo selection

Three sorting signals have been clearly identified that play a role either in the endocytocis of molecules from the cell surface or their efficient targeting within the cell: YXXØ, FXNPXY and di-leucine motifs (X stands for any amino acid and Ø stands for a bulky hydrophobic amino acid) reviewed by Le Borgne and Hoflack (1998) (Le Borgne and Hoflack, 1998). A study by Ohno et al. (1995) screened a yeast two hybrid library with the cytoplasmic tail of TGN38 (SDYQRL) to search for interacting proteins. Several clones were isolated from the library screen and two of them encoded the μ2 subunit. Alanine mutagenesis of this sequence indicated that the binding of μ2 to the TGN38 tail domain was dependent on the tyrosine and leucine amino acids. These amino acids had also been shown *in vivo* to be important for the endocytocis of TGN38. The TGN38 tail's sequence is also able to bind to μ1 in the yeast two hybrid system. In contrast, the YXXØ of the transferrin receptor only binds to μ2 and not μ1 in the yeast two hybrid system. The different affinities of the tails for either μ1 or μ2 could explain why certain molecules are trafficked by different adaptor complexes.

The crystal structure of a recombinant μ2 bound to the tail of TGN38 was resolved in 1998 by Owen and Evans (1998). The tyrosine residue and the bulky hydrophobic residue that are critical for the correct sorting of TGN38 binds into two hydrophobic pockets formed by two parallel beta strands in the medium subunit. The tyrosine binding pocket forms many hydrogen bonds with the hydroxyl group of the tyrosine residue in the peptide. These hydrogen bonds are essential for the correct binding of tyrosine containing peptides as

either changing the tyrosine to phenylalanine in the peptide or mutating an aspartic acid residue (Asp^{176} is conserved in all of the medium subunits identified) in the binding pocket inhibit the binding of the peptide (Nesterov et al., 1999; Owen et al., 1999).

Di-leucine motifs are also thought to bind to adaptor complexes but it is controversial which adaptor subunit of the complex the peptides bind to (β or μ)(Bremnes et al., 1998; Rapoport et al., 1998; Rodionov and Bakke, 1998). Crosslinking studies have indicated that di-leucine containing peptides may interact with the β subunits of the AP-1 and AP-2 complexes. This result is supported by studies performed by Marks et al. (1996). When they overexpressed proteins containing YXXØ motifs *in vivo* they found that the cells ability to endocytose molecules that contain YXXØ signals was reduced but the trafficking of proteins that contain di-leucine signals was unaffected, so suggesting that di-leucine signals do not interact with the medium subunit (Marks et al., 1996).

Targeting

In order for adaptor complexes to function in cargo selection they first must be targeted to the correct membrane. The α and γ-adaptin subunits appear to be good candidates for this role in the adaptor complex as they share only limited homology. Transfection studies using α/γ chimeras have indicated that there is some weak targeting information in the α/γ ear domains although the majority of the targeting information resides in the N-terminal head domain (Page, 1996; Robinson, 1993). Studies by Benmerah et al. (1999) have shown that the α ear domain on its own is able to correctly target to the plasma membrane (Benmerah et al., 1999), confirming the observation that there is targeting information in the ear domains. The α ear dependent targeting may in part be due to large number of interactions the α ear undertakes at the plasma membrane (discussed later). The N-terminal portion that is important in targeting the adaptor complex to the correct membrane also determines whether μ1 and σ1 or μ2 and σ2 assembled into the complex, thus it is likely

that the μ and σ subunits play a role in their complexes targeting to the correct membrane.

Binding of accessory proteins

The α and γ ear domains have been shown to play a role in the binding of a series of molecules that may modulate or regulate the budding of clathrin-coated vesicles. There are at least 4 molecules that can directly bind to α-adaptins ear domain amphiphysin: Eps15, epsin and AP180 (CALM) (David et al., 1996) (Benmerah et al., 1996; Chen et al., 1998; Hao et al., 1999). Mapping of the Eps15 and epsin α ear binding domains have revealed that these domains contain a tripeptide repeat DPF or DPW. Interestingly amphiphysin and AP180 also contain these repeats (Marsh and McMahon, 1999).

The crystal structure for the α adaptin ear domain has been published by two groups: Owen et al. (1999) and Traub et al. (1999). The α ear domain is split into two subdomains, one consisting entirely of β sheets (N-terminal) and one that consists of β sheets and α helices (C-terminal). Using the crystal structure to predict potential binding pockets both groups performed mutagenesis studies on the α ear, and investigated the ability of the mutated recombinant ears to interact with binding partners. Both groups identified residues in the same C-terminal area that when mutated affected the binding of Eps15, AP180, epsin and amphiphysin. The area they identified was very small suggesting that only one binding partner could bind to this domain at one time (Owen et al., 1999) (Traub et al., 1999). *In vitro* binding studies have indicated that the DPF peptide can bind to the α ear and the peptide is also able to inhibit the binding of amphiphysin (Owen et al., 1999).

Amphiphysin, Eps15 and epsin can also interact with several other molecules; amphiphysin interacts with dynamin (a GTP binding protein), synaptojanin (inositol-5-phosphatase; cleaves PIP_2) and endophilin (lysophosphatidic acid acyl transferase; generates phosphatidic acid) (endophilin can also interact with synaptojanin) (de Heuvel et al., 1997; Micheva et al., 1997; Schmidt et

al., 1999); epsin can also bind Eps15 and Ese1/2 (Ese1/2, DAP 160 and intersectin all share homology) (Chen et al., 1998) (Roos and Kelly, 1998; Sengar et al., 1999). Interestingly several of these molecules also contain repeated sequences.

The EH (Eps15 homology) domain is approximately 100 amino acids in length and there are three EH domains in Eps15 (Wong et al., 1995), while intersectin (Ese1/2, DAP160) contains two EH domains (Roos and Kelly, 1998; Sengar et al., 1999). The EH domain of Eps15 has been shown to bind the tripeptide NPF (Salcini et al., 1997). Interestingly epsin, AP180 and synaptojanin (non-neuronal isoforms) contain NPF repeats.

Amphiphysin, intersectin and endophilin all contain Src homology 3 domains (SH3). This domain has been shown to bind to proline-rich domains in proteins (Shupliakov et al., 1997). Interestingly, synaptojanin and dynamin have these proline-rich domains. These studies indicate that a very complex array of protein-protein interactions can be formed by the binding of very simple protein motifs. The exact function of this protein network is still not know, however overexpressing domains of several of these proteins inhibit endocytosis (Marsh and McMahon, 1999), indicating that these proteins might regulate the process of clathrin-coated vesicle formation.

A γ-adaptin ear binding protein γ-synergin has recently been described by Page et al. (1999). This molecule is the first γ ear binding protein to be identified. The protein can be alternatively spliced and ranges in size from ~110-190kD depending on the tissue examined. Like Eps15, γ-synergin contains an EH domain indicating that γ-synergin will be able to bind molecules containing NPF motifs. However, unlike Eps15 γ-synergin is not associated with AP-2, but with both cytosolic and membrane bound AP-1. Interestingly γ-synergin is mislocalised to the plasma membrane in cells expressing a construct that has had the γ ear fused onto the α-trunk. This result indicates that γ-synergin plays no role in the targeting of the AP-1 complex to membranes. The γ ear binding domain of γ-synergin has been

mapped and contains no DPF sequences, however there are 5 repeats of this sequence DDFXD/EF. The crystal structure of the γ ear has been resolved, and the structure is very similar to the N-terminal domain of the α ear. However, the γ ear does not contain the domain on the α ear that has been shown to be required for the binding of amphiphysin, Eps15 and epsin (Dr David Owen, personal communication). This observation indicates that γ-synergin will not bind to the γ ear in the same way as Eps15 binds to the α ear. The function of γ-synergin is still to be resolved.

1.6 Adaptor recruitment

Membrane binding

The precise localisation of adaptor complexes to specific membranes in the cell suggests that there must be signals on the membranes that the AP complexes can recognise and potentially bind to. Several studies have suggested that cargo molecules such as the transferrin receptor or the M6P receptor provide the specificity of for adaptor localisation (Le Borgne et al., 1993; Miller et al., 1991), however this seems unlikely as these molecules are also present in compartments that do not recruit adaptors. Several studies have indicated that there might be adaptor receptors. A study by Virshup and Bennett (1988) identified a high affinity AP-2 binding site on salt stripped membranes (Virshup and Bennett, 1988). It has also been shown that the binding of adaptors to salt stripped membranes is a saturable process, suggesting that there is only a limited number of receptors for adaptor binding (Moore et al., 1987). Several attempts have been made to identify molecules that might act as cargo receptors. Mallet and Brodsky (1996) used an AP-1 affinity column to isolate proteins of 83 and 52kD from detergent extracted cells (Mallet and Brodsky, 1996). Seaman et al. (1996) performed crosslinking studies on recruited cells, and found that several proteins (p60, p75 and p80) were specifically crosslinked to AP-1 (Seaman et al., 1996). Unfortunately in both cases the identity of these molecules still remains unknown. Synaptotagmin has been proposed to be a high affinity docking site for AP-2

(Haucke and De Camilli, 1999; Zhang et al., 1994), however there is no data that indicates that synaptotagmin is required for AP-2 recruitment *in vivo*.

Regulation

Fractionation studies have shown that adaptor complexes are distributed between the cytosol and membranes of the cell (Simpson et al., 1996). For adaptor complexes to function they must be recruited from the cytosol on to the membrane, and regulation of this process is essential if vesicular trafficking is to be controlled. Information on the regulation of adaptor recruitment has mainly come from *in vitro* studies making use of permeabilised cells and membrane fractions. These studies have indicated that regulation of adaptor recruitment is not simple and many factors influence the process.

AP-1 recruitment *in vitro* is stimulated by GTPγS and inhibited by the fungal metabolite brefeldin A. Treatment of cells with brefeldin A *in vivo* causes the AP-1 complex to redistribute into the cytosol (Robinson and Kreis, 1992). There is evidence that both GTPγS and brefeldin A are acting via the ARF family of small GTP binding proteins. GTPγS causes the ARF to become constitutively active while brefeldin A inhibits the nucleotide exchange of ARFs. The mechanism of brefeldin A action has recently been identified. Brefeldin A stabilises ARF-GDP bound to it exchange factor so creating the equivalent of a dominant negative mutant (Chardin and McCormick, 1999).

Direct evidence for the role of ARFs in AP-1 recruitment came from several studies; firstly the recruitment of AP-1 was reduced when cytosol was depleted of ARF1 by fractionation, secondly the only cytosolic components required for AP-1 recruitment *in vitro* were recombinant myristoylated ARF1 and purified AP-1 (Seaman et al., 1996; Stamnes and Rothman, 1993; Traub et al., 1993). The mechanism of ARF action is not clear and might be different for each ARF, however ARF1 has been shown to activate phospholipase D (PLD) (Cockcroft et al., 1994). Interestingly the addition of exogenous PLD or inhibitors of PLD have been shown to effect COPI recruitment (Ktistakis et al.,

1995; Ktistakis et al., 1996), however exogenous PLD or PLD inhibitors do not effect AP-1 recruitment (West et al., 1997). These results suggest that the mechanism of ARF1 action might be different for each type of coat complex.

The mechanisms involved in AP-2 recruitment are also not clearly defined. The localisation of AP-2 complex is not sensitive to brefeldin A *in vivo* and *in vitro,* however GTPγS causes the AP-2 complex to be mistargeted on to an endosomal compartment. The addition of exogenous PLD or constitutively active ARF1 also causes AP-2 to be targeted to endosomes. The addition of neomycin (PLD inhibitor, also binds PIP_2) to these experiments not only inhibits the misslocalisation of AP-2 to endosomes but also its recruitment on to the plasma membrane (West et al., 1997). These findings indicate that the generation of phosphatidic acid or PIP2 may be involved in the recruitment of the AP-2 complex onto the plasma membrane.

1.7 Novel adaptor complexes

There are many membrane traffic pathways in the cell that are not mediated by the three well characterised types of coats (clathrin, COPI and COPII), and this has led to the suggestion that there must be additional coat proteins to carry out these pathways (Robinson, 1991). This hypothesis is supported as two new adaptor complexes have been identified, AP-3 and AP-4.

AP-3

The first indication that there might be a new adaptor-like complex came from a study performed by Pevsner et al. (1994), who cloned cDNAs that had homology to μ1 (AP47) and μ2 (AP50). Pevsner et al. (1994) screened an electric ray cDNA expression library with an antiserum raised against electric ray synaptic vesicles. One of the clones isolated, ray p47, was used to screen a rat brain cDNA library, resulting in the isolation of rat p47A (μ3A) and p47B (μ3B). Northern blotting indicated that μ3A was ubiquitously expressed and that μ3B's expression was restricted to neuronal tissue. μ3A and μ3B are ~80% identical to each other and ~28-30% identical to μ1 and μ2 (Pevsner et

al., 1994). The low degree of homology of μ3A and μ3B with the conventional medium subunits suggests that they are not just isoforms of μ1 and μ2 and might be components of new adaptor-like complex.

The next adaptor-like subunit to be cloned was cloned by Newman et al. (1995), who isolated a cDNA that had homology to β1 and β2 and was named β-NAP (for neuronal adaptin-like protein). Newman et al. (1995) isolated β-NAP (β3B) by screening a human brain cDNA expression library with an autoimmune serum. Like μ3B, β3B is only expressed in neuronal tissue. β3B is ~28% identical to β1 and β2. An antiserum was raised against recombinant β3B, and the protein was found to be present both in the cytosol and associated with membranes. This result is consistent with β3B potentially being a coat component (Newman et al., 1995). If β3B is a component of a novel AP-like coat complex, the complex should consist of two large subunits (one of which is β3B) a medium subunit and a small subunit, by analogy to the known adaptor complexes.

A study by Simpson et al. (1996) raised antiseria against β3B and μ3 and used them in a range biochemical studies to investigate if μ3 and β3B are components of a novel adaptor-like complex. Gel filtration studies indicated that μ3 was a component of a larger complex as μ3 fractionated with an apparent size of ~400kD, consistent with it being a component of an adaptor-like complex. Immunoprecipitation studies using either anti-β3B or μ3 antibodies indicated that the two proteins are part of the same complex and co-immunoprecipitate with a unknown proteins of ~160kD and ~25kD. Western blotting studies using an anti-μ3 antibody indicated that even though μ3 is related to μ1 and μ2 it is not enriched in clathrin-coated vesicles.

In vitro recruitment studies indicated that the μ3/β3B complexes ability to bind to membranes is regulated in a GTP-dependent manner like AP-1 and AP-2. Immunofluorescence microscopy studies using the anti-β3B antibody indicated that the μ3/β3B complex is localised to perinuclear and more peripheral regions within the cell. Immunoelectron microscopy studies

indicated that the μ3/β3B complex is localised to a tubular vesicular network that also labelled with antibodies against AP-1 and clathrin. The μ3/β3B complex and AP-1/clathrin staining are often in very close proximity but do not colocalise, indicating that the μ3/β3B complex is not associated with clathrin.

At the time the work described in this thesis was begun the study by Simpson et al. (1996) had just been completed. However, several issues were left unresolved. Do the 160kD and 25kD proteins share homology with α/γ and σ adaptor subunits? What is the function of the AP-3 complex? Interestingly several ESTs had been identified by Dr M. S. Robinson that encode proteins that share homology to α/γ and σ1/σ2 adaptor subunits, and these proteins were candidates for the 160kD and 25kD proteins. An indication of the novel complex's function came from yeast two hybrid studies performed by Ohno et al. (1995). Ohno et al. (1995) showed that μ1 and μ2 *in vitro* were able to bind to peptides that contained tyrosine-based motifs (Ohno et al., 1995). These signals had previously been shown to be important in the trafficking of many proteins (TGN 38, LAMPI, LAMPII). As the novel complex was known to contain a medium subunit (μ3) it seemed likely that this complex would also play a role in the sorting of proteins containing tyrosine-based motifs.

AP-4

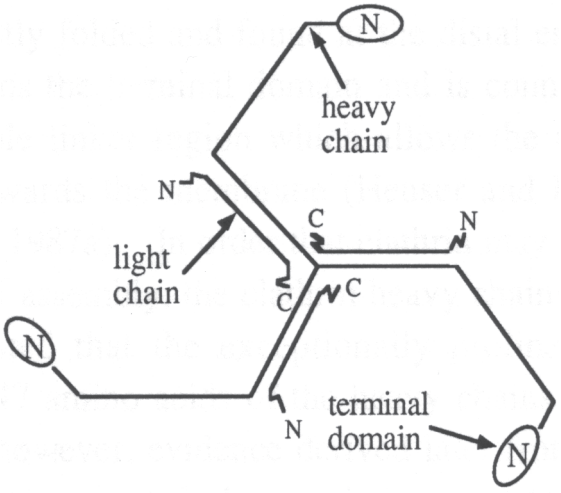
Studies by Hirst et al. (1999) and Dell'Angelica et al. (1999) have recently described the cloning and characterisation of a fourth adaptor-like complex. Like conventional adaptor complexes, the complex consists of a α/γ like subunit ε, a beta subunit β4, a medium subunit μ4, and a small subunit σ4 (Dell'Angelica et al., 1999a; Hirst et al., 1999). Northern blotting studies indicated that all four subunits of the AP-4 complex are expressed ubiquitously. The AP-4 complex, like the AP-1 and AP-3 complexes, is brefeldin A sensitive indicating that the association of the AP-4 complex with membranes is regulated in an ARF dependent manner. Immunofluorescence studies by Hirst et al. (1999) indicate the AP-4 complex is expressed at very low levels and is localised to the perinuclear region within the cell. Immuno-electron microscopy studies by Hirst et al. (1999) have indicated that the AP-4

complex is localised to the trans-Golgi network of the cell. Yeast two hybrid studies by Hirst et al. (1999), have shown that the μ4 subunit, like μ1, μ2 and μ3, is able to interact with proteins that contain tyrosine-based motifs. The ubiquitous expression and the low abundance of the AP-4 complex would suggest that the complex participates in a specialised trafficking pathway that is required in all cells.

1.8 This thesis

The aim of this thesis was to complete the characterisation of the AP-3 adaptor-like complex and to establish its function.

a



b

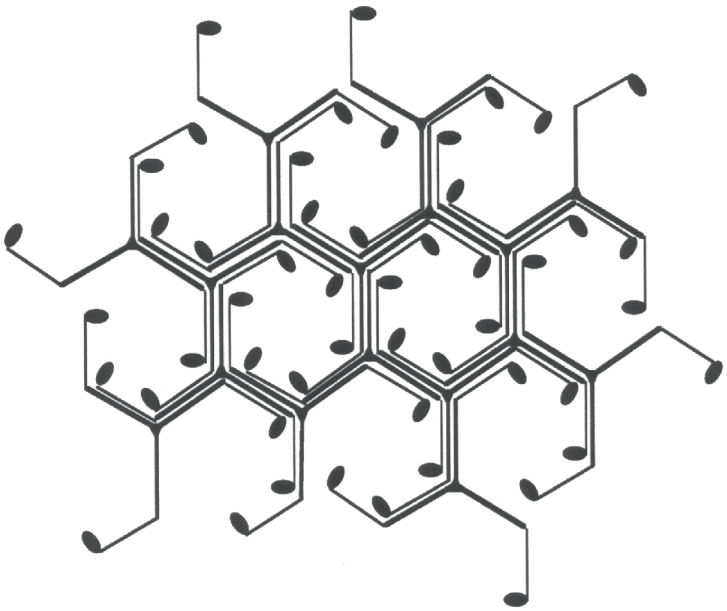


Figure 1.1

Schematic diagram of a clathrin triskelion (a) and a schematic diagram of triskelion packing to form a clathrin lattice (b). Each triskelion is composed of three clathrin heavy chains and three light chains. The globular N-terminal domain points towards the membrane. The vertex of each triskelion forms the vertex of the polyhedral coat. Diagrams obtained from C.L. Ball.

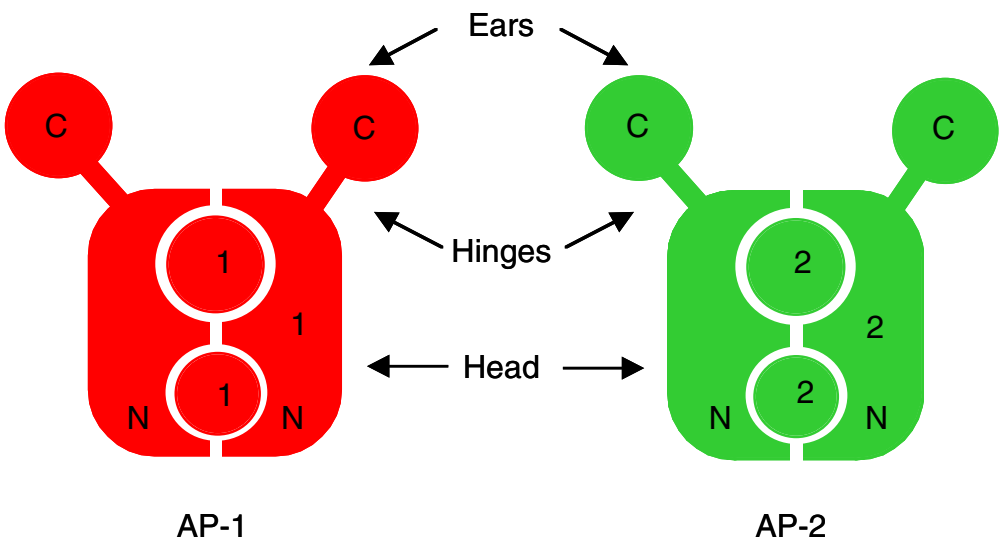


Figure 1.2

Schematic digram of the two adaptor complexes.

Chapter 2

Materials and Methods

2.1 Materials

Plasmids

pBluescript (SK/KS) was obtained from Stratagene. The pGEX (3X and 4T) family of vectors were obtained from Pharmacia Biotech. The mammalian expression vectors pCEP, pREP and β–Galactocidase pcDNA3 were originally obtained from Invitrogen. The mammalian expression vector pMEP modified from pMEP (Stratagene) was a generous gift from Dr G. Banting (Roquemore and Banting, 1998). GFP (S65T) pcDNA3 was a generous gift from Dr J. Haseloff (Siemering et al., 1996).

Bacterial strains

The bacterial strains used during this study included TG2 (plasmid amplification), MC1061 (GST fusion protein production), DH5α (plasmid amplification and GST fusion protein production), SCS110 (plasmid amplification) and C600-Hfl (λgt10 library host strain).

Bacterial culture media

Bacterial cultures were grown in 2xTY (1.6% tryptone, 1% yeast extract, 0.5% NaCl, pH 7.4), supplemented with 50μg/ml ampicillin when required.

The M.R.C Laboratory of Molecular Biology media kitchens provided bacterial growth media and plates.

Oligonucleotides

Oligonucleotides were made by Genosys Biotechnologies. The sequences of the oligonucleotides used in this study are shown below, restriction sites are underlined. The oligonucleotides used for the sequencing of β3A and β3B are not listed.

Table 2.1 Sequence of oligonucleotides

sequence	use of oligonucleotides
CGCGGATCCCAGTAGACAAGGTTCACAATA (BamHI)	PCR of C terminus of σ3A for GST fusion protein
CCGGAATTCTTTGTATGGATCACAAGTGCA (EcoRI)	"
CGCGGATCCCCGACCCATCAGTTCGA (BamHI)	PCR of β3A hinge for GST fusion protein
AGGCCCGGGATCTTTGGTAAGAGGAGT (SmaI)	"
GGTTAATTAAGCGGCCGC (PacI) (NotI)	5' polylinker for β3A
GGCCGCTTAATTAACCGC (PacI)	"
GGTGATGGCGGTGGCGC	PCR to construct missing region of β3B
TTTGATGATCTCTCCATGTTGTGCTGGCTGCA TCTGT	"
AACATGGAGAGATCATCAAACACTTGGCAAAG CTTAC	"
GGTGGGACAGTGAGCCC	"
ACGCGTCGACATGAGTAAAGGAGAAGAACTT (SalI)	PCR of GFP
ACGCGGGCCCTTATTTGTATAGTTCATCCATG CC (ApaI)	"

Antibodies

The antibodies used and generated in this study are shown on the next two pages.

Table 2.2 Antibodies

Antibody	Antigen	Source	Application	Reference
AP-6	α-adaptin	F. Brodsky	IF (1:50)	(Chin et al., 1989)
Anti-σ1	σ1	M.S. Robinson	WB (1:100)	(Page and Robinson, 1995)
Anti-σ3A	σ3A	this study	IP/ WB (1:100)	(Simpson et al., 1997)
Anti-σ3B	σ3B	F. Simpson	IP/ WB (1:100)	(Simpson et al., 1996)
Anti-μ1	μ1	M.S. Robinson	WB (1:30)	(Page and Robinson,

				1995)
Anti-μ3A/B	μ3A/B	F. Simpson	IP* (1:500) WB (1:1000)	(Simpson et al., 1996)
Anti-β3A	β3A	this study	IP/WB (1:100)	(Simpson et al., 1997)
Anti-β3B	β3B	F. Simpson	IP (1:100) / WB (1:400)	(Simpson et al., 1996)
Anti-β3B	β3B	Upstate Biotechnology	IF (1:100)	(Newman et al., 1995)
Anti-γ	γ–adaptin	M. Seaman	IF (1:400) IP (1:100) WB (1:1000)	(Seaman, 1994)
mab100/3	γ-adaptin	E. Ungewickell	IF (1:50)	(Ahle et al., 1988)
Anti-δ (N terminus)	δ	F. Simpson	IF (1:300) IP (1:100) WB (1:400)	(Simpson et al., 1997)
Anti-δ (hinge)	δ	M. Robinson	IP/WB (1:100)	
Anti-δ (hinge + ear)	δ	M. Robinson	IP/WB (1:100)	
Anti-clathrin	clathrin heavy chain	M. Robinson	IF/IP/WB (1:100)	(Simpson et al., 1996)
X22	clathrin heavy chain	F. Brodsky	IF (1:50)	(Brodsky, 1985)
Anti-GST	GST	this study	WB (1/1000)	(Page et al., 1999)
Anti-transferrin receptor	Transferrin receptor (CD77)	Serotec	IF/EA (1:100)	
Anti-LAMP I (CD107a)	cell membranes	DSHB	IF/EA (1:100)	(Chen et al., 1985)
Anti-LAMP II (CD107b)	cell membranes	DSHB	IF/EA (1:50)	(Granger et al., 1990)
Anti-alpha-2-macroglobulin receptor	cell membranes	DSHB	IF/EA (1:100)	(Hughes and August, 1981)
Pgp-1 (CD44)	cell membranes	DSHB	IF/EA (1:100	(Hughes and August, 1981)
Anti-β-galactocidase	β-galactocidase	DSHB	IF (1/20)	
Anti-mouse TR	mouse IgG	Amersham	IF (1:100)	
Anti-rabbit FITC	rabbit IgG	Amersham	IF (1:100)	
Anti-rat TR	rat IgG	Molecular Probes	IF (1:100)	
Anti-rat	rat IgG	Sigma	EA (1:1000)	

Anti-TR	TR	Molecular Probes	EA (1:1000)	

IP=Immunoprecipitation, IF=Immunofluorescence, WB=Western blotting, EA=Endocytocis assay, DSHB=Developmental studies hybridoma bank, TR=Texas red, FITC=Fluorescein iso thiocyanate

2.2 Molecular biology methods

General molecular biology procedures were performed according to standard protocols unless otherwise stated (Sambrook et al., 1989). Reagents were obtained from Boehringer Mannheim, Clontech, Invitrogen and New England Biolabs.

Polymerase chain reaction (PCR)

The standard reaction conditions used are listed below:

2μl of dNTP mix (10mM each dNTP)
10μl of Vent DNA polymerase buffer (supplied with enzyme)
1μl of template DNA (~ 5ng/μl)
2μl of each primer (~ 10pmol/μl)
0.5μl of Vent DNA polymerase
84.5μl of water (reactions final volume 100μl)

The PCR reaction was then overlaid with mineral oil and the following PCR program used:

94°C for 2 min (denaturation)
50°C for 1 min (annealing)
72°C for 2 min (extension)

This sequence was repeated 27x except the denaturation step (94°C) was reduced to 1min. The last cycle the extension time was increased to 10 minutes.

Restriction digests

Restriction digest was performed as instructed by the manufacturer using the buffers supplied with the enzymes.

Agarose gels

After restriction digest the digested DNA was run on a TBE (90mM Tris-borate, 2mM EDTA) agarose gel, with an agarose percentage ranging from 0.8% to 2% depending on the size of the DNA fragments to be resolved. The QIAEX II Kit (Qiagen) was used to extract DNA from excised gel bands if they were required for further processing.

Ligations

Cut insert and vector were ligated (ratio 10:1) by incubating overnight at 16°C (sticky ligation) or 25°C (blunt ligation) with T4 DNA ligase in the buffers supplied with the enzymes.

Blunt-end cloning of PCR products

The PCR product to be cloned was phosphorylated by incubating with T4 polynucleotide kinase at 37°C for 30 minutes (buffer supplied with enzyme). The phosphorylated PCR product was then gel purified. Cut vector was dephosphorylated by adjusting the restriction digest to optimal conditions by the addition of phosphatase buffer (supplied with the enzyme). Alkaline phosphatase was added and the reaction mixture was incubated at 37°C for 30 minutes. The dephosporylated vector was then gel purified. The phosphorylated PCR product and dephosphorylated vector were then ligated using T4 DNA ligase overnight at 25°C.

Preparation and transformation of calcium chloride competent bacterial cells.

Competent cells were prepared by inoculating 100ml of 2xTY with 1ml from a overnight culture, the cells were grown at 37°C until a OD600 of between 0.7-0.8 was reached. The cells were chilled on ice, pelleted and then resusepended in 50ml of 0.1M CaCl2. After a 30 minute incubation on ice the

cells were repelleted and resuspended in 5ml of 0.1M CaCl2 The competent cells were allowed to mature at 4°C overnight before being used or snap frozen in liquid nitrogen and stored at -80°C (10% glycerol was added to the cells before freezing).

The DNA to be transformed was mixed with 100μl of competent cells and incubated on ice for 5 minutes, the transformation mixture was then spread on 2xTY amp plates which had been prewarmed to 37°C (Pope and Kent, 1996).

Plasmid purification

Plasmid purification was carried out using the QIAprep Spin Miniprep Kit or the QIAfilter Plasmid Midi Kit (Qiagen) following the manufacturers instructions. Both kits are based on a modified alkali lysis procedure. The kits utilise a silica matrix column which only binds DNA and allows contaminating RNA and proteins to be washed through. The purified DNA is then eluted from the silica matrix by changes in salt conditions.

DNA sequencing

DNA sequencing was initially carried out using Thermo Sequenase™ or Sequenase™ V2 sequencing kits (Amersham) following the manufacturers instructions. When an automated sequencing facility became available, a Perkin Elmer fluorescent automated sequencer was used (University of Cambridge Biochemistry Department sequencing facility). DNA for automated sequencing was prepared using QIAprep Spin Miniprep Kits. The DNA for sequencing was diluted to 100ng/μl in water; primers for automated sequencing were used at to 10pmol/μl.

Labelling DNA probes

Radioactive probes were generated using the *Rediprime* (Amersham) random primer labelling kit. 25ng of denatured DNA was labelled with ^{32}P-CTP in accordance with the manufacturers instructions. The unincorporated ^{32}P-CTP was removed by passing the reaction products down a Nick column (Sephadex® G-50 DNA grade column). The labelled probe was denatured by

boiling and then snap cooled on ice before it was added to the hybridisation solution.

Library screening

A human heart λgt10 library (Clontech) was screened according to the instructions supplied by the manufacturer. The library was titred and plated out on a lawn of C600Hfl bacteria at approximately 70,000 plaque forming units per 150mm plate (10 plates were used). The plates were incubated at 37°C until the plaques had reached a suitable size (~8 hours). The plates were chilled to 4°C before duplicate filter lifts were performed, using Hbond™-N^+ (Amersham) nylon membranes. The membranes were alkali fixed in accordance with the manufacturers instructions.

The alkali fixed membranes were prehybridised in ExpressHyb™ (Clontech) hybridisation solution for 30 minutes at 65°C, as indicated by the manufacturers instructions. The prehybridisation solution was removed and replaced with prewarmed (65°C) hybridisation solution containing 25-50ng of denatured probe, and incubated at 65°C for 1 hour. The membranes were washed for 40 minutes in 2xSSC containing 0.05% SDS at room temperature, and 0.1xSSC containing 0.1% SDS for a further 40 minutes at 50°C. The wash solutions were changed several times during the incubations. The wet membranes were wrapped in plastic wrap and exposed to Kodak X-OMAT film for the appropriate length of time to obtain a signal.

Positive plaques were identified by aligning the library plates with the X-ray film, and were picked by using the large end of a Pasteur pipette. Secondary and tertiary screens were performed until plaque purified clones were obtained. The phage were amplified in liquid culture at 37°C until lysis of the bacterial host strain had occurred. The phage were manually isolated and their DNA purified using the Wizard Lambda Preps kit, as indicated by the manufacturers instructions.

Southern Blotting

Samples to be analysed were run on an agarose gel and transferred to Hbond™-N^+ nylon membranes by capillary transfer (Sambrook et al., 1989). This method entailed incubating the agarose gel in denaturation solution (1.5M NaCl, 0.5M NaOH) for 30 minutes at room temperature with gentle agitation. The gel was rinsed in distilled water and transferred to neutralisation solution (1.5M NaCl, 0.5M Tris-HCl pH7.2, 0.001M EDTA) for 30 minutes at room temperature (the neutralisation solution was changed after 15 minutes). The gel was incubated in 10xSSC (1.5M NaCl, 0.15M Na_3 citrate) for 30 minutes at room temperature before the DNA was transferred on to nylon membranes by capillary transfer over night. The membranes were alkali fixed and the same hybridisation conditions used as previously described for library screening.

Bioinformatics (DNA and protein sequence analysis)

The DNA and protein sequence manipulation program Gene Jockey (BISOFT) was used to create DNA contigs for (β3A and β3B), protein sequence alignments for (β3A, β3B, σ3A/B, μ3A/B and δ), hydropathy plots for (β3A, β3B and δ) and predicted molecular weights for (β3A, β3B,σ3A/B, μ3A/B and δ) subunits of the AP-3 complex. The SIP (Staden, 1990) and MegAlign (DNA STAR PACKAGE) software were used to compare and calculate the identities of the subunits of the AP-1, AP-2 and AP-3 complex subunits. The National Centre for Biotechnology Information (NCBI) databases were searched for novel adaptor-like proteins using the NCBI BLAST program (Karlin and Altschul, 1990).

2.3 Cloning strategies

Construction of full length β3A

β3A was isolated from a human heart cDNA library and excised from the phage using EcoRI. This generated two fragments that were cloned into pBluescript (SK). The two clones were sequenced and a cloning strategy was

designed to create a full-length clone. The two clones were joined together utilising a unique EcoRI site.

To aid the moving of β3A into several expression vectors a polylinker was added to the 5' end of the gene. The polylinker was created by annealing complimentary oligonucleotides together containing PacI and NotI restriction sites. The annealed complimentary oligonucleotides were cloned into the SacII site of the β3A cDNA in pBluescript.

Construction of full-length β3B

As the cDNA clone encoding the full-length β3B was unavailable the EST database was searched for human clones that encoded β3B. Several clones were found which could potentially encode β3B, GenBank accession numbers 175331(C1), 231345 (C3), 320375 (C2) and 165789 (C4). These clones were obtained, restriction mapped, sequenced and aligned to β3B (Fig 2.1a). None of the clones encoded the full length β3B, so a strategy was designed which would involve cutting and pasting several of these clones together using unique restriction sites (BamHI, NotI and HindIII) to create a nearly full-length clone. The missing piece was generated by overlapping PCR and inserted into the clone using unique sites (KasI and ApaI) (Fig 2.1b). To check that no cloning artefacts had occurred the complete cDNA was sequenced in one direction (the PCR fragment was sequenced in both directions).

Construction of full-length δ

δ was isolated from human heart cDNA library as two independent clones which were excised from the phage using EcoRI and cloned into pBluescript (SK) (Simpson et al., 1997). From the sequence of the clones a unique HindIII site was identified which made it possible for the two clones to be pieced together.

Construction of β3A and σ3A GST fusion proteins

To generate antisera to β3A and σ3A regions of theses proteins were expressed as GST fusion proteins. The hinge region of β3A and the C-

terminal portion of σ3A were isolated by PCR and the resulting products cloned into pGEX3X using sites engineered into the PCR products (see tables 2.1 and 2.3 showing the sites used).

Table 2.3 Constructs

cDNA	Sites used	Vector
σ3A C terminus aa 112-192	BamHI/EcoRI	pGEX3X bacterial expression vector
β3A hinge aa 644-814	BamHI/SmaI	pGEX3X "
β3A	NotI/XhoI	pMEP mammalian expression vector
β3A	NotI/XhoI	pCEP "
β3B	*NheI/SpeI/ ClaI/BamHI	pMEP "
δ	XhoI/BamHI	pMEP "
δ	XhoI/BamHI	pCEP "

The cloning of β3B into pMEP destroyed the NheI and BamHI sites of the vector.

2.4 Tissue Culture and Transfections

General tissue culture

NRK (normal rat kidney), HeLa, MDBK (Madin-Darby bovine kidney), COS-7, *mocha* (*mh*) (generated during this study), *pearl* (*pe*) (Zhen et al., 1999) and primary mouse cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 50IU/ml penicillin, 50μg/ml streptomycin, and 2mM L-glutamine at 37°C in a 5% CO_2 humidified incubator (reagents obtained from Sigma). Melan-a cells were grown in RPMI 1640 (Roswell Park Memorial Institute) with the same additions as above, except 200nM teradecanoyl phorbol acetate (TPA) was added and the cells were maintained in 10% CO_2 incubator.

Generation of cell lines

Primary fibroblasts were generated by taking tissue from 1-3 day old mice and physically disrupting the tissue using a scalpel blade or a pair of scissors. The

disrupted tissue was transferred in to DMEM supplemented with 10% fetal calf serum, 50IU/ml penicillin, 50μg/ml streptomycin, 2mM L-glutamine and mercaptoethanol (final concentration 52μM). After 3-7 days the medium was changed on the cells. Once cells had grown out from the disrupted tissue the cells were either frozen or allowed to grow until their growth rates slowed. The cells had their media supplemented with 200nM TPA. In some cases the cells changed from slow growth to fast non-contact inhibited growth. The fast growing cells were cloned by their ability to out grow the rest of the slow growing cells in the flask.

Transient and Stable Transfections

Several transfection reagents were used during this study, Fugene (Boehringer Manheim), Superfect (Invitrogen), Calcium Phosphate (Promega), Lipofectamine (GibCo). All of the procedures were carried out as described in the manufactures instructions. DNA for transfection was prepared as described in general molecular biology methods 2.2.

Table 2.4 lists which methods proved most successful for the cell types tried during this study. Transfection efficiency was estimated by transfecting cells with a control construct pcDNA3 β-Galactocidase. β-Galactocidase activity was detected by staining the cells with X-gal as described by the manufacturers instructions.

Table 2.4 Transfection methods

Cell type	Method of transfection	Approximate transfection efficiency % of cells transfected
NRK	Fugene and Lipofectamine	10
COS	Fugene, Superfect and Lipofectamine	50
HELA	Superfect	50
mh	Fugene and Calcium Phosphate	10*
pe	Superfect and Lipofectamine	1-2

*Initially up to 10% of the *mh* cells could be transfected, as the passage number of the *mh* cells increased the ability to transfect decreased to levels similar to that of *pe* cells.

For the generation of stably transfected cells the cells were transfected as above with pMEP or pCEP based constructs. The stably transfected cells were then selected by growing them in DMEM supplemented as above except hygromycin (0.22mg/ml) (Boehringer Mannheim) was added to the media.

2.5 Antibody Production

Purification of soluble fusion proteins

An overnight culture of MC1061 or DH5αcells containing either β3A or σ3A pGEX3X construct was diluted 1:10 into 2TY and grown for 1 hour at 37°C. The cells were induced to express fusion protein by the addition of 0.1M IPTG (final concentration of 0.1mM IPTG) (Sigma). The cells were grown for a further 5-7 hours and pelleted by spinning for 15 minutes at 4500 rpm. The cell pellet was resuspended in 1/100 volume of ice cold PBS. The cells were frozen (-20°C) and then thawed before being sonicated to disrupt the cell walls. The disrupted cells were incubated for 10 minutes on ice in the presence of Triton X-100 (final concentration 1%). The cell debris was pelleted by spinning for 5 minutes at 13000rpm in a bench top microfuge at 4°C. The supernatant was then transferred to an appropriate amount of PBS-washed glutathione-Sepharose (Pharmacia) and incubated for 20 minutes at room temperature. The beads were washed 3 times with ice cold PBS and then washed a further 3 times in ice cold PBS with 10 minute incubation periods. The fusion protein was eluted from the beads by incubating them for ~5 minutes at room temperature in 50mM Tris-HCl containing 10mM glutathione. This elution procedure was then repeated a further 3 times and the eluted protein analysed by SDS-PAGE.

Fusion protein injection and serum collection

For each fusion protein construct two rabbits were subcutaneously injected along the flank with 0.5mg of fusion protein. For the first injections the fusion protein was mixed with Freund's complete adjuvant (Difco) and subsequently was mixed with Freund's incomplete adjuvant (2 and 6 weeks after the initial injection). 10 days after the final injection the rabbits were terminally bled and the serum collected and allowed to clot at 37°C for 1 hour. The clot was removed by spinning at 300 rpm for 5 minutes.

Coupling of fusion protein to Sepharose

The fusion proteins to be coupled were dialysed overnight in coupling buffer (0.1M $NaCHO_3$ pH 8.3) The dialysed fusion proteins (2mg/ml) were incubated for 2 hours at room temperature with cyanogen bromide-Sepharose (1ml of packed beads) (Pharmacia), which had been activated by washing with 1mM HCl. The beads were washed once with coupling buffer and then washed with 0.1 M Tris-HCl pH 8.0 to quench the activated cyanogen bromide Sepharose. The beads were washed in 0.1M NaAc pH 4.0 containing 0.5M NaCl and then in 0.1M Tris-HCl pH 8.0 with 0.5M NaCl. This was repeated a further two times before the beads were washed once in 200mM glycine pH2.3 and then three times in PBS containing 0.02% sodium azide.

Affinity purification

10ml of serum was incubated overnight at 4°C with GST-Sepharose (2mg/ml) in order to remove antibodies raised against GST. The GST-Sepharose was removed by centrifugation and the supernatant incubated with the appropriate fusion protein bound to Sepharose beads for at least 1 hour at room temperature. The Sepharose beads were washed five times with PBS before being transferred into a chromatography column (BioRad) at 4°C. The bound antibodies were eluted from the column with 5.5ml of cold 0.2M glycine pH 2.3 containing 0.1% gelatine. The elute was collected into a tube containing 2M Tris-HCl pH 8.0 and then dialysed overnight in PBS containing 0.02% sodium azide. To ensure that all of the GST reactive antibodies were removed the

sample was incubated a further time with GST-Sepharose for 1 hour before freezing the antibodies at -80°C.

2.6 Immunofluorescence

Cells were grown on multi-well slides (ICN) and fixed by placing in -20°C methanol for 5 minutes and then in -20°C acetone for 30 seconds before allowing the slides to air-dry at room temperature. The slides were incubated in primary antibody(s) (table 2.2) diluted in 1mg/ml BSA PBS for 30 minutes, then washed five times in 1mg/ml BSA PBS. The slides were incubated with secondary antibody(s) (table 2.2) for 30 minutes and then washed as before. The slides were mounted in 100mg/L p-phenylenediamine adjusted to pH8.0 with 0.5M $NaHCO_3$ in 90% glycerol. Images were captured either using a BioRad MRC 1000 confocal microscope or a Zeiss Axioplan microscope fitted with a MicroMax 1300 cooled CCD camera (Princeton Instruments).

2.7 SDS-PAGE

Samples to be analysed by SDS-PAGE (Laemmli, 1970) were boiled in 1x sample buffer (4x sample buffer is 0.5M Tris-HCl pH 6.5, 8% SDS and 40% glycerol) containing 5% β-mercaptoethanol for 5 minutes. The samples were then resolved on mini-gels 10cm x 6cm and 0.5mm thick at a constant voltage of 140V. The stacking gel used was 50mM Tris-HCl pH 6.8 with 4% acrylamide and the separating gel used was 1.26M Tris-HCl pH 8.9 with a varying percentage of acrylamide (6.5-12%).

2.8 Western Blotting

Proteins were transferred onto nitrocellulose (Schleicher and Schuell) by electroblotting overnight at a constant current of 100mA using a wet blotting method (Towbin et al., 1979). The buffer used was 50mM Tris-HCl, 0.4M glycine, 0.1% SDS and 20% methanol. To check protein transfer and to visualise the molecular weight markers the nitrocellulose was stained with

Ponceau S in (0.1% TCA) The nitrocellulose was blocked for 1 hour at room temperature in 50mM Tris-HCl pH7.4, 150mM NaCl, 5mM EDTA, 0.25% w/v gelatine, 0.1% v/v NP40 and 0.1% NaN_3 (blocking buffer). The blot was then incubated with the appropriate antibody(s) diluted in blocking buffer (see table 2.2) for 30 minutes at room temperature before being washed 3 times quickly and 3 times slowly (10 minutes per wash) in blocking buffer. The antibodies were detected by incubating the blot with ^{125}I-protein-A (Amersham) diluted 1/1000 in blocking buffer (0.1μCi/ml) for 30 minutes before being washed as before. The blot was allowed to dry before being exposed to X-ray film or analysis on a phosphor-imager (Fuji Bas2000 Bio-Imaging Analyser).

2.9 Immunoprecipitations

Immunoprecipitations from tissue culture cells

A semi confluent 75cm^2 flask of cells was washed with 10mls of room temperature PBS, the PBS was aspirated and the cells drained on ice for 5 minutes. The cells were lysed with the addition of 1ml of NP-40 lysis buffer (150mM NaCl, 1% NP40 and 50mM Tris-HCl pH 7.5 plus protease inhibitor cocktail (Boehringer Mannheim)) for 10 minutes at 4°C. The cells were scraped on ice and the lysate cleared by centrifugation at 13000rpm in a bench top centrifuge for 15 minutes at 4°C (Harlow and Lane, 1988). For some experiments the cleared lysate was converted to more denaturing conditions by the addition of SDS (final sample concentration of 0.4%SDS). The sample was pre-cleared for 30 minutes at 4°C by the addition of 50μl of 50% slurry of protein-A Sepharose (Sigma). The protein-A Sepharose was pelleted by spinning at 13000rpm in a bench top centrifuge for 1minute at 4°C. The supernatant was transferred to a fresh tube and incubated with antibody (see table 2.2) for 1 hour at 4°C. The antigen-antibody complexes were captured by the addition of 30 μl of 50% protein-A Sepharose slurry for 1hour at 4°C. The protein-A Sepharose was peleted as before and washed 3 times quickly and then 4 times slowly (10 minutes at 4°C) in lysis buffer. The proteins were eluted from the beads by boiling in 50μl 1x sample buffer for 5

minutes at 100°C. The samples were analysed by SDS-PAGE as described in 2.6 and Western blotted as described in 2.7.

Immunoprecipitations from tissue samples

Tissue samples were homogenised in 3-4 volumes of PBS 1% NP40 or lysis buffer in a Teflon/ glass homogeniser using ~ 20 stokes. The homogenate was clarified by spinning at 100,000rpm for 15 minutes in a TL 100.3 rotor (Optima TLX ultracentrifuge Beckman Instruments). The supernatant was processed as above.

2.10 [^{32}P] orthophosphate labelling of tissue culture cells

Cells to be labelled were grown in a 150cm^2 tissue culture dish until they reached 80-90% confluency. The cells were washed with phosphate free media (DMEM) prior to labelling. The cells were labelled for 3 hours using 1mCi of [^{32}P] orthophosphate in 10ml of phosphate free DMEM. The labelling was terminated by removing the labelling media and washing the cells with ice cold DMEM three times. The cells were detergent extracted as in section 2.8 except the extraction buffer was supplemented with 1mM Na_3Vo_4 to inhibit phosphatases. Immunoprecipitations were performed from the detergent extract as previously described section 2.8.

2.11 Missorting assay

Qualitative missorting assay

mh cells were transiently transfected overnight with DNA encoding the delta subunit of the AP-3 complex, using the lipid based transfection reagent Fugene as described by the manufactures instructions. The expression vectors used were either pCEP-δ or pMEP- δ. The next day the cells were passaged onto glass mullti-well slides. If the expression vector used was pMEP the cells were induced with the addition of 2 μM cadmium.

The next day the cells were washed in 37°C DMEM and then incubated with antibodies against anti-LAMPI or CD44 diluted in DMEM for 6-7 hours (see antibodies table 2.2). In some experiments Alexa 350 wheat germ lectin (20μg/ml) was added to the incubation media in addition to the antibodies. The cells were washed six times with ice cold DMEM and fixed in –20°C methanol/acetone (if the cells were incubated in the presence of wheat germ lectin the DMEM used for washing the cells was supplemented with 50mM N-Acetyl-D-glucosamine to remove surface bound lectin). The cells were stained with an anti-delta antibody. The anti-delta antibody and endocytosed antibodies were visualised using fluorescently labelled secondary antibodies (see antibodies table 2.2). Images were captured either using a BioRad MRC 1000 confocal microscope or a Zeiss Axioplan microscope fitted with a MicroMax 1300 cooled CCD camera.

Quantitative missorting assay

pe cell stably transfected with β3B cDNA were passaged onto 24 well dishes and grown overnight (semi-confluent T75cm^2 flask for four multi-well dishes). The next day the cells were washed in 37°C DMEM and then incubated for 6-7 hours with rat anti-LAMP and CD44 antibodies diluted in DMEM (1/100). The cells were washed six times with ice cold DMEM, and fixed in –20°C methanol.

The cells were blocked in 1mg/ml BSA PBS (blocking solution) for 5 minutes, before being incubated with anti-rat or anti-Texas red antibodies for 30 minutes at room temperature (diluted 1/1000 in blocking solution). The cells were washed 3 times with blocking solution and then washed a further 3 times in blocking solution with 10 minute incubation periods. The cells were then incubated for 30 minutes with ^{125}I-Protein A (Amersham) at room temperature (diluted 1/2000 in blocking solution). The cells were washed 3 times with blocking solution and then washed a further 3 times in blocking solution with 10 minute incubation periods. The blocking solution was then removed and the cells extracted in 200μl of 1M NaOH for 1 minute. The extracted cells

were then transferred to 2ml eppendorf tubes and the amount ^{125}I-Protein A bound counted in a γ counter for 1 minute.

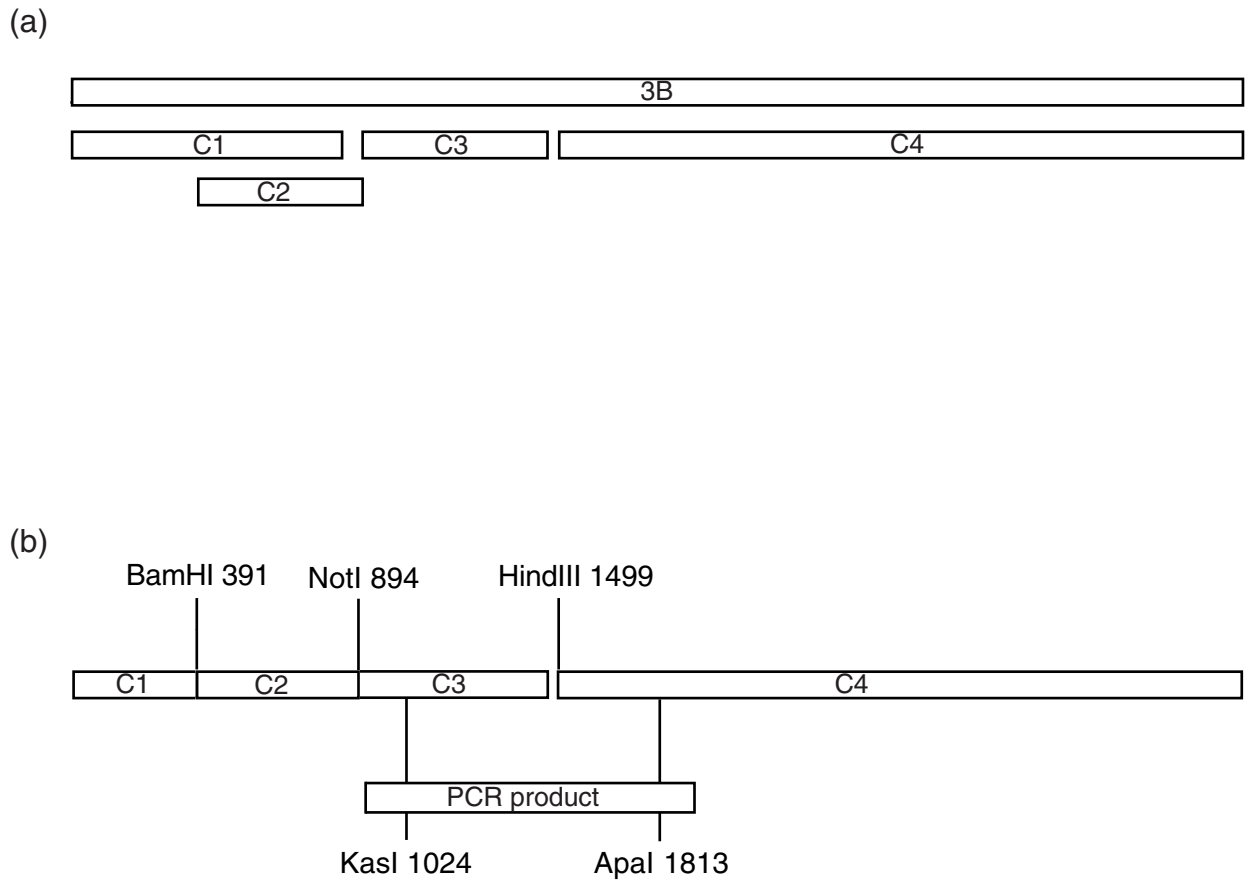


Figure 2.1

Diagram showing the aligment of 3B to the ESTs obtained from the Image consortium (a) and how 3B was assembled from the partial ESTs (b).

Chapter 3

The identification and cloning of the ubiquitously expressed isoform of β3B

3.1 Introduction

At the time this work was begun, a study by Simpson et al. (1996) had shown that p47 (μ3) and β-NAP (β3B) are components of a novel adaptor-like complex associated with two unknown proteins of 160kD and 25kD (Fig. 3.1)(Simpson et al., 1996). It seemed likely that the 160kD protein would share homology with α and γ adaptin and the 25kD protein would share homology with the σ1 and σ2 small chains, given that β3B shares homology with β1 and β2 adaptins and μ3A/μ3B with μ1 and μ2 medium subunits (Simpson et al., 1996). To identify the missing subunits of the adaptor-like complex the expressed sequence tags database (EST) was searched for clones that had homology to α and γadaptins and σ1 and σ2 small chains. The EST database was also searched for a ubiquitously expressed homologue of β3B. The reason for searching the EST database for a ubiquitously expressed isoform of β3B is there are two isoforms of μ3, one ubiquitous (μ3A) and one neuronal specific (μ3B) suggesting there is a ubiquitously expressed form of this adaptor-like complex. Five clones were identified and obtained from the IMAGE Consortium. Two clones had homology to the C-terminal portions of σ1 and σ2 and were approximately 80% identical to each other. The two sigma-like clones were isolated from human placenta and brain tissue (GenBank R23892 and R87391). One clone had homology to the N-terminal portions of α and γ and was isolated from human uterus tissue (GenBank T30164). Two clones had homology to the C-terminal region of β3B and were isolated from human liver and spleen tissue (GenBank R02669 and T98538). The σ-like ESTs were cloned by Dr Margaret S. Robinson and the α/γ–like EST were cloned and fully sequenced by Lina Christopoulou under my supervision (Simpson et al., 1997). The α/γ-like molecule was named δ and is ~15% identical to α and γ. The two σ-like molecules were named σ3A and σ3B and are ~84% identical to each other

and ~30% identical to σ1 and σ2. Multiple tissue Northern blots were probed using radiolabelled oligonucleotides corresponding to selected regions of δ, σ3A and σ3B; all three adaptor-like subunits were ubiquitously expressed (Simpson et al., 1997).

To investigate if the two ESTs that had homology to β3B encoded the same protein a multiple tissue Northern blot (Clontech) was probed sequentially with the two radiolabelled ESTs. Both ESTs bound a band of approximately the same size (4.4kb) on the Northern blot suggesting that they did encode the same protein (unpublished observations Fiona Simpson). The band that was recognised by the probes was present in all tissues on the Northern blot indicating that the isoform of β3B was ubiquitously expressed like μ3A, σ3A/σ3B and δ, suggesting that there is a ubiquitously expressed form of this complex (Simpson et al., 1997). From partial sequencing and restriction digests of the ESTs, it became apparent that the ESTs could not encode the full-length β3B so a library screen would have to be carried out to obtain the full-length clone (Fig. 3.2).

3.2 Cloning of the ubiquitously expressed isoform of β3B

To obtain a full-length clone encoding the ubiquitously expressed isoform of β3B a human heart cDNA λgt10 phage library was screened by probing duplicate filters using either radiolabelled R02669 or T98538 ESTs (described in Materials and Methods section 2.2). The library was screened using the two ESTs to increase the chances of a full-length clone being isolated. Using the above strategy nine clones were plaque purified and the DNA isolated from the phage. The isolated phage DNA was digested with EcoRI to recover the cDNA inserts. Out of the nine clones only three of the clones bound both probes, clones 1, 2 and 3, suggesting they might be full-length (Fig. 3.3). Clone 3 has an internal EcoRI site. Clone 3 was chosen for further analysis as when the two EcoRI fragments were added together they give a clone of ~3.5kb which is potentially large enough to encode the full-length β3B

homologue (β3B is encoded by ~3.3kb of DNA). The two clone 3 fragments were cloned into the EcoRI site of pBluescript (SK).

3.3 Sequencing and contig assembly

The two pBluescript clones were partially sequenced (described in *Materials and Methods* 2.2) using T3 and T7 to confirm that they encoded the β3B homologue. When it was confirmed that the 2 clones did encode the β3B homologue the clones were digested into 6 fragments and subcloned into pBluescript (SK). The fragments were sequenced as above using T3 and T7. From the sequence obtained a partial contig was assembled and the sequence used to design internal sequencing primers. From the final sequencing data a contig was assembled for the β3B isoform with complete agreement from both directions of sequencing.

The assembled contig contained 1 large predicted open reading frame (ORF) encoding a protein of 1093aa with a conserved Kozak sequence 5' to the start methione and several stop codons in all three reading frames 3' to the first stop codon (Kozak, 1987). To confirm that the correct ORF had been identified the protein sequence was compared to β1, β2 and β3B protein sequences (Fig. 3.4) (corrected β3B sequence was used for alignment; see section 3.5). The strong alignment with the homologues suggested that the correct ORF had been identified. In line with the adaptor nomenclature the newly cloned isoform was named β3A (the (A) stands for ubiquitous expression as with μ3A), GenBank accession number U91931.

3.4 Sequence comparison and hydropathy plots

The β3A and β3B are very similar with the β3A being 11 amino acids longer than β3B (Fig. 3.4). The main region of divergence between β3A and β3B is in the hinge region of the molecule, most clearly shown by a diagon plot (Fig. 3.5). β3A is related to β1 (21.6% identical) and β2 (21.4% identical) but is more homologous to β3B (62.1% identical) particularly in the N-terminals and

C-terminal domains. The homology between β3A, β1 and β2 is mainly restricted to the N-terminal domains of the proteins (Fig. 3.5). Like the other adaptins (α, γ and β) β3A contains the consensus sequences WIIGEY (WLIGEN) (Simpson et al., 1997) and KKLVY in its N-terminal portion (Fig. 3.4). The function of these conserved motifs is still unknown. β3A like other adaptins has a 3 domain structure consisting of an N-terminal domain of ~600 amino acids, a hinge domain (normally rich in acidic residues and prolines) and C-terminal ear domain. The hinge domains of β3A and β3B are very rich in acidic amino acids (D/E) and serine residues (Fig. 3.4). The very polar nature of the hinge region in β3A and β3B is most clearly shown by a hydropathy plot (Fig. 3.6) (Kyte and Doolittle, 1982). The hydropathy plots show that β3A and β3B have very hydrophilic hinge domains compared to β1 and β2 hinge domains. All of the beta subunits have several serine residues that could be potentially sites for casein kinase phosphorylation, and β3A and β3B have a very large number of these sites in the hydrophilic hinge region (Mercier, 1981) (Fig. 3.4).

3.5 Cloning of β3B

The group that cloned β3B (Newman et al., 1995) were unwilling to give access to the published β3B cDNA, so the EST database was searched for clones that could encode β3B. Several clones (GenBank 175331, 231345, 320375 and 165789) were isolated and a cloning strategy was designed to piece these ESTs together to make a full-length clone (Materials and Methods section 2.3 and Fig. 2.1). Part way through the cloning of β3B one of the sub cloning steps failed; further examination of the problem revealed that the predicted ApaI site at 1024bp was not present in the EST (GenBank 320375) (Newman et al., 1995). The EST was sequenced in both directions and did not contain an ApaI site but a KasI site at 1024bp. When the EST database was searched using the ESTs sequence several clones were identified and none of them contained an ApaI site at 1024bp confirming the sequence and suggesting there may be a sequencing error in original β3B clone. When the ESTs DNA sequence was compared to β3A and the published β3B DNA

sequences it is apparent that the published β3B DNA sequence contains 3 frame shifts starting at the predicted ApaI site (Fig. 3.7). The three frame shifts cause 14 amino acids in the region to be incorrect. When the protein sequence of the corrected β3B is compared to the sequence of β3A the two proteins are now more similar in the corrected region (Fig. 3.7). The corrected β3B sequence has the GenBank accession number AF022152.

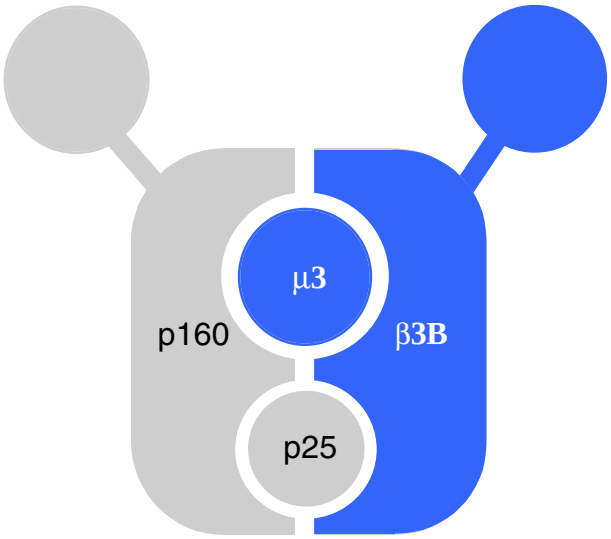


Figure 3.1

Diagram representing the proposed subunit composition of the adaptor-related complex. p160 is expected to have homology to α and γ and p25 is expected to have homology to σ1 and σ2.

Figure 3.2

Diagram showing the alignment of β3B to the ubiquitously expressed GenBank clones R02669 (a) and T98538 (b).

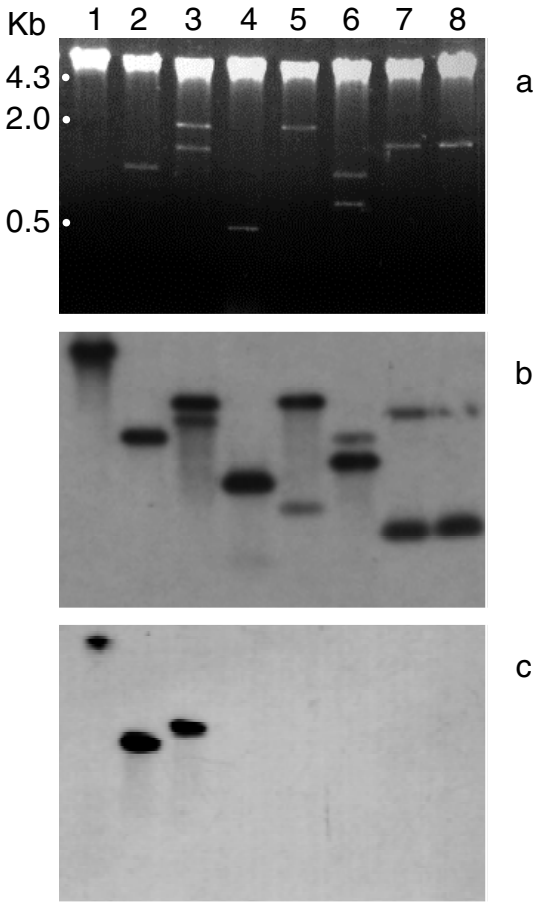


Figure 3.3

To obtain a full-length clone encoding the ubiquitously expressed isoform of β3B a human heart cDNA library was screened using the R02669 and T98538 ESTs as described in Materials and Methods 2.2. The (b) EST was excised from its vector using EcoRI and PacI, the (a) EST was isolated by PCR using T3 and T7 primers as the insert contained an internal EcoRI restriction site. The ESTs were radiolabelled as in Materials and Methods 2.2. The isolated phage were digested with EcoRI and run on a 1% agarose gel (panel a). The DNA was transferred to nylon membranes and then probed with either the R02669 EST (panel b) or the T98538 EST (panel c).

```
1    M T D S K Y - F T T T K K G E - - - - - - - - - - - - - - - - - - - - I F - - -
2    M T D S K Y - F T T N K K G E - - - - - - - - - - - - - - - - - - - - I F - - -
3A   M S S N S F P Y N E Q S G G G E A T E L G Q E A T S T I S P S G A F G L F S S D
3B   M S A A P - A Y S E D K G G S A G P - - G E P E Y G H D P A S G - - G I F S S D

1    - - - - - E L K A E L N S D K K E K K K E A V K K V I A S M T V G K D V S A L F
2    - - - - - E L K A E L N N E K K E K R K E A V K K V I A A M T V G K D V S S L F
3A   L K K N E D L K Q M L E S N K D S A K L D A M K R I V G M I A K G K N A S E L F
3B   Y K R H D D L K E M L D T N K D S L K L E A M K R I V A M I A R G K N A S D L F

1    P D V V N C M Q T D N L E L K K L V Y L Y L M N Y A K S Q P D M A I M A V N T F
2    P D V V N C M Q T D N L E L K K L V Y L Y L M N Y A K S Q P D M A I M A V N S F
3A   P A V V K N V A S K N I E I K K L V Y V Y L V R Y A E E Q Q D L A L L S I S T F
3B   P A V V K N V A C K N I E V K K L V Y V Y L V R Y A E E Q Q D L A L L S I S T F

1    V K D C E D P N P L I R A L A V R T M G C I R V D K I T E Y L C E P L R K C L K
2    V K D C E D P N P L I R A L A V R T M G C I R V D K I T E Y L C E P L R K C L K
3A   Q R A L K D P N Q L I R A S A L R V L S S I R V P I I V P I M M L A I K E A S A
3B   Q R G L K D P N Q L I R A S A L R V L S S I R V P I I V P I M M L A I K E A A S

1    D E D P Y V R K T A A V C V A K L H D I N A Q L V E D Q G F L D T L K D L I S D
2    D E D P Y V R K T A A V C V A K L H D I N A Q M V E D Q G F L D S L R D L I A D
3A   D L S P Y V R K N A A H A I Q K L Y S L D P E - - Q K E M L I E V I E K L L K D
3B   D M S P Y V R K T A A H A I P K L Y S L D S D - - Q K D Q L I E V I E K L L A D

1    S N P M V V A N A V A A L S E I A E S H P S S N L L D L N P Q S I N K L L T A L
2    S N P M V V A N A V A A L S E I S E S H P N S N L L D L N P Q N I N K L L T A L
3A   K S T L V A G S V V M A F E E V C P D R - - - - - I D L I H K N Y R K L C N L L
3B   K T T L V A G S V V M A F E E V C P E R - - - - - I D L I H K N Y R K L C N L L

1    N E C T E W G Q I F I L D C L A N Y M P K - - - - - - - - - - - - - - - - - - -
2    N E C T E W G Q I F I L D C L S N Y N P K - - - - - - - - - - - - - - - - - - -
3A   V D V E E W G Q V V I I H M L T R Y A R T Q F V S P W K E G D E L E D N G - K N
3B   I D V E E W G Q V V I I S M L T R Y A R T Q F L S P T Q N E S L L E E N A E K A

1    - - - - D D R E A Q S I C E R V T - - - - - - - - - P R L S H A N S A V V L S A
2    - - - - D D R E A Q S I C E R V T - - - - - - - - - P R L S H A N S A V V L S A
3A   F Y E S D D D Q K E K T D K K - - - - - - - - - K K P Y T M D P D H R L L I R N
3B   F Y G S E E D E A K G A G S E E T A A A A A P S R K P Y V M D P D H R L L L R N

1    V K V L M K F M E - - - M L S K D L D Y Y G T L L K K - - - - L A P P L V T L L
2    V K V L M K F L E - - - L L P K D S D Y Y N M L L K K - - - - L A P P L V T L L
3A   T K P L L Q S R N A A V V M A V A Q L Y W H I S P K S E A G I I S K S L V R L L
3B   T K P L L Q S R S A A V V M A V A Q L Y F H L A P K A E V G V I A K A L V R L L

1    S A E P E L Q Y V A L R N I N L I V Q K R P E I L K H E M K V F F V K Y N D P I
2    S G E P E V Q Y V A L R N I N L I V Q K R P E I L K Q E I K V F F V K Y N D P I
3A   R S N R E V Q Y I V L Q N I A T M S I Q R K G M F E P Y L K S F Y V R S T D P T
3B   R S H S E V Q Y V V L Q N V A T M S I K R R G M F E P Y L K S F Y I R S T D P T

1    Y V K L E K L D I M I R L A S Q A N I A Q V L A E L K E Y A T E V D V D F V R K
2    Y V K L E K L D I M I R L A S Q A N I A Q V L A E L K E Y A T E V D V D F V R K
3A   M I K T L K L E I L T N L A N E A N I S T L L R E F Q T Y V K S Q D K Q F A A A
3B   Q I K I L K L E V L T N L A N E T N I P T V L R E F Q T Y I R S M D K D F V A A

1    A V R A I G R C A I K V E Q S A E R C V S T L L D L I Q T K V N Y V V Q E A I V
2    A V R A I G R C A I K V E Q S A E R C V S T L L D L I Q T K V N Y V V Q E A I V
3A   T I Q T I G R C A T N I L E V T D T C L N G L V C L L S N R D E I V V A E S V V
3B   T I Q A I G R C A T N I G R V R D T C L N G L V Q L L S N R D E L V V A E S V V

1    V I K D I F R K Y P N K Y E S V I A T L C E N L D S L D E P E A R A A M I W I V
2    V I R D I F R K Y P N K Y E S I I A T L C E N L D S L D E P D A R A A M I W I V
3A   V I K K L L Q M Q P A Q H G E I I K H M A K L L D S I T V P V A R A S I L W L I
3B   V I K K L L Q M Q P A Q H G E I I K H L A K L T D N I Q V P M A R A S I L W L I

1    G E Y A E R I D N - A D E L L E S F L E G F H D E S T Q V Q L Q L L T A I V K L
2    G E Y A E R I D N - A D E L L E S F L E G F H D E S T Q V Q L T L L T A I V K L
3A   G E N C E R V P K I A P D V L R K M A K S F T S E D D L V K L Q I L N L G A K L
3B   G E Y C E H V P R I A P D V L R K M A K S F T A E E D I V K L Q V I N L A A K L

1    F L K K P T E T Q E L V Q Q V L S L A T Q D S D N P D L R D R G Y I Y W R L L S
2    F L K K P S E T Q E L V Q Q V L S L A T Q D S D N P D L R D R G Y I Y W R L L S
3A   Y L T N S K Q T K L L T Q Y I L N L G K Y D - Q N Y D I R D R T R F I R Q L I V
3B   Y L T N S K Q T K L L T Q Y V L S L A K Y D - Q N Y D I R D R A R F T R Q L I V

1    T D P V A - - - - - - A K E V V L A E K P L I S E E T D L I E P T L - - L D E L
2    T D P V T - - - - - - A K E V V L S E K P L I S E E T D L I E P T L - - L D E L
3A   P N E K S G A L S K Y A K K I F L A Q K P A P L L E S P F K D R D H F Q L G T L
3B   P S E Q G G A L S R H A K K L F L A P K P A P V L E S S F K D R D H F Q L G S L

1    I C Y I G T L A S V Y H K P P S - - - - - - - - - - - - - - - - - - - - - - - -
2    I C H I G S L A S V Y H K P P N - - - - - - - - - - - - - - - - - - - - - - - -
3A   S H T L N I K A T G Y L E L S N W P E V A P D P S V R N V E V I E L A K E W T P
3B   S H L L N A K A T G Y Q E L P D W P E E A P D P S V R N V E V - - - - P E W T K

1    - - - - - - - - - - - - A F - - - - - V E G G R G V V H K S L P P R T A S S E S
2    - - - - - - - - - - - - A F - - - - - V E G S H G I H R K H L P I H H G S T D A
3A   - A G K A K Q E N S A K K F Y S E S E E E E D S S D S S - S D S E S E S G S E S
3B   C S N R E K R K E K E K P F Y S D S E G E S G P T E S A D S D P E S E S E S D S
```

```
1    A E S P E T A P T G A P P G E Q P D V I P A Q G D L L - - - - G D L L N L D L G
2    G D S P - V G T T T A T N L E Q P Q V I P S Q G D L L - - - - G D L L N L D L G
3A   G E Q G E S G E E G D S N E D S S E D S S S E Q D S E S G R E S G L E N K R T A
3B   K S S S E S G S G E S S S E S D N E D Q D E D E E K G R G S E S E - Q S E E D G

1    P P V S G P P L A T S S V Q M G A V D L L G G G L D S L M G D E - - P E G I G G
2    P P V N V P Q V - - S S M Q M G A V D L L G G G L D S L V G Q S F I P S S V P A
3A   K R N S K A K G K S D S E D G E K E N E K S K T S D S S N D E S S S I E D S S S
3B   K R K T K K K V P E - - - - - - - - - - R K G E A S S S D E G S D S S S S S S

1    T - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
2    T - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
3A   D S E S E S E P E S E S E S R R V T K E K E K T K Q D R T P L T K D V S L L D L
3B   E S E M T S E S E E E Q L E P A S W S R K T P P S S K S A P A T K E I S L L D L

1    - N F V A P P T A A V - - P A N L G A P I G S G L S D L F D L T S G - - - V G T
2    - - F A P S P T P A V - - - - - - - - V S S G L N D L F E L S T G - - - I G M
3A   D D F N P V S T P V A L P T P A L S P S L M A D L E G L - H L S T S S S V I S V
3B   E D F T P P S V Q P V S P P A I V S T S L A A D L E G L - T L T D S T L V P S L

1    L S G S Y V A P K A - V W L P A M K A K G L E I S G T F T R Q V G S - - - - - I
2    A P G G Y V A P K A - V W L P A V K A K G L E I S G T F T H R Q G H - - - - - I
3A   S T P A F V P T K T H V L L H R M S G K G L A A H Y F F P R Q P C I F G D K M V
3B   L S P V S G V G R - Q E L L H R V A G E G L A V D Y T F S R Q P F S G D P H M V

1    S M D L Q L T N K A L Q V M T D F A I Q F N R N S F G L A P A A P L Q V H A P L
2    Y M E M N F T N K A L Q H M T D F A I Q F N K N S F G V I P S T P L A I H T P L
3A   S I Q I T L N N T T D R K I E N I H I G E K K L P I G M K - M H V F N P I D S L
3B   S V H I H F S N S S D T P I K G L H V G T P K L P A G I S - I Q E F P E I E S L

1    S P N Q T V E I S L P L S T V G S V M K M E P L N N L Q V A V K N N I D V F Y F
2    M P N Q S I D V S L P L N T L G P V M K M E P L N N L Q V A V K N N I D V F Y F
3A   E P E G S I T V S M G I D F C D S T Q T - - - - A S F Q L C T K D - - D C F N V
3B   A P G E S A T A V M G I N F C D S T Q A - - - - A N F Q L C T Q T - - R Q F Y V

1    S T L Y P L H I L F V - - - - - - E D G K M D R Q M F L A T W K D I P N E N E A
2    S C L I P L N V L F V - - - - - - E D G K M E R Q V F L A T W K D I P N E N E L
3A   N I Q P P V G E L L L P V A M S E K D F K K E Q G V L T G M - - - - - N E T S A
3B   S I Q P P V G E L M A P V F M S E N E F K K E Q G K L M G M - - - - - N E I T E

1    Q F Q I R D C P L N A E A A S S K L Q S S N I F T V A K R N V E G Q D M L Y Q S
2    Q F Q I K E C H L N A D T V S S K L Q N N N V Y T I A K R N V E G Q D M L Y Q S
3A   V I I A A P Q N F T P S V I F Q K V V N V A N V G A V - P S G Q D N I H R F A A
3B   K L M L P D T C R S D H I V V Q K V T A T A N L G R V - P C G T S D E Y R F A G

1    L K L T N G I W V L A E L R I Q P G N P S C T
2    L K L T N G I W I L A E L R I Q P G N P N Y T L S L K C R A P E V S Q Y I Y Q V
3A   K T V H S G S L M L V T V E L K E G S T A - Q L I I N T E K T V I G S V L L R E
3B   R T L T G G S L V L L T L D A R P A G A A - Q L T V N S E K M V I G T M L V K D

1
2    Y D S I L K N
3A   L K P V L S Q G
3B   V I Q A L T Q
```

Figure 3.4

Protein sequence alignment of 1, 2, 3A and 3B; identical residues are boxed. Potential serine phosphorylation sites are bold (**S**). Conserved consensus sequences are underlined (_). The hinge region of 3A and 3B is shaded ( ).

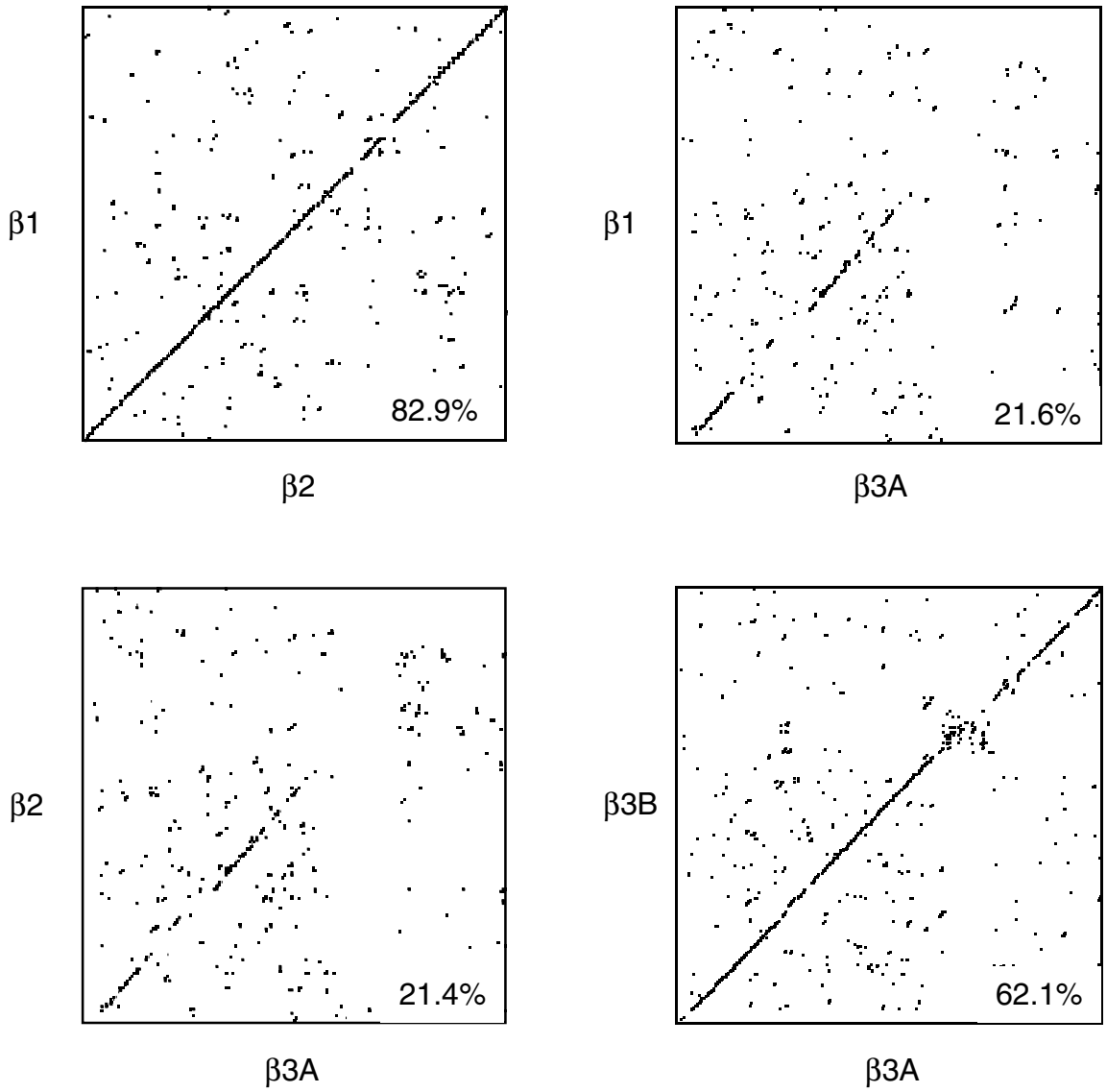


Figure 3.5

A series of Diagon plots comparing the protein sequence of β3A to β1, β2 and β3B. The greater the similarity between proteins the stronger the diagonal line.

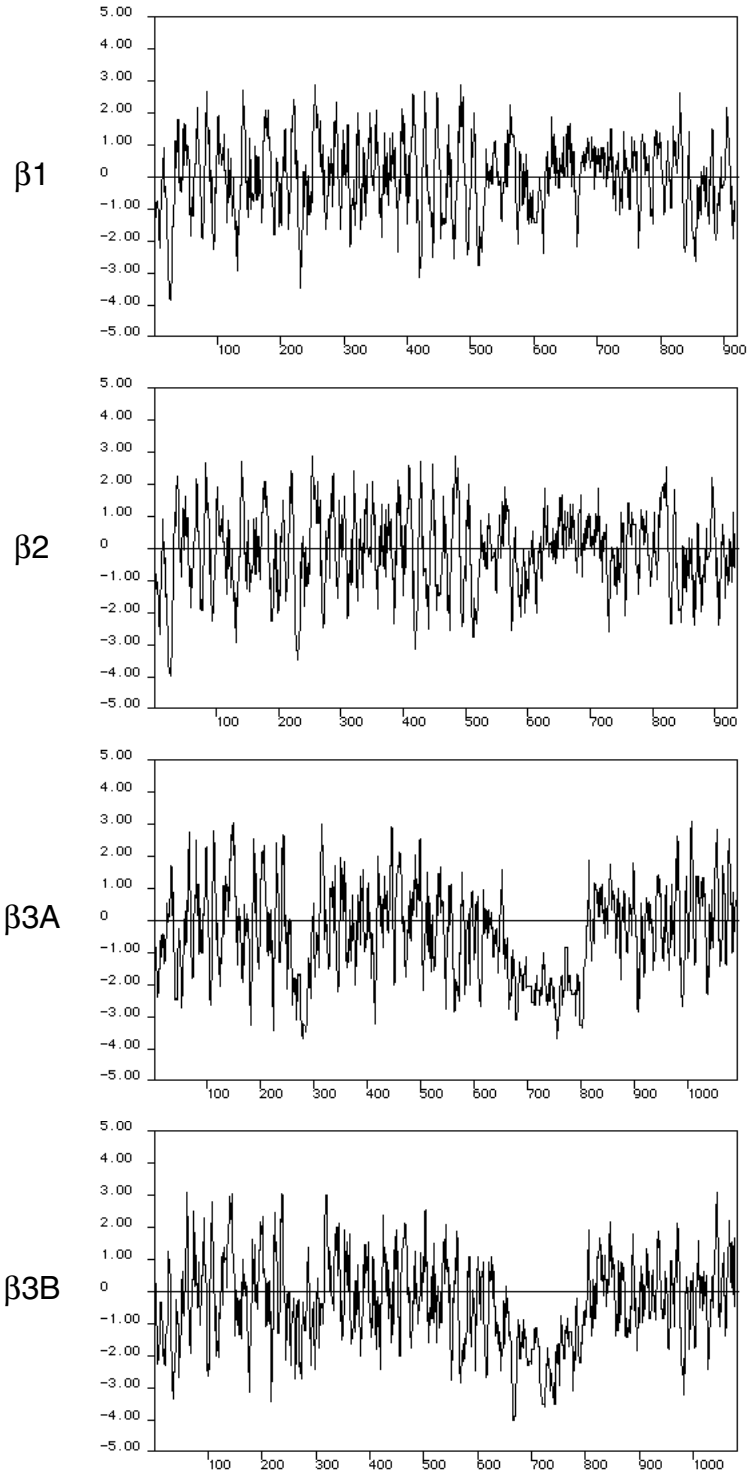


Figure 3.6

A series of hydropathy plots calculated from the protein sequences of β1, β2, β3A and β3B. A negative hydropathy index indicates a hydrophilic region in the protein.

a

```
3B   G T G A T G G C G G T G G C G C A G C T C T A C T T C C A C
3B*  G T G A T G G C G G T G G C G C A G C T C T A C T T C C A C
3A   G T T A T G G C A G T T G C T C A G C T G T A T T G G C A C

     C T G G - G C C C A A G G C G G A A G T G G - C G T C A T C
     C T G G C G C C C A A G G C G G A A G T G G G C G T C A T C
      A T A T C A C C A A A A T C T G A A G C T G G C A T A A T T

     G C C A A G G C G C T G G T G C G C - T G C T G C G C A G C
     G C C A A G G C G C T G G T G C G C C T G C T G C G C A G C
      T C T A A A T C A C T A G T G C G T T T A C T T C G T A G C
```

b

```
3B   V M A V A Q L Y F H L G P R R K W - R H R Q G A G A L L R S
3B*  V M A V A Q L Y F H L A P K A E V G V I A K A L V R L L R S
3A   V M A V A Q L Y W H I S P K S E A G I I S K S L V R L L R S
```

Figure 3.7

DNA (a) and protein (b) sequence alignment of 3A, corrected 3B (*) and published 3B.

Chapter 4

Characterisation of the adaptor-related AP-3 complex

4.1 Introduction

To further characterise the newly identified adaptor-like subunits (δ, β3A σ3A and σ3B) and to determine if they are associated with the previously described adaptor-like complex (containing β3B and μ3), portions of them were expressed as GST fusion proteins and used to raise polyclonal antibodies. Antibodies against the N-terminal portion of the δ subunit and the C-terminal portion of the σ3B subunit were raised by Fiona Simpson. The two anti-δ affinity-purified sera recognised an antigen of ~160kD on a pig brain homogenate Western blots. The antigen recognised by the anti-δ antibodies runs larger than would be predicted from the open reading frame (ORF)(130kD) for the δ subunit; this is probably due to the large amount of charged residues in its hinge domain. The anti-σ3B antibodies recognised several bands on a pig brain homogenate Western blots although both affinity-purified sera recognised a band of ~21kD, which is approximately the correct size for σ3B from its predicted ORF (21kD) (Simpson et al., 1997).

I raised antibodies against the hinge domain of the β3A subunit and the C-terminal portion of the σ3A subunit. Antibodies were raised against these domains as they have been proven to be antigenic in the cases of β1, β2, σ1 and σ2.

4.2 Characterisation of anti-σ3A and β3A antibodies

To characterise β3A and σ3A, antibodies were raised against the hinge domain (amino acids 644-814) of β3A and the C-terminal domain (amino acids 112-192) of σ3A (Materials and Methods sections 2.3 and 2.5). Two rabbits were injected with each antigen and the affinity purified sera used to probe pig brain homogenate Western blots. The anti-σ3A antibodies reacted with several antigens on the Western blot although both sera shared an

antigen in common at ~21kD which is approximately the correct size for σ3A from its predicted ORF (21kD)(data not shown). The anti-β3A sera reacted with several antigens on the Western blot although both sera shared an antigen in common at ~120kD which is approximately the correct size for β3A from its predicted ORF (121kD) (data not shown).

To confirm that the antigens anti-σ3A and β3A antibodies recognised on the Western blots were correct, native immunoprecipitations from big brain tissue followed by Western blotting were performed (Materials and Methods sections 2.8 and 2.9). After immunoprecipitation and Western blotting both the anti-σ3A and β3A antibodies recognised the same antigens as were previously recognised from the pig brain homogenate Western blots, confirming that the σ3A is ~21kD and the β3A is ~120kD (Fig. 4.1).

4.3 Subunit composition of the adaptor-related complex

To determine if δ, β3A/B, σ3A/B and μ3A/B were all part of the same complex a series of native immunoprecipitations followed by Western blotting experiments were performed as in Materials and Methods sections 2.8 and 2.9. Pig brain samples were immunoprecipitated under native conditions with antibodies against δ, β3B, σ3A/B (mixture of σ3A and σ3B antibodies were used for immunoprecipitations and Western blotting) and an anti-γ adaptin antibody to bring down the AP-1 complex as a control. The reason for using pig brain tissue was the β3B antibody is tissue specific. Rat liver was immunoprecipitated under native conditions with an antibody against β3A. Strips were cut from the Western blotted and probed in the appropriate regions for δ, β3A/B, μ3A/B and σ3A/B (μ3 antibody recognises both μ3A and μ3B on a Western blot) (Fig. 4.2).

The anti-β3B antibody not only brings down β3B and μ3, as has been previously reported, but also brings down δ and σ3A/B (which appears as a doublet, presumably as there are two isoforms). The β3B IP does not bring down γ as expected, since γ is a subunit of the AP-1 complex. Similarly the

anti-δ and σ3A/B antibodies also bring down δ, β3A/B, μ3A/B and σ3A/B and not γ (the σ3A/B IP is not as strong as the other immunoprecipitations presumably as only one of the anti-σ3 antibodies immunoprecipitates strongly). Also as expected the anti-β3A antibody also brings down δ, β3A, μ3A and σ3A/B, suggesting that the AP-3 complex is also present in non-neuronal tissues.

These results indicates that, like a conventional adaptor complex, the adaptor-like complex consists of α/γ like subunit (δ), a β subunit, a μ subunit and a σ subunit. We have named the complex AP-3.

4.4 Localisation of the AP-3 complex in non-neuronal cells

Previously the AP-3 complex had been localised by immunoflourescence and immunogold electron microscopy using antibodies against β3B. However it was only possible to localise the complex in neurons or in cells which had been permeabilised and been incubated in pig brain cytosol (*in vitro* recruitment) because of the tissue specificity of the antibodies (Simpson et al., 1996). With the production of an antibody against the δ subunit of the AP-3 complex by Fiona Simpson, it became possible to localise the endogenous complex in non-neuronal cells at the level of immunoflourescence.

Primary mouse fibroblast were fixed and stained for the δ subunit of the AP-3 complex (Materials and Methods section 2.6) (Fig. 4.3). The staining pattern is mainly perinuclear with punctate labelling extending out into the periphery of the cell. This staining is reminiscent of patterns seen with markers of the endosomal system, therefore to investigate this further cells were doubled labelled for δ and markers for the early and late endocytic systems.

NRK cells were allowed to endocytose a monoclonal anti-transferrin receptor antibody (1/100 in DMEM) for 1 hour. The cells were fixed and stained with anti-δ (Materials and Methods section 2.6) (Fig 4.4). As the cells were allowed to endocytose the anti-transferrin receptor antibody for 1 hour the early and

late endocytic systems have been labelled (Hopkins and Trowbridge, 1983). There is some staining of δ and the transferrin receptor that coincide in the perinuclear region of the cell, although there is a large amount of staining that does not coincide. At this level of resolution it is difficult to tell if the regions that do coincide are actually colocalised or not. To resolve this question the study would have to be repeated at the level of immunogold electron microscopy, unfortunately it was impossible to carry out the immunogold electron microscopy, as the anti-δ antibodies do not work under the fixation conditions required.

COS cells were fixed and stained for anti-CD63 and anti-δ antibodies (cells were processed as in Materials and Methods section 2.6) (Fig.4.5). CD63 mainly is localised to late endocytic structures and lysosomes (Metzelaar et al., 1991). The two staining patterns do not coincide so suggesting the AP-3 complex is not associated with this compartment.

4.5 The ubiquitously expressed AP-3 complex is not localised or enriched in rat clathrin-coated vesicles

Previously it had been shown that the AP-3 complex in neuronal cells did not colocalise with clathrin at the level of immunofluorescence and immunogold electron microscopy (Simpson et al., 1996). To investigate if this was also the case for the ubiquitously expressed AP-3 complex, NRK cells were fixed and stained with antibodies against anti-δ and anti-clathrin heavy chain (processed as in Materials and Methods section 2.6)(Fig. 4.6). The two patterns look very similar although there is very limited colocalisation. The small amount of colocalisation in the perinuclear region is consistent with earlier observations using immunogold electron microscopy, which revealed that β3B and clathrin were often in close proximity but were on different budding profiles (Simpson et al., 1996). This result, as with the previous study findings, suggests that the AP-3 complex is not associated with clathrin.

The Simpson et al. (1996), study had also shown that the neuronal AP-3 complex was not enriched in pig brain clathrin-coated vesicles (CCV) (Simpson et al., 1996). To investigate if this was also true for the ubiquitously expressed AP-3 complex, rat liver homogenate and rat liver CCV and were Western blotted with anti-σ3A and control σ1 antibodies (rat liver clathrin-coated vesicles were a gift from Dr M. S. Robinson)(Fig. 4.7). σ1, a subunit of the AP-1 complex, is detectable in the homogenate and is very highly enriched in the clathrin-coated vesicles, unlike σ3A which is only detectable in the liver homogenate. This result suggests that the ubiquitously expressed AP-3 complex, like the neuronal AP-3 complex, does interact with clathrin in a manner like AP-1 or AP-2.

4.6 Phosphorylation of the AP-3 complex

It has been previously shown that β3B, a subunit of the neuronal AP-3 complex is the major target for phosphorylation in the complex (Newman et al., 1995). Like β3B, β3A has many potential casein kinase phosphorylation sites in its hinge domain. To investigate if β3A is the major target for phosphorylation in the ubiquitously expressed AP-3 complex, NRK cells were labelled for 3 hours with 1mCi of ^{32}P-orthophosphate. The cells were detergent extracted and immunoprecipitated with antibodies raised against γ, σ3A, β3A and δ (γ was used as a control)(Materials and Methods section 2.10). Under the conditions used only one major phosporylated protein at ~120kD was detected in the σ3A, β3A and δ immunoprecipitations but not in the γ control immunoprecipitation (Fig. 4.8). The size of the phosporylated band and its presence only in the anti-AP-3 immunoprecipitation would suggest that the phospho-protein is the β3A subunit of the AP-3 complex.

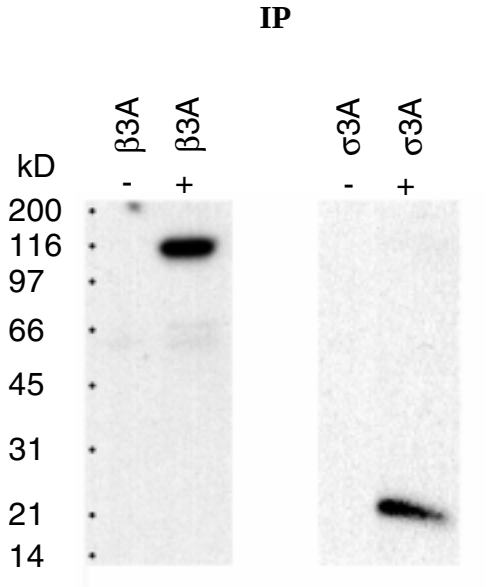


Figure 4.1

Native immunoprecipitations from pig brain tissue were performed using anti-β3A or σ3A antibodies followed by Western blotting with the above antibodies (- indicates that no antibody was added to the immunoprecipitation and the sample was only incubated with protein-A Sepharose). Immunoprecipitations and Western blotting were performed as in Materials and Methods sections 2.8 and 2.9. The immunoprecipitations indicate that β3A is ~120kD and σ3A is ~21kD.

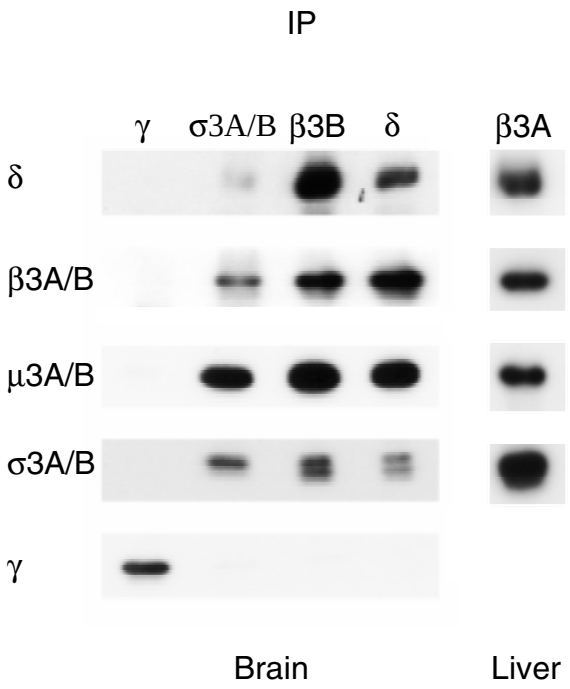


Figure 4.2

Pig brain samples were immunoprecipitated under native conditions with affinity-purified polyclonal antibodies against γ, σ3A/B, β3B and δ. A rat liver sample was immunoprecipitated under native conditions with a affinity-purified polyclonal antibody against β3A. The samples were subjected to SDS-PAGE and Western blotted, and the appropriate regions were probed with antibodies against γ, σ3A/B, β3A/B and δ (the brain immunoprecipitations were blotted with β3B and the liver immunoprecipitation blotted with β3A). Western blots and immunoprecipitations were performed as in Materials and Methods sections 2.8 and 2.9. The immunoprecipitations indicate that the AP-3 complex, like coventional adaptor complexes, consists of a α/γ like subunit (δ), a β subunit, a μ subunit, and a σ subunit.

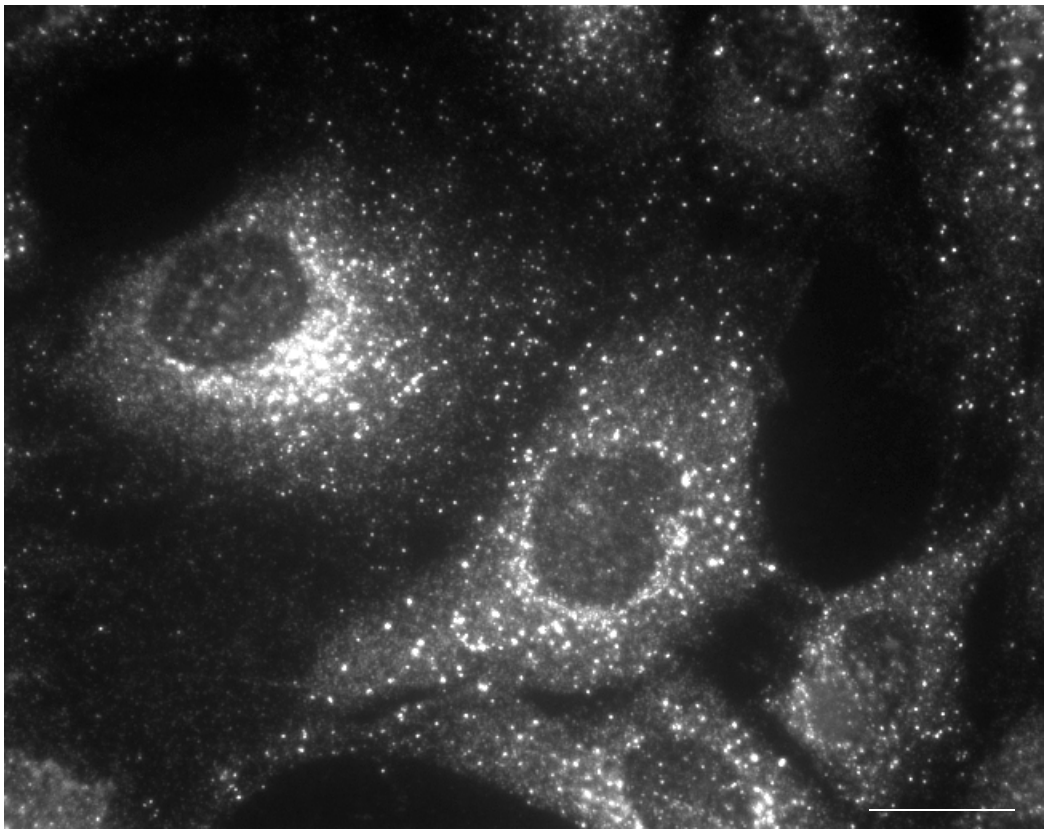


Figure 4.3

Primary mouse fibroblasts fixed with -20°C methanol/acetone and labelled with an antibody against the δ subunit of the AP-3 complex. The cells were processed as in Materials and Methods section 2.6. Scale bar 20μM.

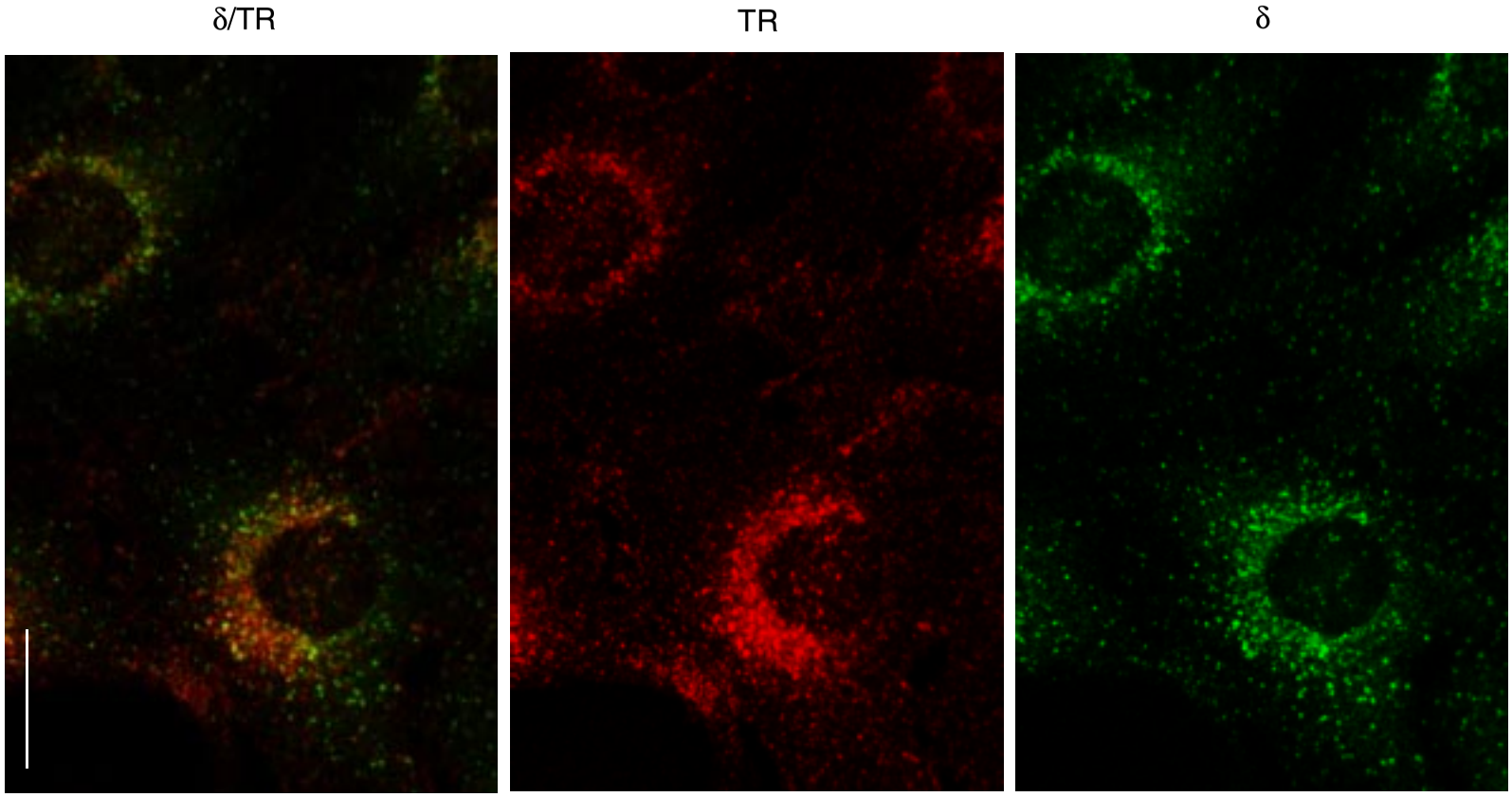


Figure 4.4

NRK cells were fed an anti-transferrin receptor antibody (1/100 DMEM) for 1 hour at 37°C. The cells were fixed and stained with an antibody against the δ subunit of the AP-3 complex (cells were processed as in Materials and Methods section 2.6). The merged image shows limited colocalisation. Scale bar 20μM

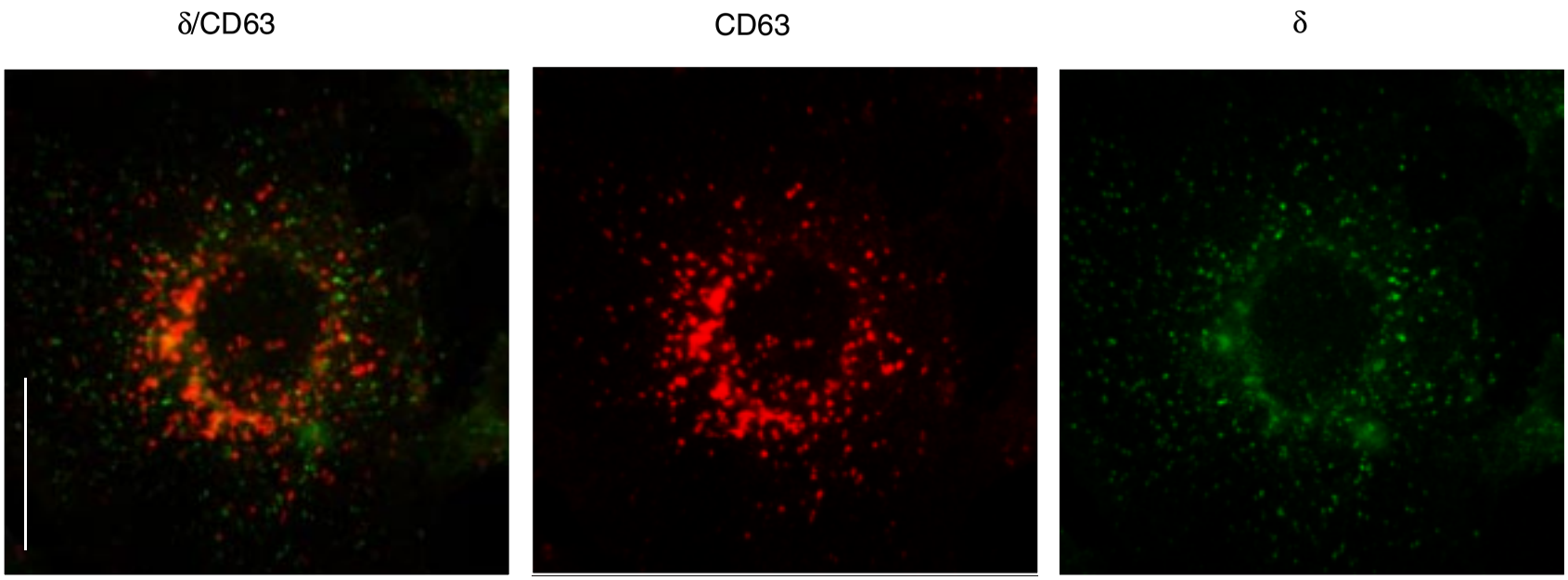


Figure 4.5

COS cells were fixed and stained with antibodies against the δ subunit of the AP-3 complex and CD63 (cells were processed as in Materials and Methods section 2.6). The merged image shows that the two patterns do not colocalise. Scale bar 20μM.

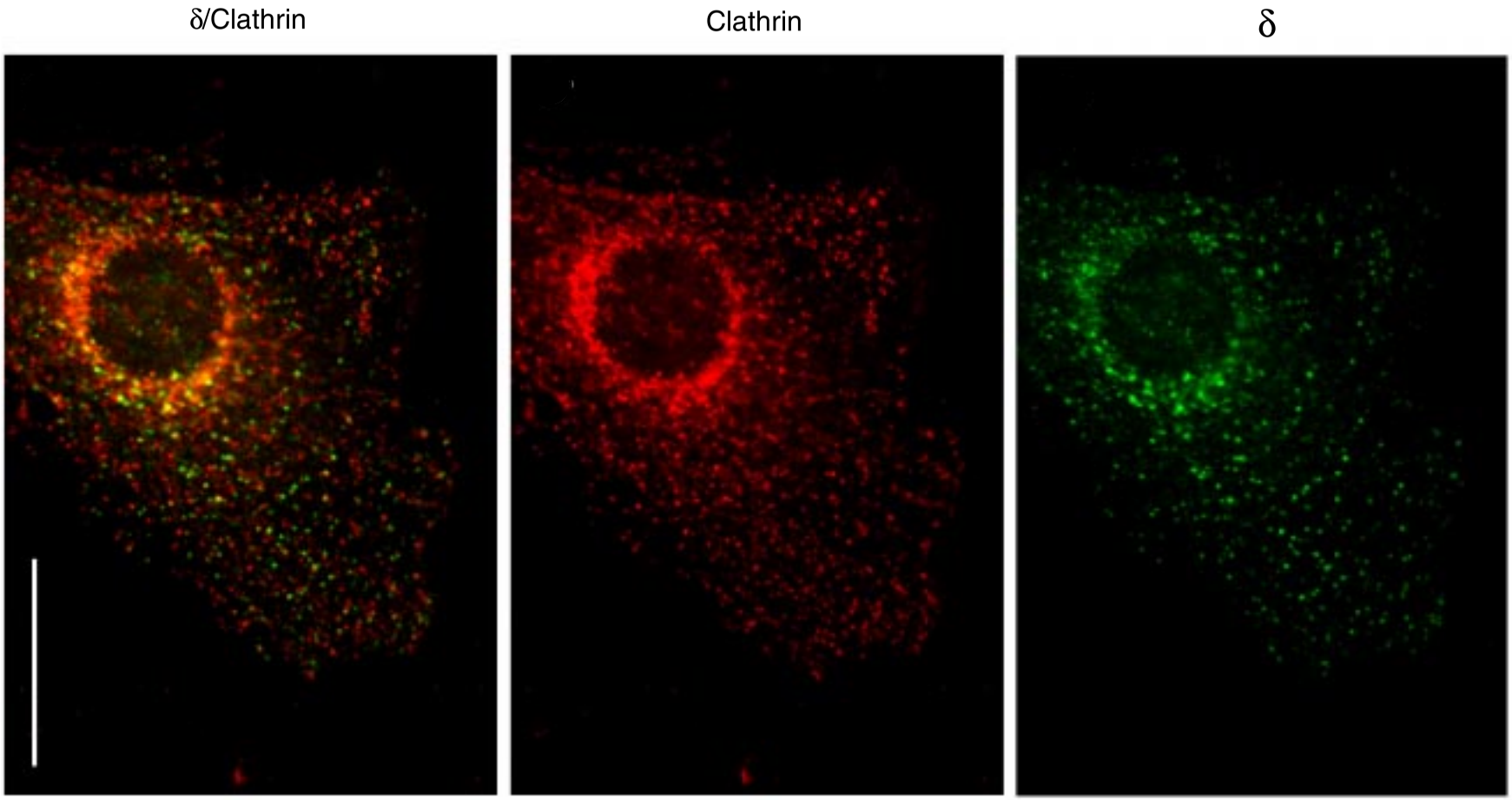


Figure 4.6

Confocal micrographs of an NRK cell labelled for δ and clathrin. NRK cells were fixed and labelled with anti-δ and a antibody against the clathrin heavy chain. Cells were processed as in Materials and Methods section 2.6. The merged images show limited overlap consistent with the two coats being on similar membranes but different budding profiles. Scale bar 20μM

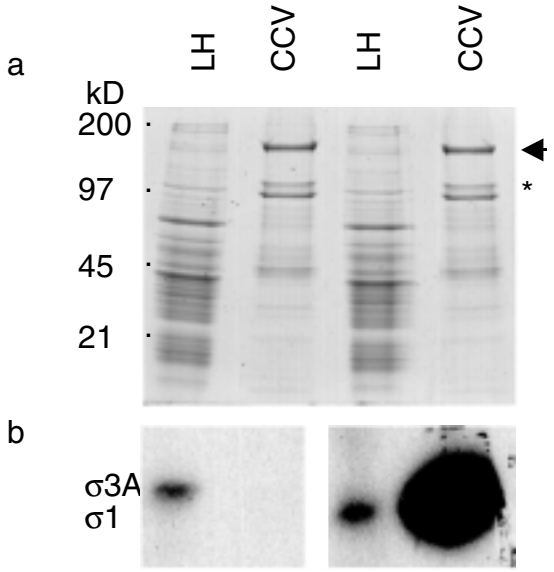


Figure 4.7

Panel (a) shows rat liver homogenate (LH) and rat liver clathrin coated vesicles (CCV) run on a 10% acrylamide gel stained with Coomasie blue (◄ indicates the clathrin heavy chain, * indicates the adaptins). There is approximately twice as much LH loaded per lane as CCV. Liver homogenate was generated by homogenising fresh rat liver in phosphate buffered saline (Materials and Methods section 2.7).

Panel (b) shows a Western blot from an equivalent gel as shown in panel (a). The appropriate region of the blot was probed with either anti-σ3A or σ1 antibodies. Western blotting was performed as in Materials and Methods section 2.8. The Western blots indicate that the AP-3 complex is notenriched inCCV.

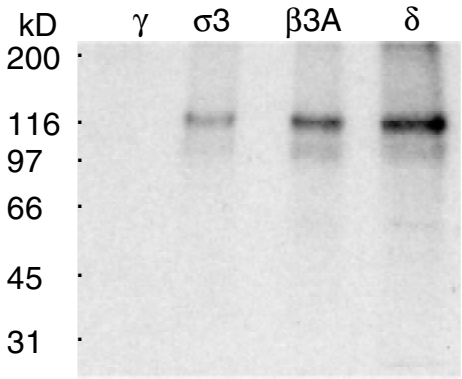


Figure 4.8

NRK cells were labelled for 3 hours with 1mCi ^{32}P-orthophosphate. The cells were detergent extracted and native immunoprecipitations with antibodies against γ, σ3A, β3A and δ were performed (Materials and Methods section 2.9). The samples were resolved on a 8.5% acrylamide gel and then stained with Coomasie blue. The gel was dried and exposed to X-ray film overnight. The immunoprecipitations suggest that the β3A subunit is phosphorylated.

Chapter 5

Insights into the function of the AP-3 complex

5.1 Introduction

Mutant Drosophilia

The first insights into the function of the AP-3 complex came from a study carried out by Dr Margaret S. Robinson, who searched the non-redundant EST database for AP-3 subunit homologues, and discovered that the human δ subunit is closely related to the protein product of the *Drosophila garnet* gene (Simpson et al., 1997). The *garnet* (*g*) locus was first described in 1916 as a fly eye colour mutant, and was cloned by P element tagging by Dr V. Llyod (GenBank DMU31351). The protein encoded by the *garnet* gene and the human δ subunit show a high degree of homology in their N-terminal domains, with a stretch of ~100 amino acids where the two proteins are ~96% identical. The high degree of homology suggests that *garnet* gene encodes the *Drosophila* δ subunit. To analyse the mutant phenotype Dr M. S. Robinson obtained wild type and *garnet* flies (two *garnet* alleles were examined, g^{3} and g^{53d}), sections were cut from their eyes and examined by bright field and phase contrast microscopy. The sections revealed that the organisation of the g^{3} and g^{53d} fly eyes appeared normal compared to wild type eyes, although there was a reduction in the pigmentation of the cells surrounding the ommatidia, especially in g^{53d} (the g^{53d} mutation only effects eye pigmentation while g^{3} effects both eye and body pigmentation). This result suggests that the AP-3 complex might be playing a role in the biogenesis of pigment granules. Pigment granules are thought to be modified lysosomes so it is possible that the AP-3 complex also is involved in the biogenesis of lysosomes and/or lysosome-related organelles (Simpson et al., 1997).

Mutant yeast

Cowles et al. (1997), performed a genetic screen for genes required for the transport of alkaline phosphatase (ALP) to the vacuole (Cowles et al., 1997a). The screen was designed to select for proteins that when over-expressed

interfered with the transport of an ALP-based construct. Two genes when over-expressed had this phenotype, ALP5 and ALP6. ALP5 encodes the gene product Apl5p that is most homologous to the human δ subunit of the AP-3 complex, and ALP6 encodes the gene product Apl6p that is most homologous to β3A subunit of the AP-3 complex. To investigate the pathway further Cowles et al. (1997) identified the other subunits of the yeast AP-3 complex (Amp3p is most homologous to μ3A and Aps3p is most homologous to σ3) and individually knocked them out and investigated their effects on ALP sorting. As expected deletion of any of the subunits interfered with the correct sorting of ALP to the vacuole. Like ALP the vacuolar t-SNARE Vam3p is also missorted, suggesting that the yeast AP-3 complex is also required for its transport. In AP-3 deleted cells both Vam3p and ALP accumulate in vesicles and membranes which do not contain vacuolar markers.

The results from both the yeast and the *Drosophila* studies suggest that in mammalian cells the AP-3 complex will probably be involved in the transport of proteins to lysosomes and lysosome-related organelles (the equivalent organelle of the yeast vacuole or the insect pigment granule).

Mutant mice

Dr Margaret S. Robinson was contacted independently by two groups led by Dr Margit Burmeister and Dr Richard T. Swank who work on mouse models of the human genetic disorder Hermansky-Pudlak syndrome (HPS). HPS is a disorder characterised by hypopigmentation, prolonged bleeding times, colitis (inflammation of the colon), decreased renal lysosomal enzymes in the urine and fibrotic lung disease. The disease occurs in diverse populations world-wide and is especially prevalent in selected regions, such as Puerto Rico. Patients with the disorder have an increased mortality rate.

There are at least 15 mouse complementation groups (*pale-ear*, *mocha*, *pearl*, *pallid*, *light-ear*, *maroon*, *ruby-eyed*, *subtle-grey*, *cocoa*, c*appuccino*, *reduced-pigment*, *muted*, *sandy*, *beige* and *ruby-eye-2J*) that have phenotypes similar to HPS, such as dilute coat and eye colouring, abnormal

platelets, lysosomal abnormalities, failure to secret lysosomal enzymes in to the urine and fibrotic lung disease. Out of the 15 mouse complementation groups, only 2 of the genes have been identified and cloned *LYST-1* and *HPS*. *LYST-1,* a gene of unknown function, is mutated in the mouse mutant *beige* and human Chediak-Higashi syndrome. The *HPS* gene, also of unknown function, is mutated in the mouse mutant *pale-ear* and in the majority of HPS patients from Puerto Rico (Swank et al., 1998).

The reason for the two groups contacting Dr M. S. Robinson was that the β3A gene mapped very closely to the *pearl* (*pe*) locus, the δ gene mapped very closely to the *mocha* (*mh*) locus. In addition the *garnet* mutation gave pigmentation defects very similar to *mh* and *pe*, making the β3A and δ genes very strong candidates for the genes mutated in the *mh* and *pe* mice.

5.2 Characterisation of the *mh* and *pe* mutations

Characterisation of the mh mutation

Dr Margit Burmeister's research group was involved in mapping the mouse mutant *mh. mh*, like other HPS model mice, has reduced pigmentation, decreased renal lysosomal enzymes in their urine, abnormal platelets, lysosomal abnormalities. *mocha, pallid and muted* mice also have inner-ear degeneration and neurological abnormalities. Figure 5.1 shows an example of a wild type litter-mate next to a *mh* mouse. The *mh* mouse is showing several phenotypes associated with HPS, reduced pigmentation and inner ear defects (note the pale coat, skin colour and balance problems).

mh was mapped to mouse chromosome 10 and an equivalent region on human chromosome 19. The *mh* allele was mapped to within ~0.5cM of several markers on chromosome 10 by several different mouse crosses. A physical map of ~2000kb encompassing the region was constructed by pulse field gel electrophoresis, and candidate genes identified by their ability to hybridise to this region (mouse chromosome 10 ESTs, chromosome 10 genes, human chromosome 19 ESTs and genes were investigated). One of

the ESTs that mapped to this region encoded the δ subunit of the AP-3 complex. To investigate if the δ gene contained the *mh* mutation, genomic digests followed by Southern blotting with probes against the δ subunit were performed from wild type, *mh* and mh^{2J} DNA (mh^{2J} is another allele of *mh*). The genomic digests and Southern blots indicated that there was a rearrangement in both *mh* and mh^{2J} DNA. The rearrangement in *mh* is caused by a 12kB deletion from the 5' end of the δ gene (the nature of the mh^{2J} will be discussed later).

To investigate what effect the rearrangement had on the δ transcript, Northern blots and RT-PCR were performed from wild type and *mh* brain tissue. The Northern blots and reverse RT-PCR indicated that the δ message was still present in *mh* tissue, except it was ~500bp shorter than the wild type message. Sequencing of the RT-PCR product indicated that there was 496bp deletion starting just shortly after the initiator ATG, that is predicted to cause an in-frame stop codon after the deletion (Kantheti et al., 1998).

Characterisation of the pe mutation

Dr Richard Swank's group was involved in the mapping of the *pe* mutation. The *pe* mouse like *mh* has many of the HPS associated phenotypes as mentioned earlier. *pe* was mapped to mouse chromosome 13 and was within ~0.5cM of several markers. The high-resolution mapping allowed a physical map of the region to be constructed from a series of yeast artificial chromosomes (YACs) and bacterial artificial chromosomes (BACs). To identify candidate genes for *pe,* several BACs mapping to the region were subjected to exon trapping. One of the exons trapped encoded C-terminal region of β3A. To investigate if the β3A gene contained the *pe* mutation, genomic digests followed by Southern blots with probes against the β3A subunit were performed from wild type, *pe* and pe^{8J} DNA (pe^{8J} is another allele of *pe*). The genomic digests and Southern blots indicated that there were rearrangements in both *pe* and pe^{8J} DNA (personal communication, Dr R. T Swank). To investigate what effect the rearrangement had on the β3A message, RT-PCR and Northern blots were performed on wild type and *pe*

tissue. The Northern blots revealed that the *pe* message was larger than expected and was decreased in intensity compared to the wild type message. Sequencing of the RT-PCR products revealed that the message contained a tandem duplication of 793bp. This duplication is predicted to cause an in-frame stop codon at the duplication site, and would cause 130 amino acid to be truncated from β3A (Feng et al., 1998).

These results, in conjunction with the mapping, indicate that the *mh* and *pe* gene encodes the δ and β3A subunits of the AP-3 complex, and rearrangements in these genes probably cause the associated phenotypes.

To further investigate the *mh* and *pe* mutations, collaborations were initiated between Dr M. S. Robinson, Dr M. Burmeister and Dr R. T. Swank's laboratories. Wild type, *mh* and *pe* tissues were sent to Dr M. S. Robinson laboratory by Dr M. Burmeister and Dr R. T. Swank. Wild type, *mh* and *pe* mice were obtained from The Jackson Laboratory and breeding colonies established in Cambridge. R. T. Swank also sent a *pe* immortal fibroblast cell line to Dr M. S. Robinson laboratory. To further aid the analysis of the *mh* mutation I generated an immortal fibroblast cell line from the *mh* mice (Materials and Methods section 2.4).

Western blots from mh and pe

To characterise the effects of the *mh* and *pe* mutations on the AP-3 complex, *mh* and *pe* samples were homogenised and boiled in sample buffer (Materials and Methods section 2.7). The samples were resolved by SDS-PAGE and then blotted overnight. The blots were probed with antibodies against the subunits of the AP-3 complex.

Figure 5.2 shows a series of Western blots from *mh* brain tissue, control brain tissue, *mh* and control cells (melan-a cells), which were probed with antibodies raised against various AP-3 subunits and an antibody against the γ subunit of the AP-1 complex as a control. The anti-δ antibody (raised against the N-terminal region) gave no signal at any position on the *mh* brain and *mh*

fibroblasts sample Western blots, suggesting the out-of-frame deletion in *mh* causes a null. To confirm that no truncated δ was being made from another initiator site, Western blots were probed with antibodies raised against the C-terminal region of δ (see Table 2.2 Antibodies) and no signal was detected (data not shown). Interestingly, the other subunits of the AP-3 complex are also greatly reduced or undetectable from *mh* sample Western blots. A Northern blot for σ3A indicated that σ3A was still being synthesised (performed by Dr M. Burmeister). These results indicate that the *mh* mutation is a null, and that the δ subunit is essential for the stability of the other subunits of the AP-3 complex (Kantheti et al., 1998). Interestingly the σ3 subunit appears to be more stable than the other subunits, as trace amounts of σ3 can be detected in the *mh* samples.

Figure 5.3 shows a series of Western blots from control cells (melan-a), *pe* fibroblasts, control brain tissue and *pe brain* tissue (see above for antibodies used). The anti-β3A antibody gave no detectable signal from *pe* fibroblasts. The levels of the δ and σ3 subunits do not seem to be severely affected by the absence of β3A in *pe* fibroblasts, although μ3A has become undetectable. A Northern blot for μ3A indicated that μ3A was still being synthesised (performed by Dr R. T. Swank). The levels of β3B and μ3 in brain tissue appear to be unaffected by the *pe* mutation, suggesting that there is functional AP-3 complex in *pe* brain tissue. These results indicate that *pe* allele causes a severe reduction in the amount of β3A in *pe* mice (possibly due to mRNA and protein instability), and that β3A is essential for the stability of the μ subunit in the AP-3 complex (Zhen et al., 1999).

The pearl mutation is not a null

Western blotting and native immunoprecipitation experiments from *pe* fibroblast have proven to be uninformative in indicating whether *pe* is a true null or whether a small amount of β3A is still being expressed. To try and resolve this problem spleens from control and *pe* mice were used for native immunoprecipitations and Western blotting experiments (Materials and Methods sections 2.8 and 2.9). *pe* and control spleens were

immunoprecipitated with antibodies raised against β3A and γ as a control. The samples were subjected to SDS-PAGE and then Western blotted, and appropriate strips cut from the blots and probed with anti-δ, β3A, μ3A, σ3A and γ as a control.

Figure 5.4 shows an example of such a series native immunoprecipitation and Western blotting experiments from *pe* and control spleens (Materials and Methods sections 2.8 and 2.9). The results indicate that β3A is present in *pe* tissue at very low levels and that the truncated β3A is able to incorporate into the AP-3 complex, as β3A brings down δ, μ3A and σ3A in the native immunoprecipitation.

Immunoprecipitations from mh and pe fibroblasts

It has been previously shown, that there are strong interactions between the β/μ subunits and σ/α,γ subunits of the AP-1 and AP-2 complexes using the yeast two-hybrid system (Page and Robinson, 1995). The data from the *pe* and *mh* Western blotting experiments (Fig. 5.3 and 5.4) suggested that a σ/δ sub-complex might be being formed in *pe* cells, as the δ and σ3 subunits appeared to be more stable than expected (in *mh* all of the subunits are severely reduced). To investigate this, native immunoprecipitation and Western blotting experiments were performed from *pe* and *mh* cells, as described in Materials and Methods sections 2.8 and 2.9.

Figure 5.5 show an example of a series of native immunoprecipitation and Western blotting experiments from *pe* fibroblasts. *pe* fibroblast were immunoprecipitated with antibodies against μ3, σ3, δ and an antibody against γ as a control. The samples were subjected to SDS-PAGE and then Western blotted, and appropriate strips cut from the blots and probed with the above antibodies (Materials and Methods sections 2.8 and 2.9). The σ3 and δ immunoprecipitations indicate that a σ/δ sub-complex is being formed in the *pe* cells, as the σ3 immunoprecipitation brings down δ, and conversely the δ immunoprecipitation brings down σ3. The σ3 and δ immunoprecipitation also brings down a non-stoichometric amount of μ3A. The μ3A is probably not

directly interacting with δ and σ3 but with trace amounts of β3A brought down in the immunoprecipitation (the μ3 antibody is more sensitive at detecting itself on a Western blot than the β3A antibody, this would explain why μ3A can be detected in the σ3 and δ immunoprecipitations, while β3A can not).

To investigate if μ3A, β3A, σ3 subunits are being expressed in *mh* fibroblast and if β3A and μ3A interact, native immunoprecipitations and Western blotting experiments were performed as above.

Figure 5.6 show an example of a series native immunoprecipitation and Western blotting experiments from *mh* fibroblasts. *mh* fibroblast were immunoprecipitated with antibodies against δ, μ3, β3A, σ3 and an antibody against γ as a control. The samples were subjected to SDS-PAGE and then Western blotted, and appropriate strips cut from the blots and probed with the above antibodies. The immunoprecipitations indicated that μ3A, β3A and σ3 are all being synthesised in *mh* fibroblasts, and β3A and μ3A interact with each other as μ3A comes down in the β3A immunoprecipitation. In contrast, σ3 appears to not to be free.

The *mh* and *pe* immunoprecipitation results biochemically confirm the yeast two hybrid studies previously performed by Page et al. (1995), and extend them to the AP-3 complex (Page and Robinson, 1995).

Localisation of the AP-3 complex in mh and pe

To investigate the effect of the *pe* and *mh* mutations on the localisation of the AP-3 complex, control (melan-a), *mh* and *pe* fibroblasts were fixed and stained with anti-δ antibodies (Materials and Methods section 2.6) (Fig. 5.7). The *mh* fibroblasts showed no staining for δ, as would be expected, however the *pe* cells showed diffuse staining for δ, indicating that the δ/σ sub-complex described in Fig. 5.5 is unable to associate with membranes in *pe* cells. This result indicates that the δ/σ sub-complex is not functional.

5.3 Characterisation of the *mocha* ^{2J} (*mh*^{2J}) mutation

mh^{2J} is another allele of *mh*, which has an overall less severe phenotype than *mh* (less severe pigment dilution, clotting problems and balance problems) except *mh*^{2J}has more pronounced neurological abnormalities (hyperactivity and handling-induced seizures). The mutation has not been mapped completely, although it is known that *mh*^{2J} allele, like *mh,* has a genomic rearrangement (Kantheti et al., 1998). Analysis of *mh*^{2J} Northern blots revealed that there are two mRNAs for δ instead of one in wild type tissues. The most abundant mRNA is much larger than wild type message and the other is the same size as the normal message. Sequence analysis of RT-PCR products, has revealed that the larger message contains a large insertion which would lead to a in-frame stop codon in the hinge domain, at amino acid 858 of the δ subunit. Sequencing of the normal sized RT-PCR product has revealed that it encodes the wild type δ protein. It is thought that the wild type message is created by splicing-out the insertion from the mutant message (Dr M. Burmeister, personal communication).

Immunoprecipitations from mh^{2J}

To characterise the *mh*^{2J} mutation further, native immunoprecipitations were performed from control and *mh*^{2J} liver, brain and spleen tissue, using antibodies against δ and σ3 (Materials and Methods sections 2.8 and 2.9) (Fig. 5.8). The native immunoprecipitations indicate that in *mh*^{2J} tissue, there is both wild type and truncated δ subunit expressed, as anti-δ immunoprecipitations not only brings down wild type sized δ (~160kD) but also a band at ~120kD. The truncated δ subunit is also able to incorporate into the AP-3 complex, as σ3A immunoprecipitations brings down not only wild type δ but also truncated δ. In addition these results indicate that there is relatively less wild type δ in brain tissue compared to the other tissues examined.

Localisation of the AP-3 complex in mh^{2J}

To investigate the mh^{2J} mutation further, wild type and mh^{2J} primary fibroblasts were generated from 2-3 day old decapitated mice, sent on ice from Dr M. Burmeister. The primary fibroblasts were fixed and stained with anti-δ N and C-terminal antibodies (Materials and Methods section 2.6) (Fig. 5.9). The anti-N-terminal antibody recognises both wild type and truncated protein and the C-terminal antibody only recognises wild type protein. The staining pattern produced with the N-terminal anti-δ antibody was similar to wild type cells, except the intensity of the staining was decreased. Very little staining was observed with the C-terminal anti-δ antibody (very faint labelling was observable in some cells, data not shown). This result indicates that there is very little wild type δ in mh^{2J} fibroblasts, and that the AP-3 complex containing the truncated δ subunit is able to recruit on to membranes and might be functional.

5.4 β3B is able to incorporate into non-neuronal AP-3 complexes

pe cells stably expressing β3B were generated by transfecting *pe* cells with β3B pMEP DNA, and selecting with hygromycin as described as in Materials and Methods section 2.4. Hygromycin resistant colonies were picked and screened by immunofluorescence using an anti-β3B antibody (Table 2.2 Antibodies). Figure 5.10 shows a series of fluorescence micrographs from untransfected *pe* cells and *pe* cells stably expressing β3B, fixed and stained for anti-β3B and δ antibodies (Materials and Methods section 2.6). The fluorescence micrographs show that when β3B is transfected into *pe* cells the δ staining goes from being diffuse to punctate, suggesting that the β3B is able to incorporate into the non-neuronal AP-3 complex (containing μ3A) and then associate with membranes. To confirm this result, native immunoprecipitations were performed from untransfected and stably expressing β3B *pe* cells, with antibodies raised against δ and β3B. The samples were Western blotted and strips cut from the appropriate regions and probed with anti-δ, β3B, μ3 and σ3 antibodies (Materials and Methods sections 2.8 and 2.9) (Fig. 5.11). The native immunoprecipitations indicate

that the β3B subunit is able to incorporate into the non-neuronal AP-3 complex, as the β3B and δ immunoprecipitations bring down δ, β3B, μ3 and σ3 from stably transfected *pe* cells.

5.5 LAMPI is missorted in *mh* fibroblasts

Distribution of LAMPI and LIMPII in mh and pe fibroblasts

While this work was under way, a paper from Dr B. Hoflack laboratory was published, suggesting that the AP-3 complex might be involved in trafficking of LAMPI and LIMPII (Le Borgne et al., 1998). Le Borgne et al. (1998) used antisense oligonucleotides to decrease the levels of μ3A in tissue culture cells. The anti-sense oligonucleotides decreased the levels of μ3A by ~50%. The antisense did not grossly affect the steady state localisation of LAMPI and LIMPII, but affected the amount of LAMPI and LIMPII trafficking via the cell surface (detected by antibody uptake experiments). The treatment of cells with antisense oligonucleotides had no effect on the processing of Cathepsin D.

To investigate if the *mh* and *pe* mutations cause a similar missorting of LAMPI and LIMPII, as the anti-sense studies, control (primary mouse fibroblasts), *mh* and *pe* fibroblasts were fixed and stained anti-LAMPI and LIMPII antibodies (see Table 2.2 Antibodies) (Fig. 5.12). The fluorescence micrographs indicate that there is no gross missorting of LAMPI and LIMPII in *pe* and *mh* fibroblasts compared to control primary fibroblasts.

Transient transfections in mh

From the steady state labelling of LAMPI and LIMPII in wild type, *mh* and *pe* cells it became apparent that any differences in localisation of the markers due to the *mh* and *pe* mutations would be subtle, therefor an antibody uptake approach would be required.

It was decided that the best approach to reveal any differences in LAMPI and LIMPII trafficking, would be to transiently transfect the *mh* fibroblasts with wild

type δ cDNA in pMEP, followed by antibody uptake experiments. The transient transfections would allow the comparison of antibody uptake between transfected and untransfected cells, so providing an in-built control.

Figure 5.13 shows and example of such an transfection and antibody uptake experiment. The *mh* cells were transfected with δ in pMEP (Table 2.3 constructs) using Fugene overnight (Materials and Methods section 2.4). 48 hours after transfection the cells were allowed to endocytose Alexa 350 labelled wheat germ lectin as an endocytocis control and an antibody against LAMP1 (Materials and Methods section 2.11). The cells were fixed and stained with anti-δ antibodies (Materials and Methods section 2.6). The fluorescence micrographs indicate several results. Firstly, the anti-δ staining indicates that cells transfected with δ cDNA are able to make AP-3 complex that can recruit on to membranes. Secondly, the transfection process does not inhibit endocytocis, as wheat germ lectin positive structures are present in transfected cells. Thirdly, LAMPI traffics less via the cell surface in these cells as less LAMPI internal staining is present in transfected cells. To confirm this result, 30 micrographs from this experiment (35 transfected cells) were examined. It was found that in the majority of transfected cells (>80% 30 out of 35) the trafficking of LAMPI was less via the cell surface. In the five cases where a reduction LAMPI trafficking via the cell surface was not observed, the cells were either expressing very low or very high levels of the δ subunit. This type of experiment was repeated several times and similar results were obtained (data not shown). These results indicate that in *mh* cells expressing the δ subunit, LAMPI traffics less via the cell surface than in *mh* cells lacking functional AP-3 complex.

Unfortunately this type of experiment is not possible with the *pe* cells, as high enough transfection efficiencies could not be obtained, even after trying several different transfection reagents.

5.6 Development of a quantitative assay for AP-3 function

The development of a quantitative missorting assay would not only allow the more accurate determination of LAMPI missorting, it would also allow the comparison of wild type and mutant constructs stably transfected into *mh* and *pe* cells, so giving a better insight into AP-3 function.

The quantitative missorting assay is based on the same immunofluorescence antibody uptake assay, as used in section 5.4, except mock and transfected cells are grown on multi-well plates and ^{125}I-protein A is used to detect the endocytosed antibodies. The amount of antibody bound or endocytosed is compared between the transfected and mock-transfected cells (as described in Materials and Methods section 2.11). Figure 5.14 shows a flow diagram of the protocol.

The development of this assay has been hindered, as making stables expressing the δ subunit in *mh* or the β3A subunit in *pe* cells has been more difficult than expected, although *pe* cells stably expressing the neuronal isoform β3B have been established. To try and resolve these problems, several new constructs have been generated by Rachel Rudge. These constructs are now being tested.

Preliminary uptake experiments

To ascertain if the assay was feasible, several preliminary experiments were performed on *pe* and *pe* cells stably expressing β3B. To determine the appropriate concentrations of antibodies and cells to use in the assay, a crude titration experiment was performed (Fig. 5.15). The concentrations of anti-LAMPI (2.04μg/ml) and anti-CD44 (2.92μg/ml) (CD44 is not endocytosed) antibodies were kept constant and the concentration of cells varied. The titration experiment indicated, that the concentration of anti-CD44 antibody became limiting before the concentration anti-LAMPI antibody, for the same number of cells. This experiment indicates how important it is for the correct

concentration of antibodies and cells to be used, if the assay is to be meaningful.

Once the appropriate concentrations of anti-CD44 and anti-LAMPI antibodies were determined, a preliminary quantitative LAMPI missorting assay was performed on *pe* cells stably expressing β3B (Materials and Methods section 2.11). The experiment was performed at both 37°C and 4°C (4°C inhibits endocytocis)(Fig. 5.16). The anti-CD44 panel indicates that approximately the same numbers of mock and stably transfected β3B *pe* cells (*pe**) were used in each experiment. This experiment indicates that the *pe* cells expressing β3B have less LAMPI trafficking via the cell surface, as both the levels of cell surface (4°C) and internalised (37°C) LAMPI were reduced compared to mock transfected *pe* cells. However the amounts of LAMPI in rescued and non-rescued cells need to be measured, as in some lysosomal storage diseases the levels of LAMPI expression changes (Meikle et al., 1999).

It is worth noting that the quantitative *pe* β3B rescue experiment does not appear to be as striking (not as large as a difference between mock and transfected cells) as the *mh* rescue experiment (Fig. 5.10). The differences in the levels of rescue between the *mh* and *pe* experiments are probably due to the mock transfected *pe* cells being heterogeneous in their uptake of LAMPI, as observed in preliminary immunofluorescence experiments (data not shown). This heterogeneity would make the difference between mock and transfected cells smaller, however it can be not ruled out that the β3B might only be able to partially compensate for the absence of β3A. To resolve this problem clonal mock transfected cells and *pe* cells expressing β3A are being generated. This result suggests that β3B is able to form functional AP-3 complex in non-neuronal cells, thus at least partially rescuing the *pe* mutant phenotype.

Figure 5.1

Wild type mouse next to a *mocha* littermate. Note the reduced pigmentation and balance problems of the *mocha* mouse.

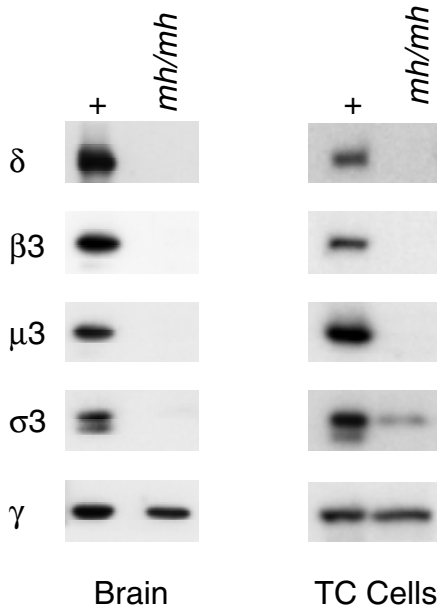


Figure 5.2

The *mh* and control samples were homogenised, subjected to SDS-PAGE and Western blotting (Materials and Methods sections 2.7 and 2.8). Blots were probed with antibodies to various AP-3 subunits and with an antibody against the γ subunit of the AP-1 complex as a loading control. In the *mh* samples, absence of the δ subunit leads to severe reduction in the other subunits of the AP-3 complex.

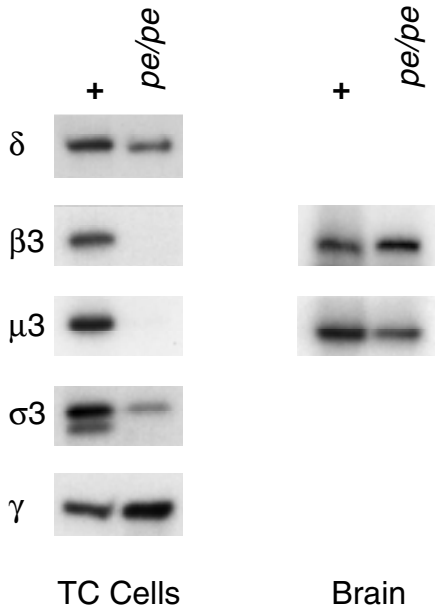


Figure 5.3

The *pe* and control samples were homogenised, subjected to SDS-PAGE and Western blotting (Materials and Methods sections 2.7 and 2.8). Blots were probed with antibodies to various AP-3 subunits and with an antibody against the γ subunit of the AP-1 complex as a loading control. In the *pe* samples, absence of the β3A subunit leads to severe reduction of the μ3 subunit.

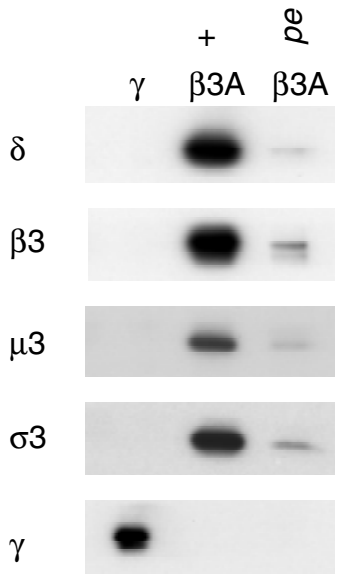


Figure 5.4

Native immunoprecipitations from *pe* spleen were performed using antibodies raised against β3A and the γ subunit of the AP-1 complex as a control (Materials and Methods sections 2.8 and 2.9). The *pe* mutation is not null, as a small amount of truncated β3A is still detectable. The truncated β3A can incorporate into the AP-3 complex as the other AP-3 subunits are brought down in the anti-β3A immunoprecipitation.

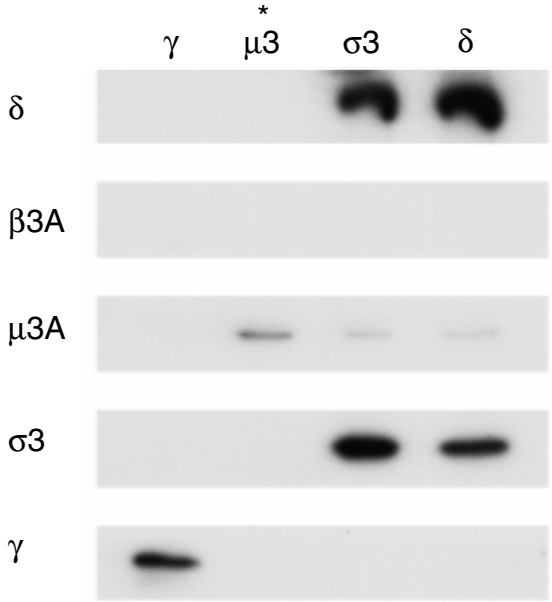


Figure 5.5

pe fibroblasts were immunoprecipitated with antibodies against δ, β3A, σ3 and an antibody against γ as a control under native conditions. The μ3A (*) immunoprecipitation was performed under denaturing conditions (immunoprecipitation buffer was supplemented with 0.3%SDS) as the antibody only works under these conditions. The samples were subjected to SDS-PAGE and then Western blotted, and appropriate strips cut from the blots and probed with the above antibodies (Materials and Methods sections 2.8 and 2.9). The immunoprecipitations indicate that δ and σ3 are forming a subcomplex, despite tha absence of β3 and μ3.

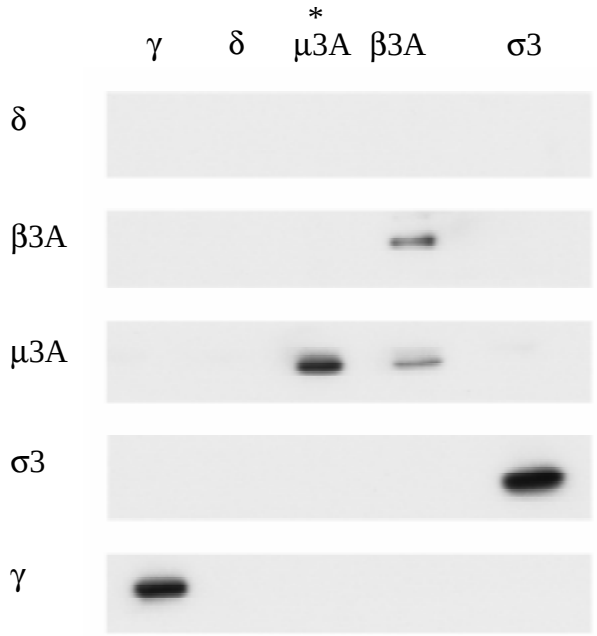


Figure 5.6

mh fibroblasts were immunoprecipitated with antibodies against δ, β3A, σ3 and an antibody against γ as a control under native conditions. The μ3A (*) immunoprecipitation was performed under denaturing conditions (immunoprecipitation buffer was supplemented with 0.3%SDS) as the antibody only works under these conditions. The samples were subjected to SDS-PAGE and then Western blotted, and appropriate strips cut from the blots and probed with the above antibodies (Materials and Methods sections 2.8 and 2.9). The immunoprecipitations indicate that μ3A, β3A and σ3A are all being synthesised in *mh* fibroblasts, and β3A and μ3A interact with each other, but not with σ3.

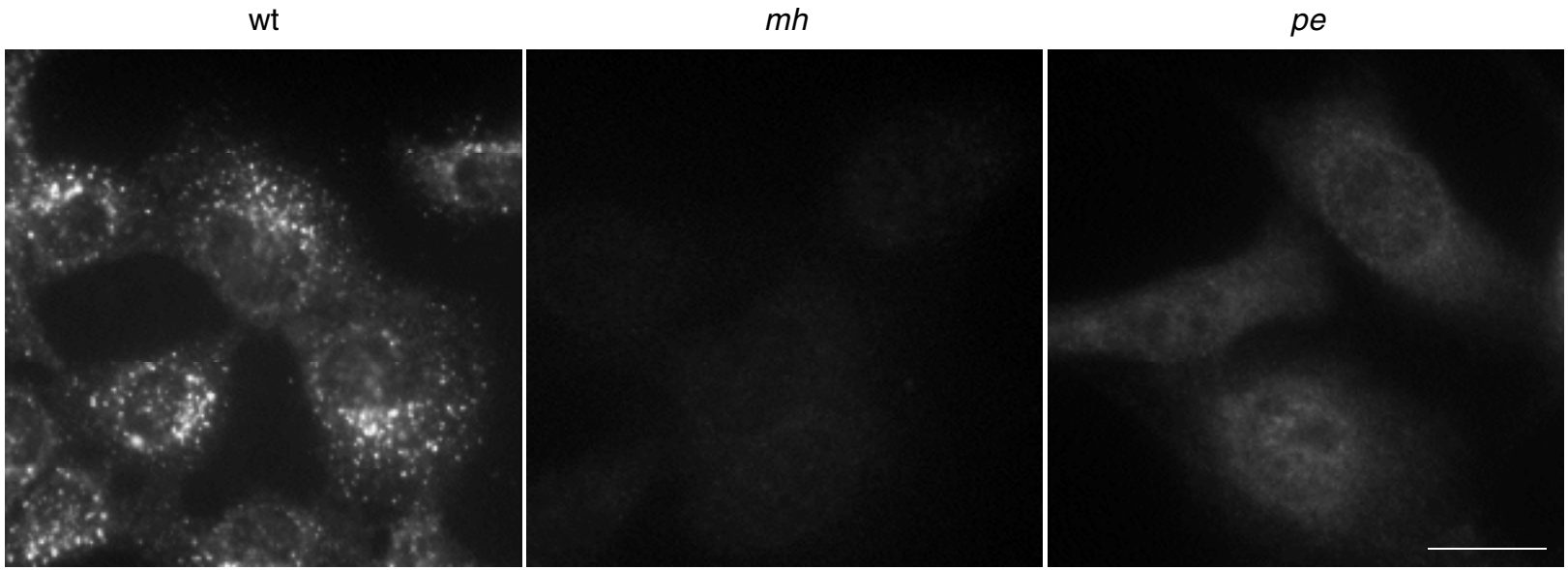


Figure 5.7

Control (melan-a), *mh* and *pe* cells were fixed and stained with an anti-δ antibody (Materials and Methods section 2.6). There is no staining for the δ subunit in *mh* cells, however *pe* cells show diffuse staining which is significantly above background staining. Scale bar 20μM.

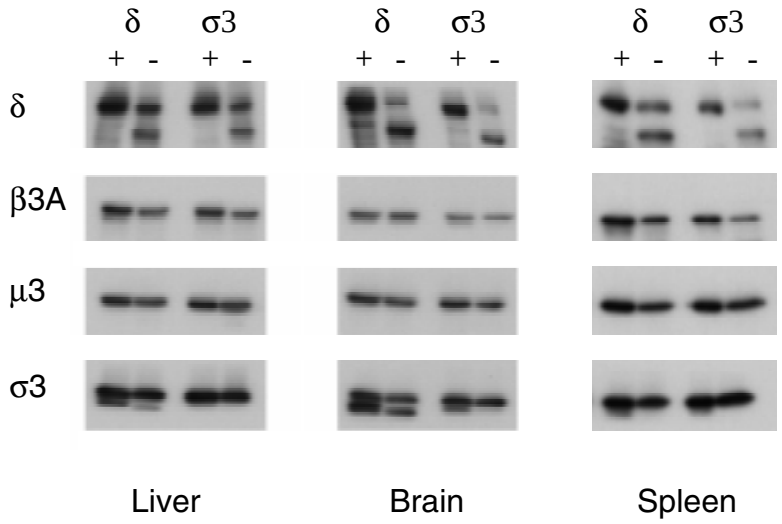


Figure 5.8

Native immunoprecipitations were performed from control (+) and *mh*^{2J} (-) liver, brain and spleen tissue using anti-δ and σ3 antibodies (Materials and Methods sections 2.8 and 2.9). Both the wild type and truncated δ are expressed in *mh*^{2J} tissue, and the truncated protein is also able to incorporate into the AP-3 complex.

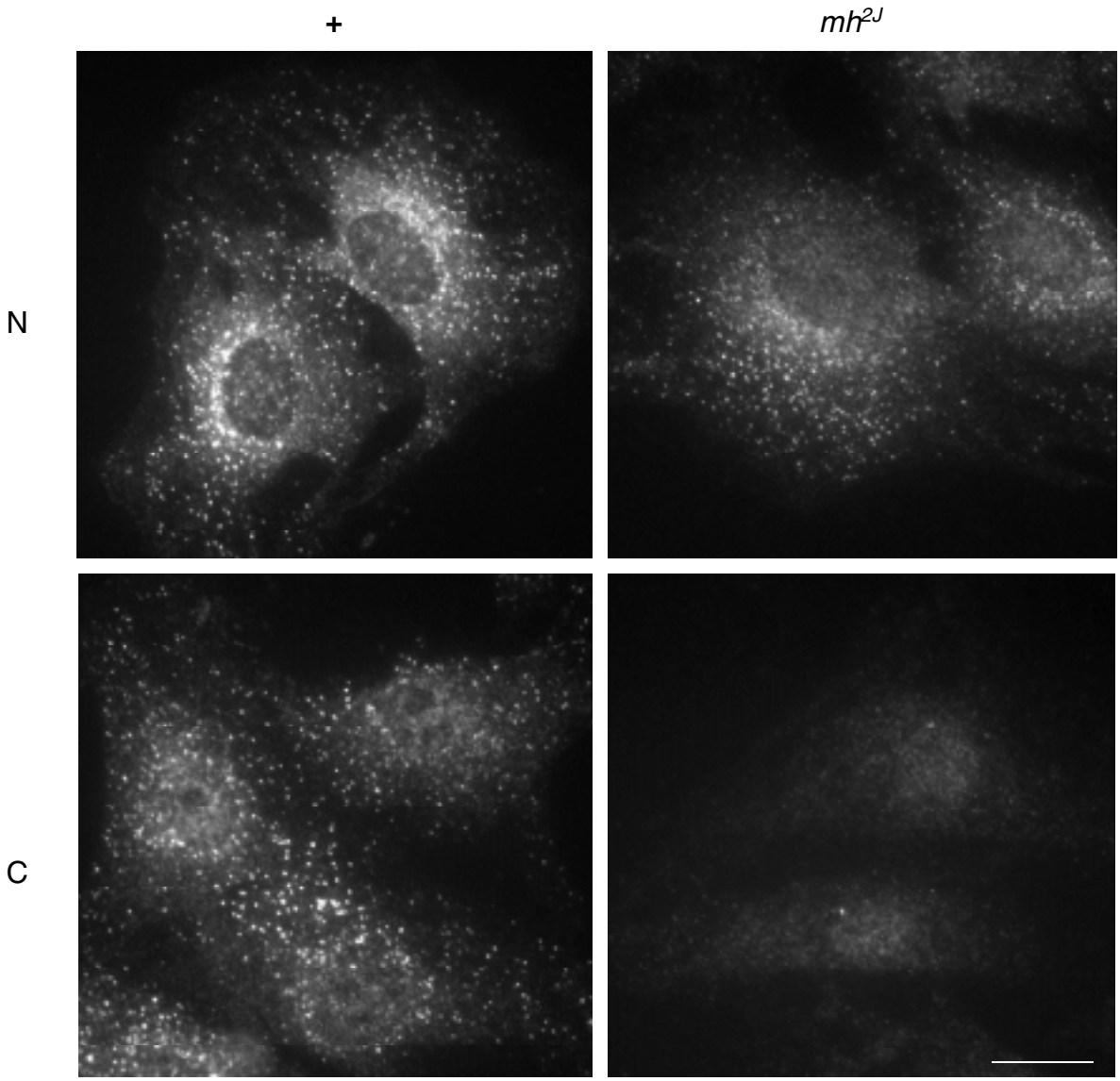


Figure 5.9

Primary mouse *mh*^{2J} and control fibroblasts were fixed and stained with N and C-terminal anti-δ antibodies (Materials and Methods section 2.6). The staining pattern produced with the N-terminal anti-δ antibody in *mh*^{2J} was similar to wild type fibroblasts, except the intensity of the staining was decreased. Very little staining was observed in *mh*^{2J} fibroblasts with the C-terminal anti-δ antibody. This result indicates that there is very little wild type δ in *mh*^{2J} fibroblasts and that the AP-3 complex containing the truncated δ is able to bind to membranes.

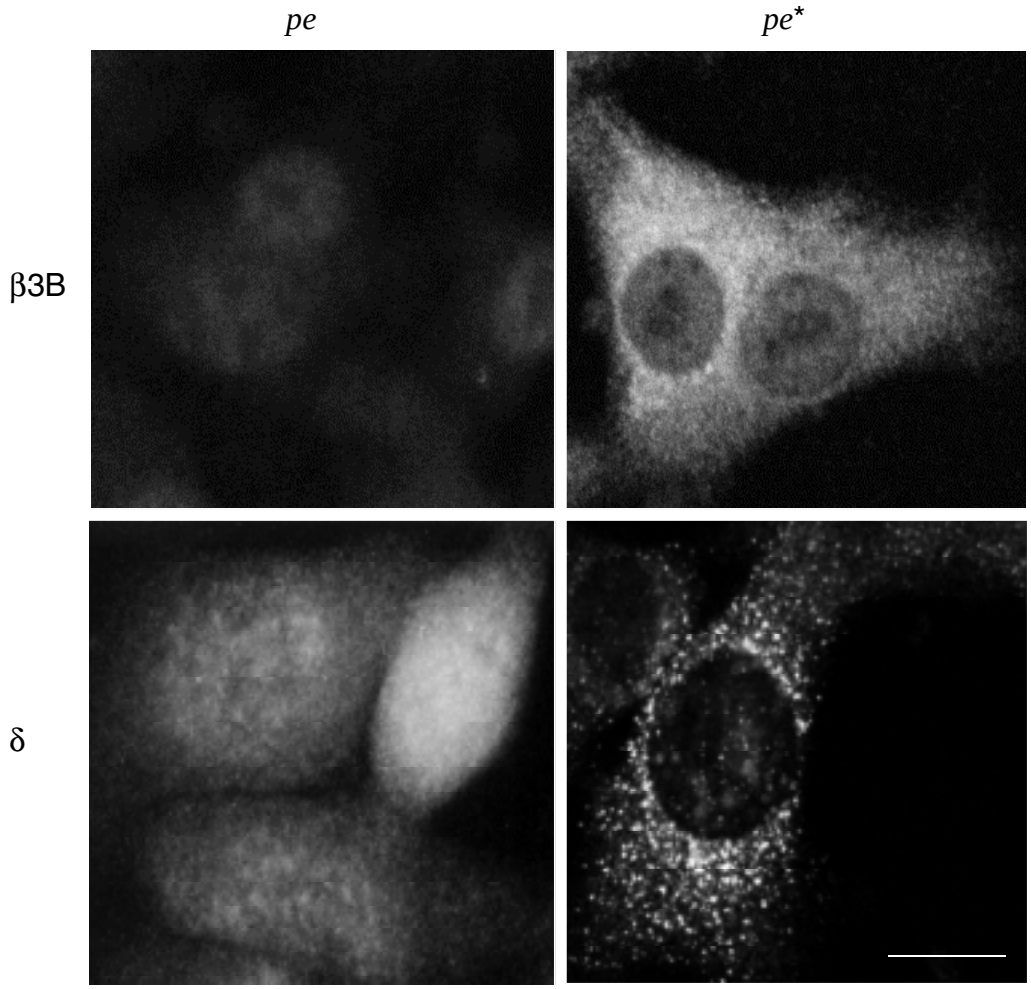


Figure 5.10

pe and *pe** stables expressing β3B were fixed and stained with antibodies against β3B and δ (Materials and Methods section 2.6). Micrographs show that β3B is able to incorporate into the non-neuronal AP-3 complex and the complex is able to recruit on to membranes. Scale bar 20μM.

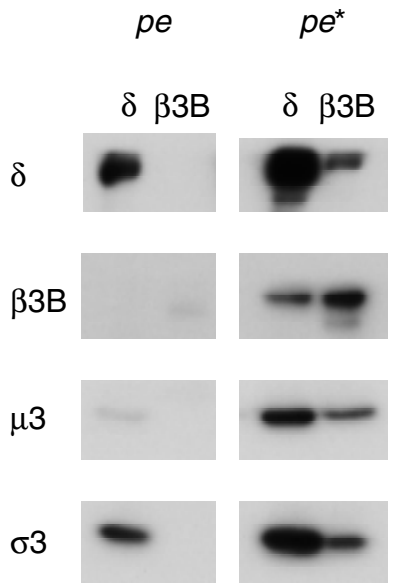


Figure 5.11

Native immunoprecipitations using antibodies against δ and β3B were performed from untansfected *pe* and *pe* * cells stably expressing β3B. The samples were Western blotted and strips cut from the appropriate regions and probed with anti-δ, β3B, μ3 and σ3 antibodies (as described in Materials and Methods section 2.8 and 2.9). The native immunoprecipitations indicate that β3B is able to incorporate into the non-neuronal AP-3 complex.

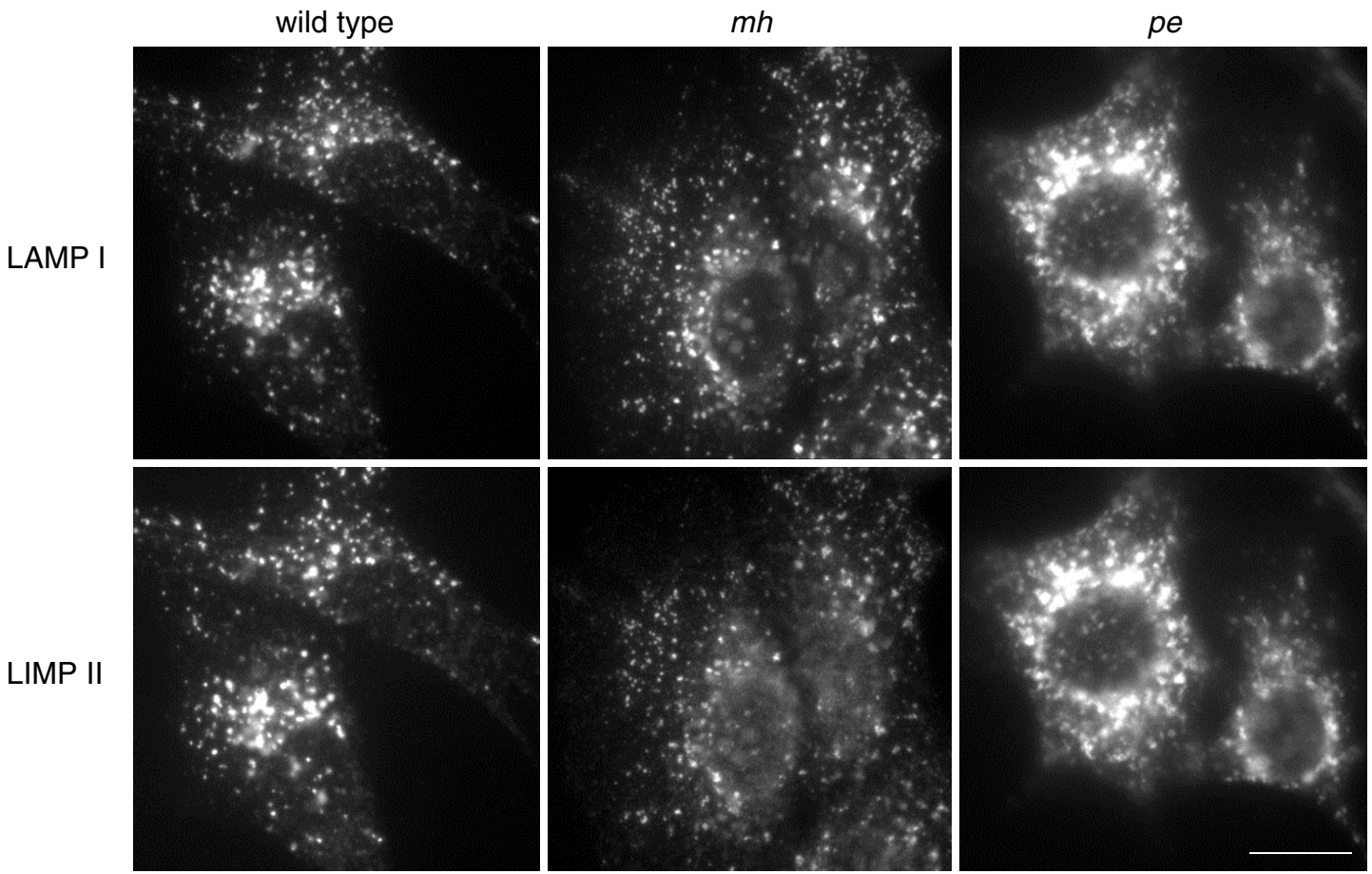
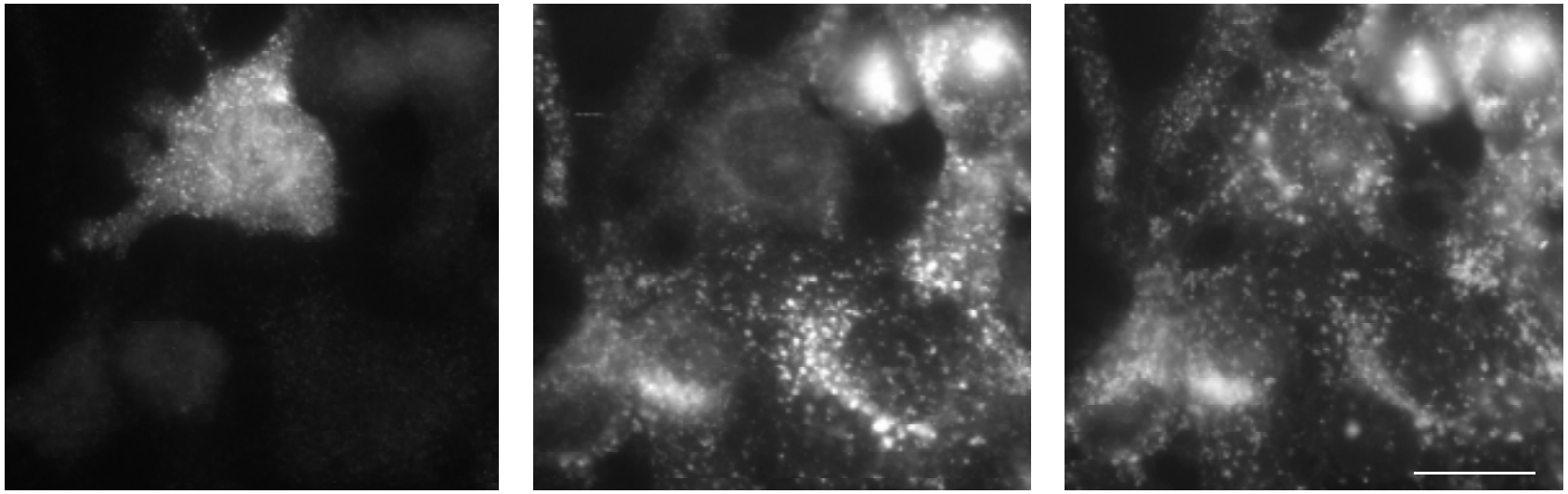


Figure 5.12

Wild type (mouse primary fibroblasts), *mh* and *pe* fibroblasts were fixed with -20° methanol for 5 minutes and stained with anti-LAMPI and LIMPII antibodies (cells were processed as in Materials and Method section 2.6). Staining patterns do not appear to be grossly different between wild type, *mh* and *pe* cells. Scale bar 20μM.



δ | Endocytosed anti-LAMP1 | Endocytosed WGA

Figure 5.13

mh fibroblasts were transiently transfected overnight with δ in pMEP (Materials and Methods section 2.4). 48 hours after transfection the cells were fed Alexa labelled wheat germ lectin, as a control, and anti-LAMPI antibodies (Materials and Methods section 2.11). The cells were fixed and stained with anti-δ antibodies to reveal transfected cells (Materials and Methods section 2.6). These micrographs indicate, that *mh* cells expressing the δ subunit traffic LAMPI less via the cell surface and the endocytocis of wheat germ lectin is not affected by this process. Scale bar 20μM.

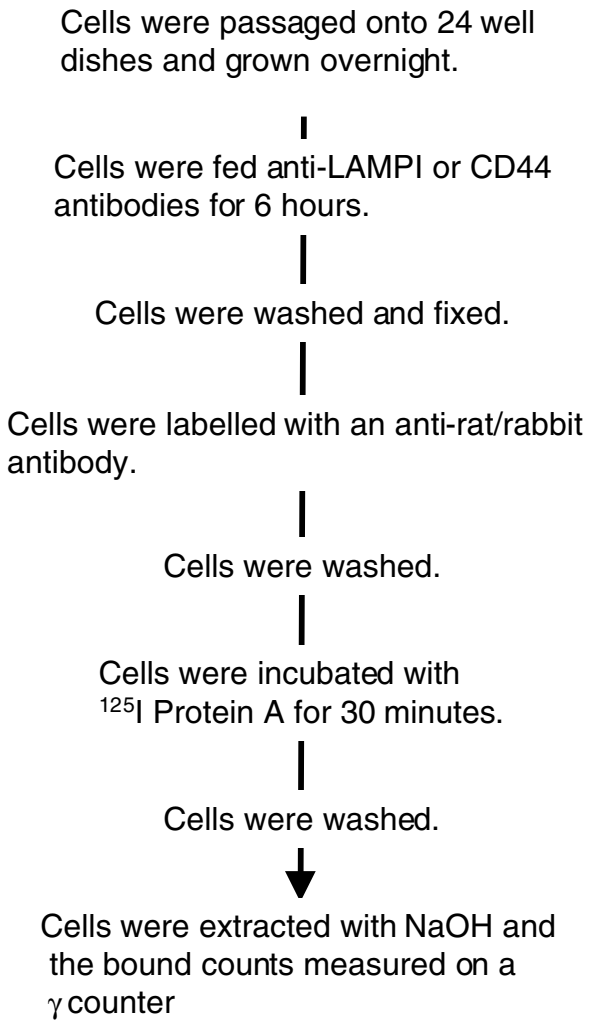


Figure 5.14

Flow diagram of the quantitative mis-sorting assay (Materials and Methods section 2.11).

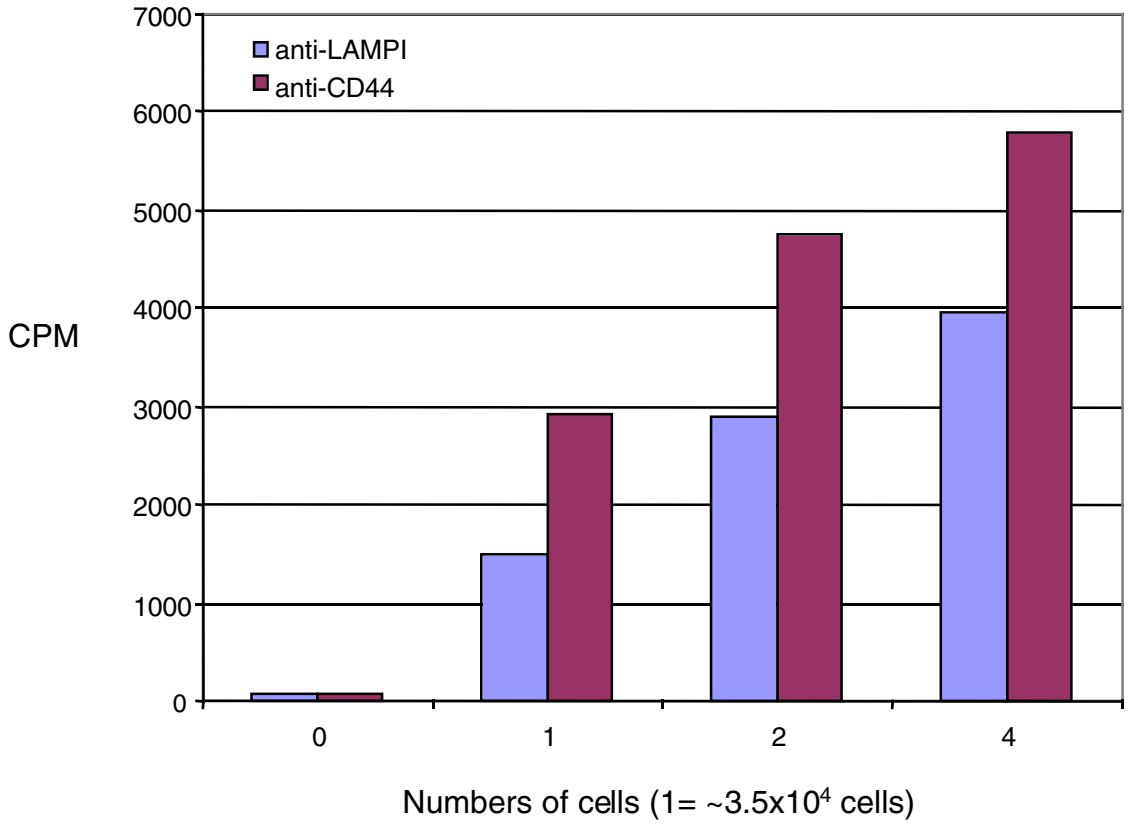
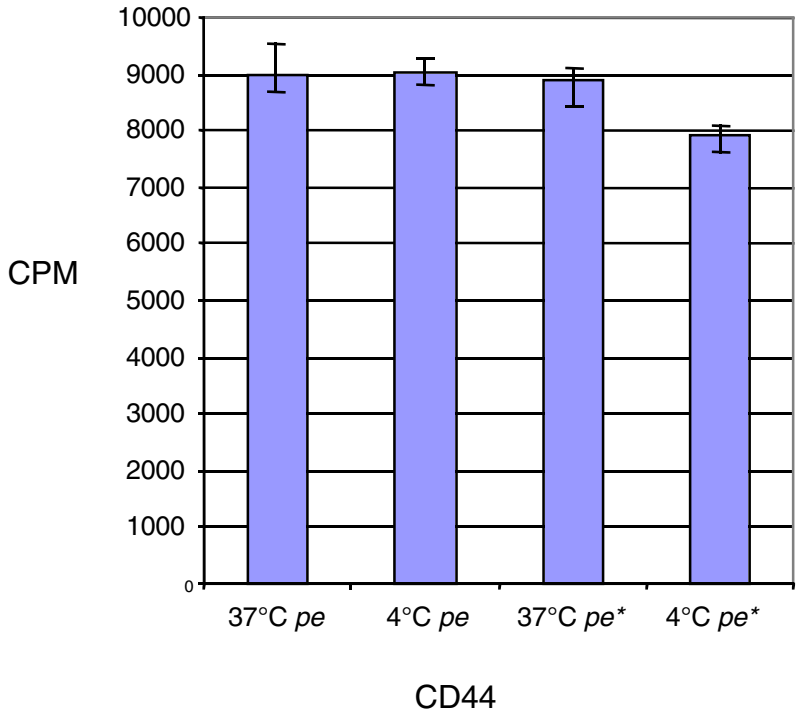
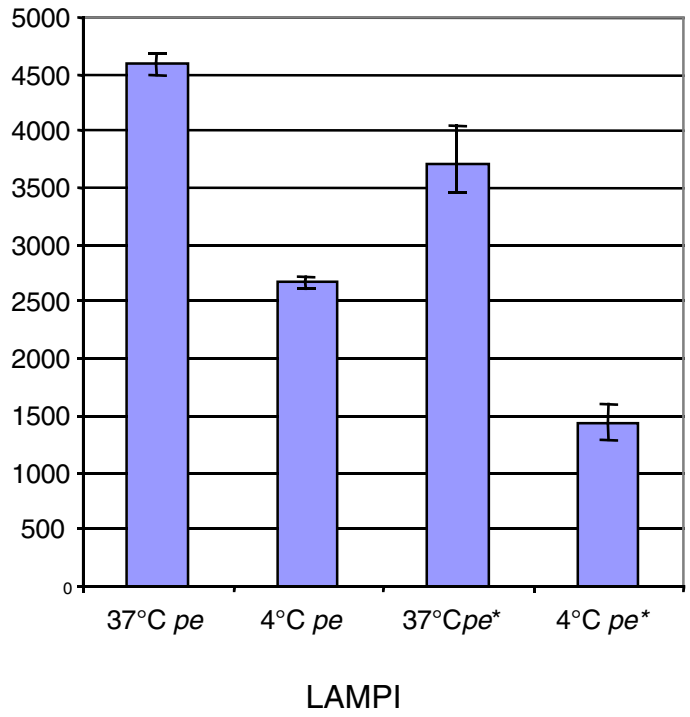


Figure 5.15

To determine the appropriate concentrations of cells and antibodies to use in the mis-sorting assay, a crude titration experiment was performed in duplicate on *pe* cells. *pe* cells were plated onto multi-well dishes at varying concentrations and then fed anti-LAMPI (2.04µg/ml) and anti-CD44 (2.92µg/ml) antibodies for 6 hours, and then processed as described in Materials and Methods section 2.11. The experiment indicates that CD44 antibody has become limiting, between $3.5x10^{04}$ and $7x10^{04}$ cells, and that the anti-LAMPI antibody has becoming limiting between $7x10^{04}$ and $1.4x10^{05}$ cells.





A preliminary antibody uptake experiment was performed on β3B transfected *pe* (*pe**) and mock transfected *pe* cells as described in Materials and Methods section 2.11. The cells were either given control anti-CD44 antibody (anti-CD44 is not endocytosed) or an anti-LAMPI antibody at 4°C or 37°C. This experiment indicates that *pe* cells transfected with β3B have less LAMPI trafficking via the cells surface, as the amount of antibody bound in *pe* cells expressing β3B is reduced compared to the *pe* fibroblasts at 37°C and °4C.

Chapter 6

Discussion

This thesis has focused on the cloning and characterisation of the AP-3 complex. The characterisation of the complex has been greatly facilitated by the discovery of *Drosophilia* and mice with mutations in several of the AP-3 subunits. These mutations have allowed experiments to be devised and performed in mammalian cells that had previously only been possible in genetically pliable model systems, such as yeast.

6.1 Composition and subunit interactions of the AP-3 complex

The cloning and immunoprecipitation studies indicate the AP-3 complex, like conventional adaptor complexes, consists of a α/γ subunit (δ). a β subunit, a μ subunit and a σ subunit. The AP-3 complex is complicated by the fact that there are two β isoforms β3A/β3B, two μ isoforms μ3A/μ3B and two σ isoforms σ3A/σ3B. δ, β3A, μ3A, σ3A and σ3B are all expressed ubiquitously, although σ3A and σ3B are expressed at different levels in different tissues (Pevsner et al., 1994) (Simpson et al., 1996) (Simpson et al., 1997) (Dell'Angelica et al., 1997a) (Dell'Angelica et al., 1997b). β3B and μ3B are only expressed in neuronal tissue (Newman et al., 1995) (Pevsner et al., 1994). The complexity of the AP-3 complex compared to conventional adaptor complexes, raises several interesting questions. Are there neuronal isoforms of the δ and σ subunits, can the neuronal and non-neuronal subunits interact "mix and match" and why are there neuronal specific isoforms of β and μ? Characterisation of the *mh* and *pe* mice has given insights into several of these questions.

Are there neuronal isoforms of the δ and σ subunits?

Searching the EST database with δ and σ subunits has not led to the identification of any neuronal isoforms, although their existence can not be ruled out. However it seems very unlikely that there is a neuronal isoform of the δ subunit, as *mh* mice have neuronal abnormalities, in contrast to *pe* mice

which do not have neuronal abnormalities, probably as the neuronal isoform of β3A, β3B, is able to compensate for the absence of β3A.

Subunit interactions

The characterisation of *mh* and *pe* fibroblasts has given several insights into AP-3 subunit interactions. Western blotting and immunuoprecipitation studies from *mh* and *pe* fibroblasts have biochemically confirmed yeast two hybrid studies indicating that the β and μ, and α/γ and σ subunits can interact (Page, 1996), as β3A and μ3A, and δ and σ3A immunoprecipitate as heterodimers. These studies have also indicated that the δ subunit is essential for the stability of the whole complex as in its absence the other AP-3 subunits are degraded. The β subunit is also essential for the stability of the μ subunit as when the β subunit is absent the μ subunit becomes degraded. The most definitive way to increase our understanding of adaptor subunit interactions is to resolve the adaptor complex's structure at atomic resolution. A collaboration has been established with Dr David Owen to determine the structure of the adaptor subunits.

Can the neuronal and non-neuronal subunits interact "mix and match"?

Transfection studies using *pe* fibroblasts has given an insight into whether the neuronal and non-neuronal subunits can interact i.e. can β3A interact with μ3B and β3B interact with μ3A. Immunoprecipitation studies from *pe* cells transfected with β3B indicated that β3B is able to interact with μ3A and form potentially functional complexes. Yeast two hybrid studies by Winnie Lui, a PhD student in Dr M. S. Robinson's laboratory, have also indicated that β3B is able to interact with μ3A and that β3A is able to interact with μ3B (personal communication). These results suggest that the neuronal and ubiquitously expressed subunits are promiscuous as β3B (or β3A) can interact with μ3A or μ3B. However these experiments could be misleading as only one subunit out of each pair of isoforms is present in each experiment, so subtle differences in affinity would not be detected, thus it is still possible that there could be two separate AP-3 complexes in neuronal tissue i.e. one containing β3B/μ3B and the other β3A/μ3A. It is also not clear whether the same cell can co-express

both μ3A and μ3B. One way this question can be resolved, is if μ3A and μ3B specific antibodies can be generated.

6.2 Phosphorylation of the AP-3 complex

Immunoprecipitation studies indicated that the β3A subunit is the major target for phosphorylation in the ubiquitously expressed AP-3 complex. Support for this result has come from a study performed by the laboratory of Dr J. S. Bonifacino, that indicates that the β3A subunit is phosphorylated, and that the phosphorylation is restricted to serine residues (Dell'Angelica et al., 1997b). The sequence analysis of β3A and β3B indicates that there are many consensus casein kinase phosphorylation sites in the hinge domain. Interestingly, several of these sites are conserved between the mammalian and yeast β3 subunits and a genetic interaction has been detected between yeast casein kinase 1 and the AP-3 complex, suggesting that β3A phoshporylation might important for the function of the AP-3 complex (Dell'Angelica et al., 1997b) (Panek et al., 1997). The precise role of β3A and β3B phosphorylation in AP-3 function has still to be resolved. A study by the laboratory of Dr R. Kelly has suggested that the phosphorylation of the β3 subunit might affect the regulation of the AP-3 complex's ability to be recruited onto membranes (personal communication, Dr V. Faúndez).

The β subunits of the AP-1 and AP-2 complexes are also phosphorylated *in vivo* but it is not clear what role phosporylation plays in AP-1 and AP-2 function (Wilde and Brodsky, 1996). A study by Wilde et al. (1996) indicated that the phosporylation of the β2 subunit inhibited the ability of the AP-2 complex to bind to clathrin *in vitro,* however their study did not conclusively indicate if the phosporylation of β2 modulates clathrin adaptor interactions *in vivo*.

A possible way to precisely determine if the phosphorylation of the β3 subunit is essential for AP-3 function would be to transfect *pe* cells with β3A or β3B

constructs that no longer contained consensus phosphorylation sites in their hinge domains, and then assay for function of the AP-3 complex.

6.3 Does the AP-3 complex interact with a structural scaffold?

As the AP-3 complex is related to AP-1 and AP-2, it was important to determine if AP-3 complex interacts with clathrin. Previous studies by the laboratory had shown that the neuronal AP-3 subunits were not localised or enriched in clathrin-coated vesicles (Simpson et al., 1996). To investigate if this was also true for the ubiquitously expressed AP-3 subunits, Western blotting and immunofluorescence studies were performed on clathrin-coated vesicles and tissue culture cells from non-neuronal sources. The studies indicated that the AP-3 not was enriched or localised to clathrin-coated vesicles (Simpson et al., 1997). These results remain controversial as two studies performed by the laboratory of Dr J. S. Bonifacino added support but also contradicted our findings (Dell'Angelica et al., 1998; Dell'Angelica et al., 1997b). In the first study by Dell'Angelica et al. (1997) concluded that the AP-3 complex was not enriched in clathrin-coated vesicles based on Western blotting experiments. In the later study by Dell'Angelica et al. (1998) the group used GST-β3A based constructs to carry out *in vitro* "fishing" experiments from cytosol. The β3A based constructs fished out the clathrin heavy chain from cytosol. They mapped the binding domains of β3A and clathrin, to amino acids 817 to 825 of the C-terminal "ear" domain of β3A, and the N-terminal portion of clathrin. The group also used an anti-β3A antibody to carry out immunofluorescence and immunoelectron microscopy studies. Their studies revealed that ~65% of anti-β3A positive structures were also positive for anti-clathrin staining. The Dell'Angelica et al. (1998) results are unexpected and contradictory to the previous findings. There could be several reasons why their findings are contradictory. The ability of β3A to bind to clathrin *in vitro* may not reflect an interaction that occurs *in vivo*. Indeed several papers have been published indicating strong *in vitro* interactions between adaptors and other proteins (IRS1 and synaptotagmin), which have proven difficult to substantiate *in vivo* (Zhang et al., 1994) (VanRenterghem et al., 1998). The

colocalisation of β3A with clathrin is very surprising and it could be explained if the β3A antibody is cross-reacting with β1 or β2. This is possible as the region Dell'Angelica et al. (1998) used to raise the antibody against has many domains that are highly conserved between the β1, β2 and β3.

To try and resolve if β3A interacts with clathrin *in vivo*, several experiments have been undertaken in the Robinson laboratory. The β3A subunit has been FLAG tagged to allow the localistaion of the AP-3 complex in *pe* cells. β3A constructs missing the proposed clathrin binding domain (Dell'Angelica et al., 1998) and a construct containing the β2 clathrin binding domain (Shih et al., 1995) will be generated and transfected into *pe* cells. These constructs will help determine if the proposed clathrin-binding domain in β3A is essential for AP-3 function. The role of clathrin in AP-3 function will also be investigated by perturbing clathrin's ability to bind to adaptors, this will be achieved by transfecting cells with a clathrin hub construct that acts as a dominant negative (Liu et al., 1998). Together, these studies should indicate whether AP-3 is able to interact with clathrin *in vivo*.

Alternative scaffold proteins

If the findings from our laboratory are correct, the AP-3 complex does not use clathrin as a structural scaffold. Are there any other candidate molecules which might act like a scaffold?

The*VPS41* gene encodes a protein that could be a potential scaffold for the AP-3 complex. The *VPS41* gene encodes a hydrophilic protein of 992 amino acids. Analysis of the sequence revealed that the protein contains one clathrin heavy chain repeat at amino acid 753-853 (zigzag motif) (Radisky et al., 1997; Ybe et al., 1999). Mutations in *VPS41* cause missorting of alkaline phosphatase, similar to mutations in AP-3 subunits (Cowles et al., 1997b; Radisky et al., 1997). Mutations in the *Drosophila VPS41* gene, called *light,* also cause a phenotype similar to mutations in *garnet* (*Drosophilia* AP-3 δ) and the two genes have been shown to genetically interact (Lloyd et al.,

1998). Experiments performed in the laboratory of Dr S. Emr have sown that yeast *VPS41* is able to bind to the δ hinge/ear *in vitro* (Rehling et al., 1999).

To characterise *VPS41* in mammalian cells, antibodies have been raised against a GST fusion protein encoding the C-terminal portion of *VPS41*. The antibodies label a band of the appropriate sise on Western blots (data not shown) and now can be used to determine in mammalian *VPS41* binds to δ hinge/ear, and whether it is missing in any of the mouse mutants.

Another potential source of scaffold molecules for the AP-3 complex might come from the further mapping and identification of the genes that cause mouse (at least 11 genes unidentified) and *Drosohilia* (8 genes unidentified) pigmentation mutants, similar to *garnet*, *mocha* and *pearl.*

6.4 Localisation of the AP-3 complex

This study has confirmed and extended the previous study performed by Simpson et al. (1996) The ubiquitously expressed AP-3 complex is associated with both perinuclear and more peripheral membranes (Simpson et al., 1996) (Simpson et al., 1997). The nature of the more peripheral membranes remains elusive as there is only very limited colocalisation with early and late endosomal markers. The lack of colocalisation of AP-3 with endosomal markers and the predominance of AP-3 labeling on the trans Golgi network, might suggest that the peripheral labeling is more likely to be associated with a biosynthetic compartment. Another indication that the peripheral AP-3 labeling is associated with a biosynthetic compartment comes from a preliminary study performed by Alex C. Theos in Dr M. S. Robinson's laboratory. COS cells were transiently transfected with ZnT-3-GFP (Palmiter et al., 1996), fixed and then stained with either anti-δ or anti-CD63 antibodies (this neuronal specific zinc transporter is mislocalised in *mh* mice, see Discussion section 6.4). The ZnT-3-GFP construct labeled both perinuclear and more peripheral structures. The smaller peripheral structures labeled with ZnT-3-GFP partially colocalised with AP-3 staining, and the larger structures

with anti-CD63 staining. This preliminary experiment suggests that the AP-3 complex is associated with a compartment that is accessible from the biosynthetic pathway and that ZnT-3 is the first molecule to show significant levels of colocalisation with the AP-3 complex (personal communication, Alex C. Theos). Although ZnT-3 can be colocalised with AP-3 it has not been possible to colocalise AP-3 with LAMPI, another potential cargo molecule. There could be several reasons why there is partial colocalisation with ZnT-3 and AP-3, and not LAMPI. Firstly, the expression levels of ZnT3-GFP are much higher than endogenous LAMPI, secondly ZnT3 is normally expressed in neuronal cells so might not be efficiently sorted in non-neuronal cells (no μ3B and β3B) and thirdly ZnT-3 shows the most striking missorting defect out of any of the potential AP-3 cargo molecules investigated, so might be more dependent on AP-3 sorting. A possible way to trap LAMPI in an AP-3 positive compartment, is to transiently transfect GFP tagged LAMPI into cells and investigate if it partially colocalises with AP-3 staining.

6.4 Function of the AP-3 complex

Insights into the function of the AP-3 complex have come from the analysis of three genetic models systems flies, yeast and mice. Mutations in the AP-3 complex in all three systems lead to similar mutant phenotypes in lysosome related organelles. The similarity of the mutant phenotypes would suggest that the AP-3 complex has a similar function in all three organisms.

Analysis of the garnet allele

The first insight into the AP-3 complexes function came from the analysis of several *Drosophilia garnet* alleles (g^3 and g^{53d}). The *garnet* gene encodes the δ subunit of the fly AP-3 complex (Simpson et al., 1997). Mutations in the *garnet* gene affect both body and eye pigmentation. Analysis of *garnet* fly eyes revealed that the overall organisation of the mutant eyes was normal compared to wild type eyes, although there was a reduction in the pigmentation of pigment granules surrounding the ommatidia (Simpson et al., 1997). A study by Ooi et al. (1997) confirmed and extended the study by

Simpson et al. (1997) by further characterising g^3 and another mutant allele, g^{50e}, of *garnet*. A Nothern blot from g^3, g^{50e} and wild type flies, revealed that the g^3 mutation causes a truncation and g^{50e} causes a severe reduction in the δ mRNA. Dr J. S. Bonifacino's laboratory has also characterised several of the other fly eye pigmentation mutants that genetically interact with *garnet.* Interestingly 3 of these complementation groups (*orange*, *carmen* and *ruby*) encode the other subunits of the AP-3 complex (personal communication, Dr J. S. Bonifacino). These results indicate that the AP-3 complex is playing a role in pigment granule biogenesis and/or in the trafficking of cargo to pigment granules. It is thought that pigment granules are related to lysosomes so this suggests that the AP-3 complex is also involved in the trafficking of lysosomal proteins. This hypothesis is supported as there is another fly eye pigment granule mutant, *deep-orange*, which is homologous to *VPS18* (Shestopal et al., 1997), and has been shown to be involved in the sorting of proteins to the vacuole (in yeast) (Robinson et al., 1991).

Analysis of yeast lacking AP-3 subunits

Cowles et al. (1997) identified that the AP-3 complex in yeast is essential for the correct sorting of alkaline phosphatase and the vacuolar t-SNARE Vam3p to the vacuole (Cowles et al., 1997b). This result was also confirmed by a similar study performed by Stepp et al. (1997). The only other mutant identified as being essential for the correct sorting of alkaline phosphatase was *VPS41.* Interestingly deletion of the clathrin heavy chain yeast does not effect the sorting of alkaline phosphatase or Vam3p, indicating that AP-3 and clathrin do not interact (Huang et al., 1999; Panek et al., 1997).

Analysis of mutant mice (mh, pe and mh^{2J})

Mapping and Western blotting experiments in collaboration with Dr R.T Swank and Dr M. Burmeister have revealed that the *pearl* phenotype is caused by a mutation in β3A, and the *mocha* phenotype is caused by a mutation in the δ subunit of the AP-3 complex (Feng et al., 1998; Kantheti et al., 1998; Zhen et al., 1999). The *mocha* mutation is a null, as the *mocha* rearrangement leads to an in-frame stop codon just after the start codon. Western blotting

experiments from *mh* tissues and fibroblasts indicated that in the absence of the δ subunit the other subunits of the AP-3 complex become destabilised. Western blotting experiments from *pe* tissues and fibroblasts revealed that the *pe* mutation leads to a severe reduction in the amount of detectable β3A, probably due to combined mRNA and protein instability (β3A was only detectable after enrichment by immunoprecipitation). In the absence of β3A the μ3 subunit becomes unstable, however the δ and σ3 subunits are still detectable. The δ/σ sub-complex formed in *pe* cells is unable to associate with membranes indicating that the sub-complex is not functional.

Antibody uptake studies have indicated that LAMPI is trafficked more via the cell surface in *mh* and *pe* fibroblasts compared to rescued cells, expressing β3B and δ. To further characterise the sorting of LAMPI in *mh* and *pe* cells, stable cell line expressing β3A in *pe* and δ in *mh* will have to be generated. It has proved very difficult to generate stables expressing full-length δ. It appears that unregulated expression of the wild type δ subunit is to toxic to *mh* cells. To try and overcome this problem the δ subunit has been moved into an inducible expression vector that is tetracycline responsive (Zeng et al., 1998).

These results confirm the study performed by Le Borgne et al. (1998) which stated that perturbations in AP-3 complex function cause LAMPI and LIMPII to be trafficked more via the cell surface (Le Borgne et al., 1998). Interestingly, a study by Dell'Angelica et al. (1999) has identified a human patient with HPS that has mutations in the β3A subunit of the AP-3 complex. As in *mh* and *pe* fibroblasts, the trafficking of LAMPI is perturbed in the mutant human fibroblasts. The Dell'Angelica et al. (1999) study has also identified that CD63, another late endocytic marker, is also trafficked more via the cell surface in these fibroblasts. The trafficking of CD63 has also been investigated in *mh* and *pe* cells by Dr Brian A. Rous in the laboratory of Dr P. Luzio. *mh* and *pe* cells were transfected with wild type and mutant CD63 constructs and the localisation of these constructs observed. Interestingly, the localisation of wild type CD63 was not grossly affected in *mh* and *pe* cells although a mutant

CD63 construct, which bound poorly to μ2 in the yeast two hybrid system, was found to be mainly on the cell surface and not in lysosomes (wild type tail sequence GYEVM and mutant GYEVI). This construct in wild type mouse fibroblasts or *pe* cells transfected with β3B was mainly found in lysosomes and not on the cell surface (personal communication, Dr B. A Rous).

These results support the hypothesis that AP-3 is involved in the sorting of lysosomal transmembrane proteins to the lysosome. Perturbations in AP-3 function cause lysosomal proteins to be missorted to the cell surface, where they are re-internalised by the AP-2 complex and then delivered to lysosomes. However these results are indirect, and do not indicate whether the AP-3 complex is involved in the direct binding and trafficking of LAMPI, LIMPII and CD63, or another factor required for their correct sorting. It is plausible that the unidentified factor could be a molecule such as a SNARE since mistargeting of the SNARE in AP-3 deficient cells could then lead to vesicles being targeted to the wrong compartment. Data to support this idea already exists as the vacuolar t-SNARE Vam3p is mislocalised in AP-3 deficient yeast (Cowles et al., 1997a). To investigate this possibility, antibodies against several SNAREs have been obtained from Dr R. H. Scheller.

Does the AP-1 complex have any role in the sorting of LAMPI and LIMPII ?

The data from several studies indicate that lysosomal transmembrane proteins (LAMPI and LIMPII) in the absence of functional AP-3 complex are trafficked via the cell surface, and might suggest that LAMPI and LIMPII are not sorted directly by the AP-1 complex (Dell'Angelica et al., 1999b; Le Borgne et al., 1998). It has been postulated that the AP-1 complex is involved in the sorting of lysosomal enzymes and transmembrane proteins (LAMPI and LIMPII) to the lysosome via an endosomal intermediate. Many studies have been performed that implicate the AP-1 complex in the trafficking of the M6P receptor and its cargo to the endosomal system, reviewed by Le Borgne et al. (1998), however there have been very few studies actually indicating that AP-1 complex is involved in the trafficking of LAMPI and LIMPII to the lysosome.

The main evidence suggesting that the AP-1 complex is able to traffic lysosomal transmembrane proteins comes from *in vitro* binding studies (Honing et al., 1996) (Honing et al., 1998). The Honing et al. (1998) study used plasmon surface resonance to investigate adaptor binding to lysosomal tail motifs (dileucine and tyrosine based). The binding experiments indicated that AP-1 was able to bind to LAMPI and AP-3 was not. These results do not reflect the *in vivo* analysis of LAMPI trafficking in cells defective in AP-3 function. It is possible that the *in vitro* binding studies were misleading, as components normally present in the cell, which might provide specificity or increase the affinity, would not be present in the *in vitro* experiments. Another indication that AP-1 complex is not involved in the trafficking of LAMPI and LIMPII is that the μ1 "knock out" mice show defects in mannose6-phosphate receptor trafficking, and not in LAMPI and LIMPII trafficking (Dr Peter Schu, personal communication). These results would suggest that there is little strong evidence for the role of AP-1 in the trafficking of LAMPI and LIMPII to lysosomes.

Is the "ear" domain of δ required for AP-3 function?

Immunoprecipitation and mapping experiments in collaboration with Dr M. Burmeister have revealed that the *mocha*^{2J} allele is caused by a genomic insertion (possibly a transposable element). The insertion causes the δ mRNA to contain a stop codon just after the codon for amino acid 858. The truncation causes most of the C-terminal portion to be missing from the δ subunit ("hinge and ear" domains). In all tissues examined so far, the insertion can be excised by splicing. The removal of the insertion from the mRNA leads to both wild type and truncated protein being expressed in the same tissues, although brain tissue contains lower levels of wild type δ compared to other tissues examined. As both wild type and truncated protein are expressed in *mh*^{2J} cells, examination of the truncation's effect on LAMPI trafficking would be difficult to interpret. To simplify the system Rachel Rudge, a PhD student in Dr M. S. Robinson's laboratory, has taken *mh* fibroblasts and stably transfected them with a construct encoding the truncated cDNA. Surprisingly, the truncated protein is able to reduce the amount of LAMPI trafficking via the cell surface

(personal communication, Rachel Rudge). To determine if the truncated δ construct is fully functional, a stable cell line expressing the wild type δ subunit is required. However the rescue by truncated δ was unexpected as the ear domain of α-adaptin has been shown to be involved in the binding of a series of proteins which are thought to be essential for AP-2 complex function (Eps15, epsin and amphiphysin) (Traub et al., 1999). The γ-adaptin ear domain also has at least one binding partner (γ-synergin) but it is not clear if it is required for AP-1 function (Page et al., 1999). This result might be indicating that adaptor complexes can function without interacting with these other molecules and these other molecules may only play a role in the modulation of adaptor complex function. Another interpretation of the result is that the δ ear domain is not involved in the binding of other proteins, but this seems unlikely as "fishing" experiments with the yeast δ hinge/ear have isolated *VPS41* as a binding partner (Rehling et al., 1999). A third possibility is that the interacting proteins may be capable of binding to other sites in the coat. This is supported by the observation that two of the α ear binding partners, amphiphysin and epsin can also bind to clathrin (McMahon et al., 1997) (Wendland et al., 1999).

Why does the AP-3 complex have a neuronal specific β and μ subunit?

The precise function of the AP-3 complex in neuronal cells has still to be firmly established. Several studies have given insights into the possible function of the AP-3 complex in neurons. The AP-3 complex is localised to a perinuclear compartment in the cell body, and also more peripheral staining in the neurites of primary cultured neurons (anti-β3B antibody) (Simpson et al., 1996). The AP-3 complex has been shown to play a role in the generation of synaptic vesicles *in vitro,* from PC12 enriched endosomes (Faundez et al., 1998). Budding in the assay can be completely reconstituted by the addition of purified ARF-1, AP-3 and endosomal membranes. Interestingly, the budding assay only works if AP-3 has been purified from brain tissue and not from a non-neuronal source (personal communication, Dr Victor Faúndez). This data would suggest that the budding assay is dependent on the AP-3 complex containing the neuronal isoforms of β3B or μ3B. The nature of the

assay (endosomal membranes) and the dependence on neuronally purified AP-3 complex would suggest that the AP-3 complex containing β3B or μ3B has a different function from the non-neuronal AP-3 complex. The Faúndez et al. data, taken in conjunction with the *pe* rescue experiments, might suggest that β3B and β3A can perform non-neuronal tasks and that only β3B is able to perform neuronal tasks.

Interestingly, the budding assay is clathrin independent and the addition of β3A-GST fusion proteins, that bind clathrin *in vitro* (Dell'Angelica et al., 1998), do not inhibit this assay but inhibit an assay for the generation of synaptic vesicles from PC12 plasma membranes (AP-2 dependent) (Shi et al., 1998). These results suggest that the AP-3 complex functions independently from clathrin and supports claims made in this thesis (see section 4.5)

Can loss of functional AP-3 complex be correlated to the pleiotrophic phenotypes observed in the mutant mice?

There are a very large numbers of phenotypes associated with the *mh, mh^{2J}* and *pe* mice. These include hypopigmentation, prolonged bleeding, decreased secretion of lysosomal enzymes in the urine, colits, fibrotic lung disease and the *mh* and *mh^{2J}* mice, specific neurological abnormalities (hyperactivity and handling induced seizures). Can all of these phenotypes be directly related to the missorting of proteins in the absence of functional AP-3 complex? In several of the mutant phenotypes, there is enough known about the normal and diseased states to make a educated guess at the molecules that might be mis-sorted in the AP-3 deficient mice. Two of these HPS associated phenotypes are being investigated, the *mh* and *pe* hypopigmentation defects and the *mh* specific neurological abnormalities.

Neurological defects in mh mice

To try and determine why the loss of functional AP-3 complex causes neurological abnormalities in *mh* mice, brain sections were prepared from *mh* and control mice, and the morphology and vesicular zinc staining analysed in the sections (Kantheti et al., 1998). Interestingly, the zinc staining was

undetectable in the mossy fibers of the *mh* sections compared to control mice sections. The gross morphology of the *mh* mossy fibers appeared normal compared to control mice, with similar numbers of clear core vesicles present. The absence of vesicular zinc staining in the *mh* sections suggested that a zinc transporter might be getting missorted. A candidate molecule is the neuronal zinc transporter ZnT-3. ZnT-3 is a multi-spanning transmembrane protein that contains both a conserved dileucine and a tyrosine type motif near its C-terminal (Palmiter et al., 1996). The *mh* sections were stained for the ZnT-3, using a rabbit polyclonal antibody. In the control sections, ZnT-3 staining was present in synaptic vesicles of the mossy fibers but in the *mh* sections the staining was absent. Interestingly, is some *mh* neurons a perinuclear reticular staining pattern was present in the cell bodies. These results suggest that the ZnT-3 zinc transporter is mislocalised in the *mh* brains, possibly being retained in the trans Golgi network. It is unclear if the absence of vesicular zinc cause the *mh* associated neurological abnormalities. Mice have been generated that are deficient in the ZnT-3 transporter (Cole et al., 1999), and as in the Kantheti et al. (1998) study there is no zinc staining in the mossy fibers. Interestingly, the ZnT3 deficient mice do not appear to have such striking neurological phenotypes as the *mh* mice. This might suggest that the *mh* phenotypes are probably caused by the missorting of other AP-3 dependent cargo molecules and not just ZnT-3 alone.

Hypopigmentation in mh and pe mice

Both *mh* and *pe* mice have a reduction in the amount of eye, skin and hair pigmentation, although these tissues still contain pigmented melanosomes. As the mice have reduced pigmentation, the sorting of the key melanogenic enzyme tyrosinase has been investigated. Tyrosinase is a type I transmembrane protein that contains potential dileucine and tyrosine based sorting motifs (Honing et al., 1998). A study by Honig et al. (1998) suggested that the dileucine motif in tyrosinase is able to bind to the AP-3 complex. The sorting of tyrosinase does not appear to be grossly effected in primary melanocytes derived from *mh* and *pe* mice, with active tyrosinase present in

late endocytic structures (Alex C. Theos, personal communication). Interestingly, there is a very early paper that shows tyrosinase activity in clathrin-coated vesicles (Maul and Brumbaugh, 1971). This paper in conjunction with the above findings would suggest that tyrosinase is not sorted via AP-3 but possibly by the AP-1 complex to the melanosome, since our results indicate that AP-3 is not associated with clathrin-coated vesicles (Simpson et al., 1996) (Simpson et al., 1997). These findings would suggest that the defects in pigmentation in *mh* and *pe* mice are not due to the direct mislocalisation of tyrosinase but possibly other factors that might be required for the activity of tyrosinase. Alternatively there might be somewhat less efficient sorting of tyrosinase and other melanosomal markers that could lead to decreased melanin biosynthesis, without affecting the overall distribution of tyrosinase.

Both these studies indicate that the analysis of the mutant phenotypes will provide a large amount of information about the molecules that can and can not interact with the AP-3 complex, but it will take a lot more research to directly relate the loss of functional AP-3 complex to the many phenotypes observed in these mice.

In conclusion, the AP-3 complex, like the other adaptor complexes consists of a β subunit, a α/γ (δ) subunit, a μ subunit and a σ subunit. Analysis of organisms defective in AP-3 complex have revealed that the AP-3 complex is involved in the trafficking of proteins to lysosomes or lysosome like organelles. Interestingly, there are a large group of mutants in *Drosophilia* and mice that give phenotypes similar to the loss of functional AP-3 complex. Analysis of these other mutants should hopefully give further insights into the AP-3 dependent pathway.

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