Title
Studies of the roles of the light chains in clathrin function

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Studies of the roles of the light chains in clathrin function

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Susan L. Acton

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To my husband and family

Preface

To avoid as much repetition as possible, commonly used procedures in this thesis are described in full in a materials and methods chapter (Chapter 7) and these procedures are often referenced. Details concerning individual experiments are described along with each experiment in Chapters 1 through 5.

Acknowledgments

Thanks are truly due to Frances Brodsky who took me on as a naive young graduate student. I have learned a great deal from her during my five years and I believe I have become a better scientist because of it.

I would also like to thank some of the members of the laboratory who kept me sane during the rough times: Inke Näthke, Darren Wong, and Deirdre Crommie. The advice and discussions of Bruce Koppelman and Mike Chang are also greatly appreciated. A special thanks to Don Simonetti whose outlook on life always made me smile.

I am particularly grateful to have worked with Dr. Tony Jackson. Our collaboration with him was very fruitful and I thoroughly enjoyed our interactions.

I would like to give a special thanks to Dr. Marvin Schulman of Merck whose advice on graduate school, scientific research and life in general was always greatly appreciated.

I am forever indebted to my parents without whom none of this would have been possible. Throughout my many years of education their support, both emotional and financial, was well in excess of what any child could have expected or hoped for and I am eternally grateful to them. I only hope that one day I can be as supportive of my children.

Most importantly, I could not have endured the five year period during the preparation of this work were it not for my husband, Douglas Dominic Buechter. His love and support helped me through the day to day struggles. I am indebted to him for his patience and understanding.

Some of the results reported herein have already been published as of the date of this thesis and were reproduced from the Journal of Cell Biology by copyright permission of the Rockefeller University Press. The coauthor directed and supervised the research which forms the basis for Chapters 2 and 3.

> Acton S.L. and F.M. Brodsky (1990) Predominance of clathrin light chain LCb correlates with the presence of a regulated secretory pathway. *J. Cell Biol.* 111:1419-1426.

The text of Appendix 1 of this thesis is a reprint of the material as it appears in:

> Chin D.J., R.M. Straubinger, S. Acton, I. Nathke, and F.M. Brodsky (1989) 100-kDa polypeptides in peripheral clathrin-coated vesicles are required for receptor-mediated endocytosis. *Proc. Nat. Acad. Sci. USA* 86:9289-9293.

This work was supported in part by NIH grants GM 38093 and GM 26691, NSF grant DCB8711317, and the Pew Charitable Trusts. S.A. was supported by an NIH training grant to the Pharmaceutical Chemistry PhD program and by the American Foundation for Pharmaceutical Education.

Abstract

The light chain subunits of clathrin, LCa and LCb, have been implicated in the regulation of coated vesicle assembly and disassembly in cells. Regions of structural dissimilarity between LCa and LCb indicate possible functional differences. To determine how LCa and LCb might differentially influence clathrin function, characteristics of the two types of clathrin light chains were studied. First, expression levels of each light chain were compared in tissues and cells with and without a regulated secretory pathway to determine if the additional clathrin functions in these cells preferentially require one light chain. LCb was found to predominate in cells and tissues maintaining a regulated secretory pathway, suggesting that LCb plays a specialized role in either secretory granule formation and/or rapid membrane retrieval after secretion. This is the first evidence for a differential function for LCb. A second difference between LCa and LCb was investigated to follow up earlier studies indicating the possibility of differential turnover of the two light chains. Although half-lives of LCa and LCb were found to be different, both half-lives are sufficiently long to allow participation in many rounds of endocytosis before degradation. This minimizes the possibility that turnover contributes to regulation of clathrin function. To gain further insight into the roles of the light chains in clathrin function, an expression system was devised in which light chain levels could be altered in cells. Reduced and abolished levels of LCa were obtained in C1R cells and PC12 cells, respectively. Surprisingly, the loss of LCa resulted in no observed differences in clathrin functions. Characteristics examined included percentage of clathrin assembled, localization of clathrin and proteins sorted by clathrin, rates of endocytosis and recycling of transferrin, and secretion of norepinephrine and secretory granule proteins. These studies indicate that LCa is unnecessary for cell survival or growth and that LCb may substitute for LCa. These data suggest that, rather than being highly specialized, LCa and LCb can perform many of the same functions and that their different structural properties, such as phosphorylation, may allow these common functions to be differentially regulated for each light chain.

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Abbreviations

BME	ß-mercaptoethanol
BSA	Bovine serum albumin
DAB	3',3'-Diaminobenzidine
DEX	Dexamethasone
dFCS	dialyzed fetal calf serum
DMF	dimethylformamide
DNA	Deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EGTA	ethylene glycol bis(ß-aminoethyl ether) tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
G418	Geneticin
GAM	Goat anti-mouse
HC	Clathrin heavy chain
HOAc	Acetic acid
HRP	Horse radish peroxidase
HRP-GAM	Horse radish peroxidase goat anti-mouse Ig
HRP-GARb	Horse radish peroxidase goat anti-rabbit Ig
HS	Horse serum
^{125}I-RAM	Iodinated rabbit anti-mouse Ig Fab
kD	Kilodaltons
KLH	keyhole limpet hemocyanin
LCa	Clathrin light chain a
LCb	Clathrin light chain b
mAb	Monoclonal antibody
MBS	*m*-maleimidobenzoic acid N-hydroxysuccinimide ester
MeOH	Methanol
milk	Dry non-fat milk
NE	Norepinephrine
NGF	Nerve growth factor
NP40	Nonidet P-40 detergent (same as triton X-100)
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate-buffered saline
pMAMneo	a mammalian expression vector
PMSF	phenylmethylsulfonyl fluoride
RIA	Radioimmunoassay
r.t.	room temperature
RSB	Reducing sample buffer
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TE	Tris-EDTA buffer

Chapter 1: Introduction to clathrin

1.1 Clathrin function in endocytosis and transport

Cells require the internalization and transport of receptors and their ligands for normal growth and survival (Goldstein et al., 1985; Payne and Schekman et al., 1985). Nutrients, hormones, cellular proteins, and other factors (Table 1.1) are transported via a specialized system present on the plasma membrane and in the Golgi apparatus of cells. This transport system binds and concentrates the receptors and ligands on specialized regions of the membrane that can be distinguished from the surrounding membrane by their distinctive 'coated' appearance. This coat is due to a polyhedral network of protein composed of polymerized clathrin (see Brodsky, 1988 for review). Clathrin is found in two regions of the cell: at the plasma membrane and near the trans Golgi apparatus (Figure 1.1).

Ligand	Characteristics of receptor
nutrient	
transferrin	Dimer of 90 kD
low density lipoprotein	160 kD
transcobalamin II	50 kD
intrinsic factor	180 kD
effector	
epidermal growth factor	190 kD
insulin	130-135 kD and 49-90 kD
chorionic gonoadotropin	unknown
immunoglobulin-Fc	family of receptors (45-90 kD)
clearance/transport across cell	
man-6-P glycoproteins	150 kD
asialoglycoproteins	42-59 kD
oxidized (or acetylated) LDL	trimer of 70 kD
immunoglobulin-Fc	family of receptors (45-90 kD)

Table 1.1 Examples of receptors and ligands transported in clathrin-coated structures

Ligands are separated into functional categories. This is only a partial list of ligands transported by clathrin (see Steinman et al., 1983 for a more complete list).

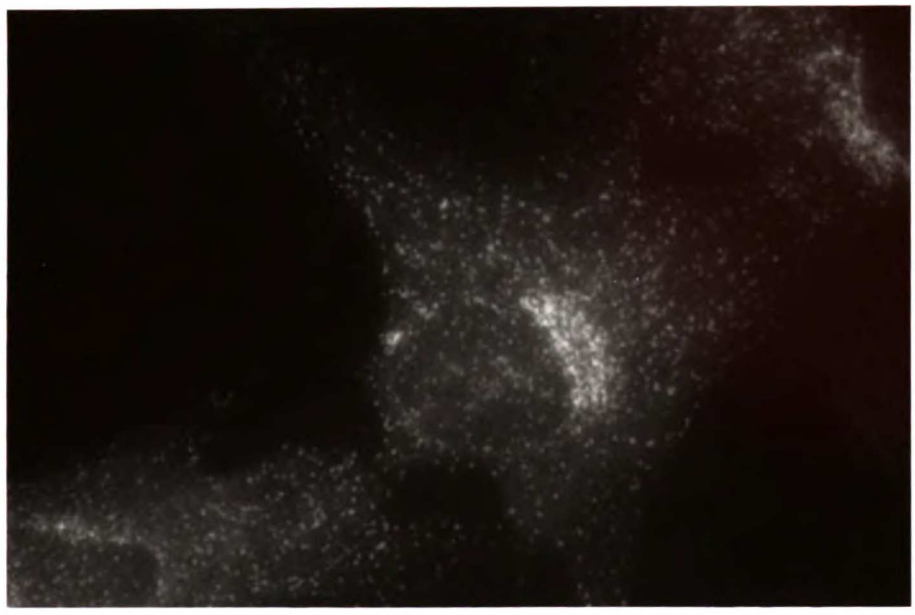


Figure 1.1 Clathrin localization in cells by immunofluorescence.

Monoclonal antibody X22 (anti-clathrin heavy chain) was used to detect clathrin in fixed and permeabilized human fibroblast cells. Fluorescein conjugated goat-anti-mouse IgG was used to detect the anti-clathrin mAb. See Chapter 7 for details of the methodology. Magnification is 2000X.

At the plasma membrane, clathrin is primarily involved in the endocytosis of nutrients, hormones, and their receptors. During the early stages of endocytosis, the coated membrane is thought to be flat (Larkin et al., 1986). The membrane in this region then invaginates to form a coated pit and finally pinches off to form a coated vesicle (Figure 1.2). Inside this vesicle are found the receptors and their ligands.

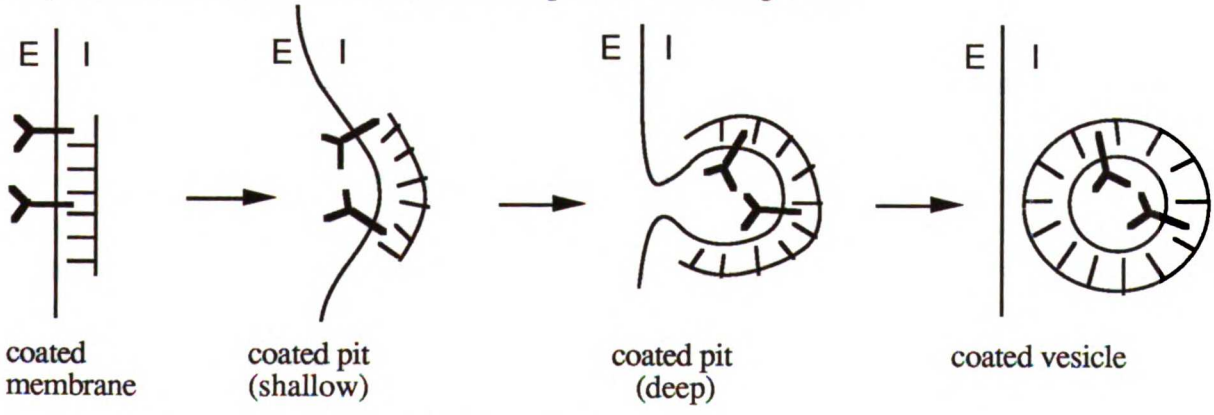


Figure 1.2 Stages of coated vesicle formation

E and I indicate extracellular and intracellular space, respectively. Receptors are indicated by a bold 'y'.

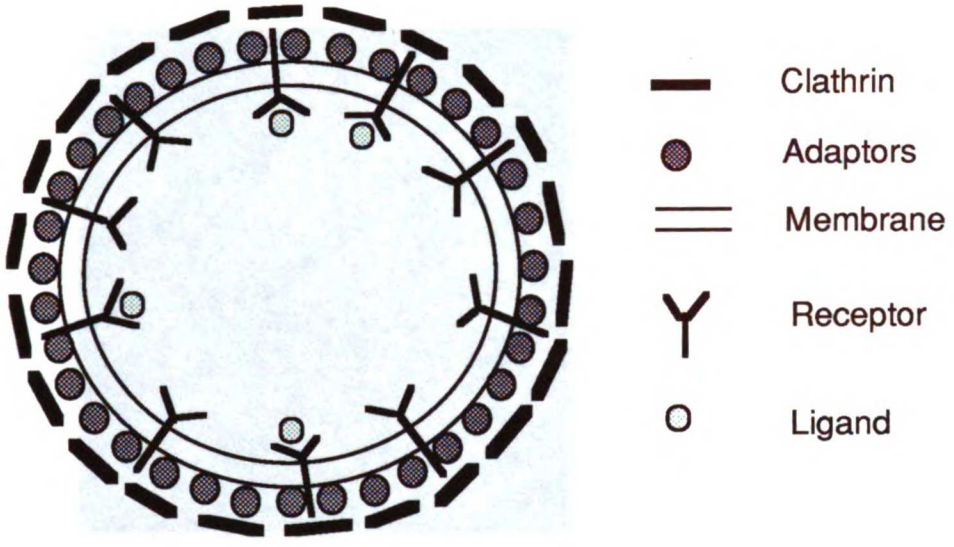


Figure 1.3 Schematic of the cross-section of a coated vesicle

In addition to clathrin, a second major component of coated vesicles is a complex of proteins known as assembly proteins or adaptors. These proteins are located between the clathrin coat and the membrane (Fig 1.3) and have been termed 'assembly proteins' due to their ability to cause clathrin assembly *in vitro* (Zaremba and Keen, 1983; Pearse and Robinson, 1984). They have also been called adaptors as a result of data which indicates that they may serve as the linker between clathrin and receptor tails (Pearse, 1988). This group of proteins shall be referred to here as assembly complexes. There are two major types of assembly complexes in cells (Ahle et al., 1988; Keen, 1987; Robinson, 1987); the first is termed AP1 and is located in the Golgi region and the second, AP2, is located at the periphery (near the plasma membrane) of cells (Figure 1.4). The two types of complexes are similar in protein number and size, but the individual constituent proteins are unique in each (Figure 1.5).

Within approximately one minute after formation, the coat of a clathrin-coated vesicle depolymerizes (Pearse and Bretscher, 1981). A member of the heat-shock protein family, hsc70, can catalyze the uncoating of coated vesicles *in vitro* (Schlossman et al., 1984), and it has been suggested that this also occurs *in vivo* (DeLuca-Flaherty et al., 1990). It is not known whether the AP complexes dissociate from the membrane at this time. The internalized receptors and ligands are then sorted by an unknown mechanism for further transport within the cell. Some receptors, such as the transferrin receptor, are recycled back to the plasma membrane where they undergo further rounds of endocytosis

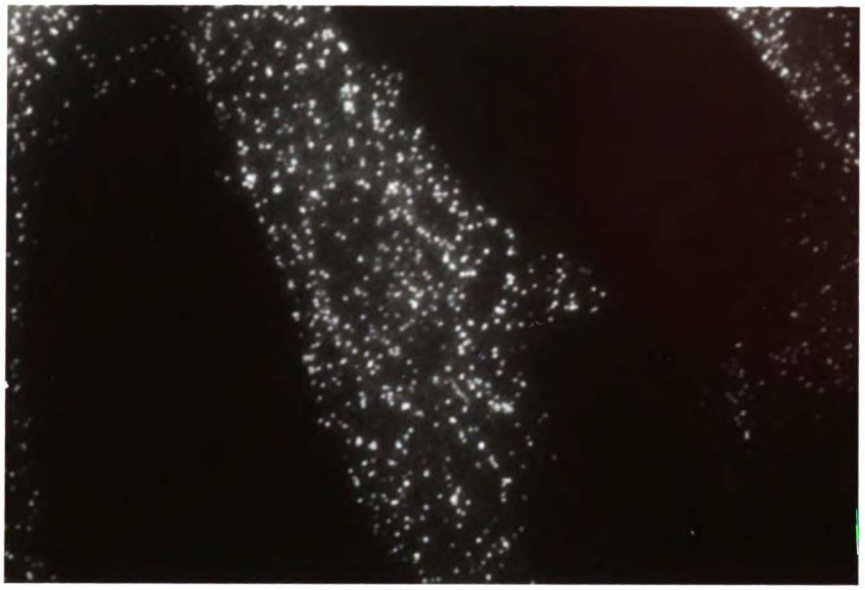


Figure 1.4 Immunofluorescence localization of AP2.

Distribution of the assembly complex in human fibroblasts. Monoclonal AP.6 and fluorescein conjugated goat-anti-mouse IgG were used to localize AP2 (see Chapter 7 for details). Magnification is 2000X.

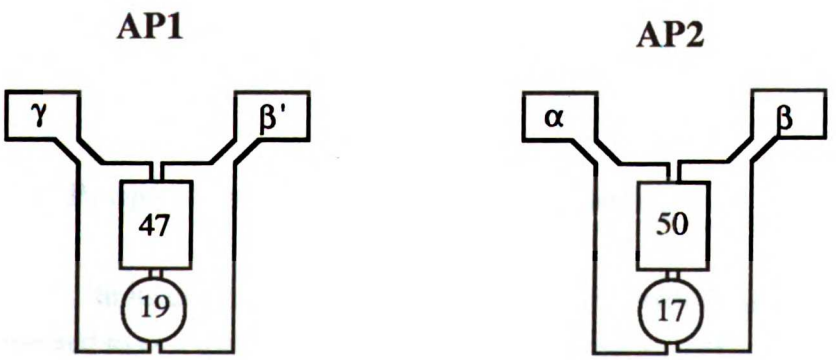


Figure 1.5 Proposed subunit structure of the adaptor complexes

Subunits named with greek symbols all run at approximately 100 kD in SDS-PAGE. The smaller subunits are labeled according to their molecular weights (in kD).

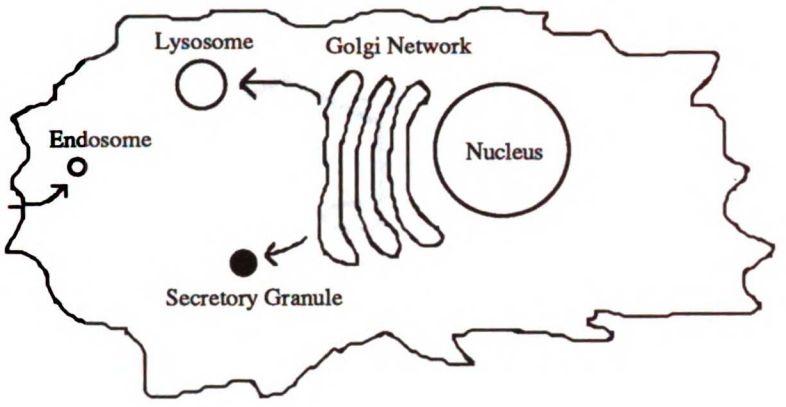


Figure 1.6 Trafficking by clathrin coated vesicles
Arrows indicate pathways in which clathrin participates in the trafficking of proteins.

(Karin and Mintz, 1981). Other receptors (e.g. EGF receptor) are transported to lysosomes where they undergo degradation (Dunn and Hubbard, 1984). In the endocytic transport process, clathrin is thought to be involved only in the very early stages.

The clathrin located in the Golgi apparatus serves two functions (Fig. 1.6). One of these is to assist in the transport of newly synthesized lysosomal enzymes from the trans Golgi network to a prelysosomal compartment. The mannose-6-phosphate receptor binds and carries lysosomal enzymes (Kornfeld, 1987) and clathrin has been shown to transport this receptor from the trans Golgi to a prelysosomal compartment. Clathrin also plays a role in the formation of secretory granules in some specialized secretory cells. The exact role of clathrin in this process is not clear but it appears that it is involved in concentrating and packaging secretory granule proteins (Orci et al., 1984 ; Tooze and Tooze, 1986).

1.2 Properties of clathrin light chains

In its unassembled state, clathrin has the geometry of a three-legged pinwheel and is referred to as a triskelion (Ungewickell and Branton, 1981; Kirchhausen and Harrison, 1981) (Figure 1.7). The triskelion is composed of six polypeptide chains: three heavy chains of 190 kD form the long arms, and three light chains of between 26-33kD are bound to the proximal arms of the heavy chains (Brodsky, 1988). Each heavy chain and light chain is oriented with its carboxy terminus towards the vertex. In mammalian cells,

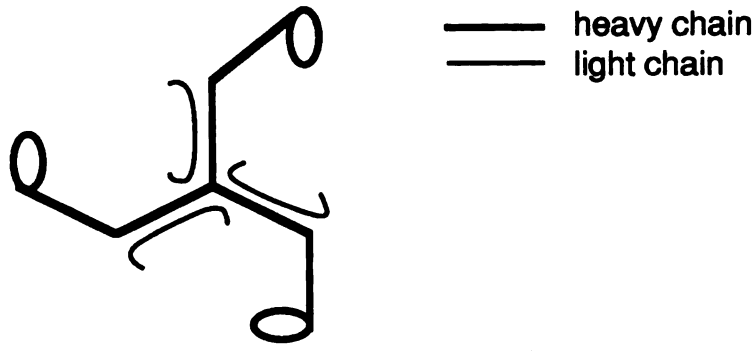


Figure 1.7. Structure of unpolymerized clathrin

Each triskelion is formed from three clathrin heavy chains and three light chains. The large circles at the ends of the 'arms' represent the heavy chain amino-terminal domains.

```
                1         10         20         30         40         50         60
HUMAN LCA       MAELDPFGAPAGAPGGPALGNGVAGAGEEDPAAAFLAQQESEIAGIENDEAFAILDGGAPGPQPHG
BOVINE LCA      --------V--***---------**-------------------------------------S----
RAT LCA         ------------------------------------------------------------A--
HUMAN LCB       ********MADDFGFFSSSES-APE-A----------------------G-GAPA-SHAA-AQP-
BOVINE LCB      ********MADDFGFFSSSES-APE-A----------------------G-GAPA-SQG-LAQP-
RAT LCB         ********MAEDFGFFSSSES-APE-A----------------------SG-GAPAASQVASAQP-

                     70         80         90         100        110        120
HUMAN LCA       EPPGG*PDAVDGVMNGEYYQESNGPTDSYAAISQVDRLQSEPESIRKWREEQMERLEALDANSRKQ
BOVINE LCA      ----I*-------T--D-----------------------------------T-------------
RAT LCA         -----*-------------E-----------------------------------T-------------
HUMAN LCB       PTS-AGSEDMGTTV--DVF--A---A-G----A-A---TQ------------RK--QE---A-KVT
BOVINE LCB      PAS-A*SEDMGATV--DVF--A---A-G----A-A---TQ------------RK--QE---A-KVM
RAT LCB         LAS--GSEDMGTTV--DVF--A---A-G----A-A---TQ------------KK--QE---A-KVT

                   130        140        150        160        170        180        190
HUMAN LCA       EAEWKEKAIKELEEWYARQDEQLQKTKANNRVADEAFYKQPFADVIGYVTNINHPCYSLEQAAEEA
BOVINE LCA      ------------D---------------S-------------------------------------
RAT LCA         ------------------------------------------------------------------
HUMAN LCB       -Q--R---K-D----NQ--S--VE-N-I---I--K---Q--D--I----************-S---
BOVINE LCB      -Q--R---K-D----NQ--S--VE-N-I---I--K---Q--D--I----************-S---
RAT LCB         -Q--R---K-D----NQ--S--VE-N-I---I--K---Q--D--T----************-S---

                      200        210        220        230        240
HUMAN LCA       FVNDIDESSPGTEWERVARLCDFNPKSSKQAKDVSRMRSVLISLKQAPLVH
BOVINE LCA      -----E---------------------------------------------
RAT LCB         -------------------------------------------------
HUMAN LCB       --KESK-ET------K--Q-----------C-----L----M----T--SR
BOVINE LCB      --KESK-ET------K--Q-----------C-----L----M----T--SR
RAT LCB         --KESK-ET------K--Q-----------C-----L----M----T--SR
```

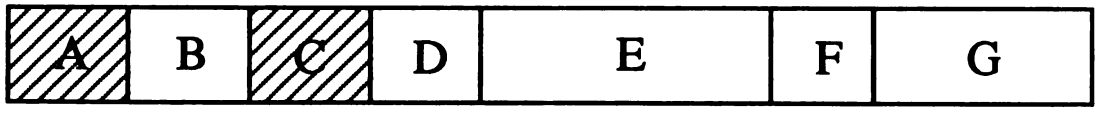
Figure 1.8 Primary sequences of the clathrin light chains

The amino acid sequences of human, bovine, and rat LCa and LCb as deduced by cDNA sequencing. Dashed lines represent amino acids identical to human LCa. Stars represent positions with no corresponding amino acid. Numbering is based on bovine brain LCa.

the light chains are of two types, LCa and LCb, which are encoded by different genes (Jackson et al., 1987; Kirchhausen et al., 1987). Yeast, however, has only one type of light chain (Silveira et al., 1990). The distribution of LCa and LCb on triskelions appears to be random (Kirchhausen et al., 1983), thus four types of triskelions can be found in cells: 1) triskelions containing only LCa 2) triskelions with two LCa polypeptides and one LCb, 3) triskelions containing one LCa and two LCb polypeptides, 4) and those with all LCb. Mammalian LCa and LCb are 60% identical in protein sequence (Figure 1.8) (Jackson et al., 1987).

The light chains are extended molecules and can, in primary sequence, be divided into several domains (Figure 1.9) Some of these regions are very similar in sequence in LCa and LCb (regions B, D, E, F, and G) indicating that they are involved in the common functions of the two light chains. LCa and LCb also have domains that are dissimilar (regions A and C) suggesting that these regions are important for their differential roles.

The most highly conserved region of the light chains is region B. It is termed the consensus sequence, since it is identical in both LCa and LCb and in all mammalian species examined (rat, bovine, human) (Jackson et al., 1987; Kirchhausen et al., 1987; Jackson and Parham, 1988). In addition, the yeast light chain has a similar region which is 45% homologous to the mammalian consensus sequence (Silveira et al., 1990). It is exposed on the surface of triskelions and coated vesicles (DeLuca-Flaherty et al., 1990; Chapter 5) and may therefore interact with other cellular components. The exact function of this



A = amino terminal region, phosphorylated in LCb but not LCa
B = consensus sequence (identical in all mammalian light chains)
C = proline-glycine rich region, hsc70 binding site
D = calcium binding region
E = heavy chain binding region
F = brain insert region
G = carboxy terminal region

Figure 1.9 Domains of clathrin light chains

The light chains are divided into linear domains according to functional and sequence properties. Hatched regions are sequences very dissimilar in LCa and LCb. Open areas indicate regions which show significant identity.

region is unknown but it is likely to be universally important since it is so highly conserved. Region E is also similar in LCa and LCb and has been shown to be the heavy chain binding region (Brodsky et al., 1987; Kirchhausen et al., 1990). This domain may form a coiled-coil motif (Kirchhausen et al., 1987) and may bind to a similar motif on the heavy chain (Blank and Brodsky, 1987). LCa and LCb compete for the heavy chain binding site (Winkler and Stanley, 1983; Brodsky et al., 1987) with dissociation constants estimated to be less than 10^{-11} M (Winkler and Stanley, 1983).

Clathrin assembly *in vitro* can be stimulated by calcium and the light chains have been shown to bind calcium with a K_d of 25-50 μM (Mooibroek et al., 1987). The calcium binding site has been localized to residues 85-96 (numbering system based on human brain LCa) (Näthke et al., 1990). This domain may assume a similar structure to the EF hand motif of other calcium binding proteins (Näthke et al., 1990). The calcium binding domain lies between two regions (heavy chain and hsc70 binding regions) that may be important in the assembly/disassembly of coated vesicles. It has been suggested that calcium may play a role in modulating clathrin assembly *in vivo*.

LCa and LCb are differentially spliced in neurons (Jackson et al., 1987; Wong et al., 1990) resulting in one additional exon in LCb and one or two additional exons in LCa (Figure 1.10) This region (region F) is relatively hydrophobic compared to the rest of the light chains and is located on the carboxy side of the heavy chain binding region (region E). The function of these exons is not known; however, it has been proposed that these regions may be important for transport of clathrin in axons (Wong et al., 1990). Molecular weights estimated by SDS-PAGE for the neuronal and nonneuronal forms of the clathrin light chains are much greater than those calculated from the predicted amino acid sequence (Figure 1.10). This anomalous behavior has been shown to be due to a short sequence of negatively charged amino acids (residues 22-25) (Näthke et al., 1990; Scarmato and Kirchhausen, 1990).

Although mammalian LCa and LCb are 60% identical, they contain two regions that are very diverse: regions A and C (Figure 1.9). Region A contains a phosphorylation site present in LCb, but not LCa. Phosphorylation of LCb *in vitro* has been shown to be catalyzed by a casein kinase II enzyme (Schook and Puszkin, 1985; Bar-Zvi and Branton, 1986). Since the 47 kD subunit of the AP1 complex has been shown to copurify with casein kinase II activity (Meresse et al., 1990), it may be the enzyme that phosphorylates LCb. LCb phosphorylation may itself play a role in modulating the phosphorylation state of the 50 kD subunit of the AP2 protein (Hanson et al., 1990). Phosphorylated LCb inhibits phosphorylation of the 50 kD polypeptide and stimulates the dephosphorylation of

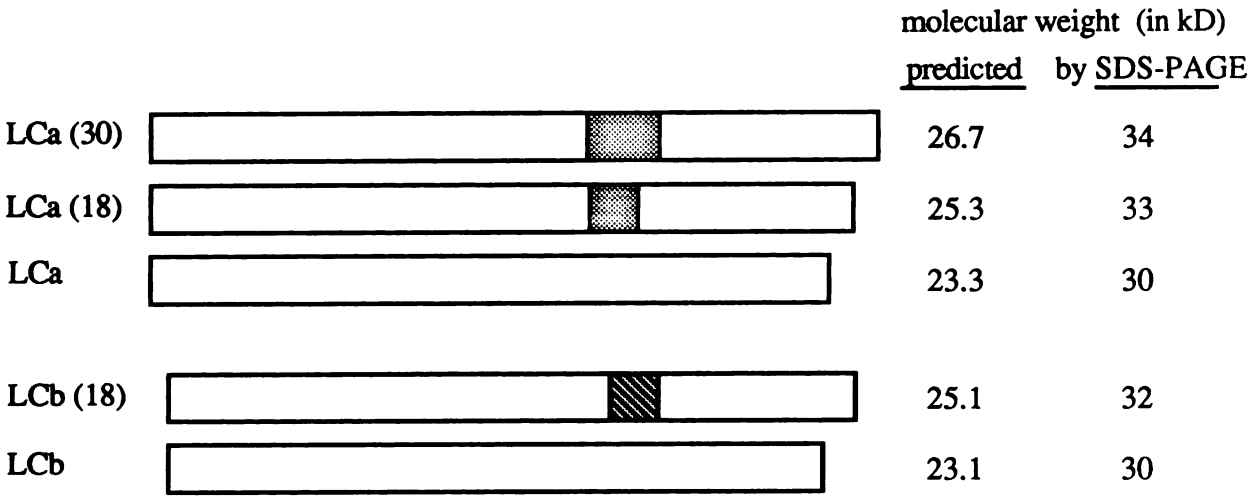


Figure 1.10 Peripheral and neuron forms of clathrin light chains

Numbers in parenthesis indicate number of amino acids in the neuron-specific exons. Shaded regions indicate the relative position of the neuron-specific exons in the sequence.

the 50 kD subunit. Thus, the phosphorylation of LCb may play a role in the interactions of clathrin with the assembly polypeptide complexes.

DeLuca-Flaherty and coworkers (1990) have shown that hsc70 (the uncoating protein) will hydrolyze ATP in response to a peptide in LCa from region C (residues 47-71), but not the peptide from LCb in this region. The conformation of this region in LCa can be influenced by ions, particularly calcium, and it has been suggested that calcium binding may play a role in the uncoating process. The data indicate that LCa may play a specialized role in the uncoating of coated vesicles.

1.3 Significance and aims

Mammalian light chains, LCa and LCb, have properties suggesting that they serve the role of regulatory subunits of the clathrin molecule. The experiments described in this thesis were designed to investigate the differential properties of the light chains in an effort to determine how each might be influencing clathrin function. It was also hoped that these studies might explain why yeast, unlike mammalian cells, has only one type of light chain. The aims of this work were:

Aim 1: Determine if the relative levels of expression of LCa and LCb are tissue or cell type specific.

Aim 2: Determine if turnover rates of clathrin subunits could play a role in clathrin regulation.

Aim 3: Develop a system for altering LCa:LCb ratios *in vivo*.
Aim 4: Determine the effects of changing LCa:LCb ratios *in vivo* on clathrin function.

Each of these aims is described in a separate chapter of this thesis.

From these studies we hoped to gain a more thorough knowledge of the process by which clathrin modulates the internalization and transport of receptors and their ligands. In addition to adding to our understanding of the normal process of receptor trafficking, this work may be useful in the future for utilizing these pathways for the delivery of drugs into cells.

Chapter 2: Differential tissue distribution of clathrin light chains

2.1 Introduction

Cells utilize clathrin to endocytose nutrients and hormones and to transport lysosomal enzymes from the Golgi to a prelysosomal compartment (Griffiths et al., 1988). In cells which specialize in regulated secretion, clathrin participates in two additional functions: packaging of secretory granules (Tooze and Tooze, 1986; Orci et al., 1984) and rapid retrieval of membrane after stimulated secretion (Heuser and Reese, 1984). The contribution(s) of the light chains in these processes are not yet defined, although many of their properties suggest they play a regulatory role (Schmid et al., 1984; Bar-Zvi et al., 1988). Previous studies have indicated that coated vesicles isolated from bovine brain tissue have more LCb than LCa (Lisanti et al., 1982). In contrast, reticulocytes were shown to contain approximately a 10:1 LCa/LCb ratio (Bar-Zvi and Branton, 1986). Thus the relative amount of light chains expressed in tissues varies greatly; however, the purpose of this variation is unknown.

A method for light chain quantitation in tissue and cell samples was developed to determine if a correlation exists between light chain ratio and tissue function. Because of the role of clathrin in regulated secretion, it was of particular interest to determine if cells with and without a regulated secretory pathway have different relative levels of light chains. These studies revealed a correlation between the predominance of LCb and the presence of a regulated secretory pathway. In addition, these studies showed that cells utilize the same ratio of light chains (LCa/LCb) in assembled structures as they express.

2.2 Results

Antibody characterization

To quantitate the levels of the light chains it was necessary to obtain antibodies that were either specific for one of the light chains in RIA or could recognize either light chain well in a immunoblot. Monoclonal X16 is LCa specific and binds well in immunoblots, but does not bind well in RIAs of cell lysates since it binds in the heavy chain binding region (Brodsky et al., 1987) (Figure 2.1). X43 recognizes both light chains in immunoblots, but only poorly. Thus, it was necessary to find an antibody against LCb that recognizes immunoblots sufficiently well to allow the quantitation of the two light chains

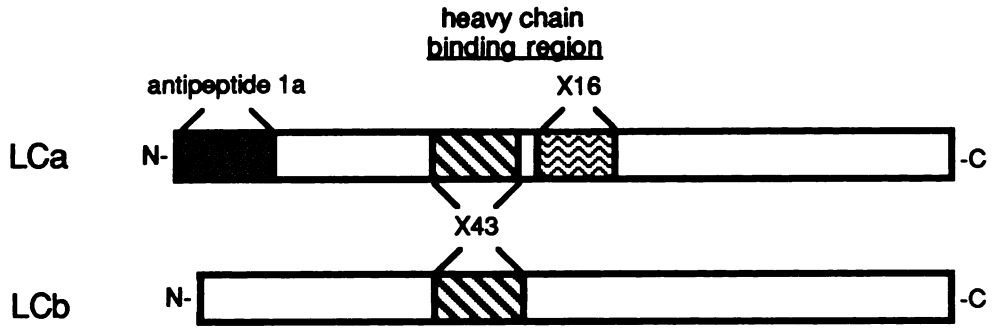


Figure 2.1 Antibody binding sites on clathrin light chains

Binding sites of X16 and X43 were determined by peptide binding studies (Brodsky et al., 1987). The antipeptide 1a serum was prepared against synthesized peptide (DeLuca-Flaherty et al., 1990).

by immunoblotting. Monoclonal LCB.1 was a possibility because it immunoblots well; however, this antibody had not been sufficiently characterized and it was not clear whether LCB.1 was completely LCb specific. While LCB.1 appeared to react with brain and non-brain LCb, it also appeared to react with non-brain LCa. It did not react, however, with brain LCa. To further clarify the specificity of LCB.1, adrenal clathrin LCa and LCb were purified and immunoblots were performed. These studies revealed that LCB.1 is specific for LCb. In addition, it was determined that the reactivity of LCB.1 with purified non-brain LCa in RIAs is due to a small amount of contaminating brain form of LCb in the preparation. This finding clarified the specificity of LCB.1 for LCb and indicated that it would be a useful tool for quantitating LCb by immunoblotting. The finding of brain LCb in adrenal tissue was later confirmed when it was established that 'brain' light chains are actually 'neuronal' light chains (Wong et al., 1990). The neurons which innervate the adrenal glands may contain a sufficient amount of neuronal light chains to be recognized in RIA and immunoblots. Conversely, since adrenal and brain have a common embryological origin, the neuronal exon may be present but vestigial in adrenal tissue.

To characterize the LCB.1 antibody further, immunoblots of brain and non-brain tissues from various animals were performed (Table 2.1). As expected, the antibody recognized a higher molecular weight form of LCb in brain tissue than in non-brain tissue. The antibody recognized bovine, human and chicken LCb, but not mouse or rat LCb.

To determine the binding site of LCB.1 on the light chains, an RIA was performed on peptides derived from the sequence of human LCb (Figures 2.2, 2.3). These peptides were synthesized in the laboratory of P. Parham (Stanford University). Since LCB.1 recognizes human and bovine LCb, but not rat LCb, the search was narrowed to those peptides which contain at least one different amino acid in the rat sequence than in the

tissue	low MW LCb	high MW LCb
rat liver	-	-
rat brain	-	-
mouse liver	-	-
mouse brain	-	-
chicken liver	+	-
chicken brain	+	+
bovine	+	-
bovine brain	+	+
human	+	-
human brain	+	+

Table 2.1 LCb antigens of mAb LCB.1 in tissue homogenates

Tissue lysates were electrophoresed by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with monoclonal LCB.1 and enzyme-coupled second antibody. + (binding detected), - (no binding). High MW and low MW refers to LCb with and without the neuronal insert, respectively.

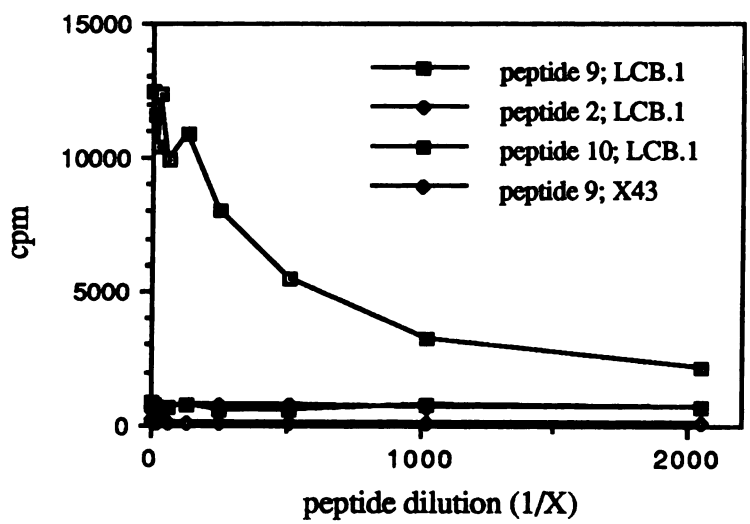


Figure 2.2 LCB.1 binding to LCb peptides

Peptides were bound to RIA plates, blocked with BSA, and probed with the antibodies shown. Antibody binding was detected with iodinated rabbit-anti-mouse IgG. The key indicates the peptide and antibody tested. Peptide 9: LCb aa 9-22; peptide 2: aa 23-43; peptide 10: LCb aa 44-70. Amino acid numbering is based on the bovine brain LCa sequence (Figure 1.8).

```
             9                    22
peptide 9    MADDFGFFSSSESGAPEAA
               E
```

Figure 2.3 Monoclonal LCB.1 binding site on clathrin LCb

The bovine and human sequence (identical) is shown above with the amino acid difference in the rat sequence shown below. Numbers indicate amino acids in the sequence using bovine brain LCa as a reference. LCb starts at amino acid 9 in the sequence of bovine LCa (see Figure 1.8), thus this is the amino terminus of LCb.

human or bovine sequence. Only the peptide encoding the amino-terminal 21 amino acids of LCb (designated peptide 9) gave a strong response in RIA (Figure 2.3). This region of LCb contains the phosphorylation site.

An unexpected finding from the experiments characterizing LCB.1 was that this antibody also recognized a protein of approximately 50 kD in brain tissue, but not non-brain tissue of mice and rats (Figure 2.4). LCB.1 appeared to react equally well with the 50 kD protein as with LCb. The significance of this is unclear. The 50 kD protein is not resistant to boiling (the light chains are) so it is unlikely that the 50 kD protein is an additional form of light chain. In addition to being found in brain tissue, the 50 kD protein was also present in lysates of PC12 cells, a rat adrenal pheochromocytoma line. These cells have properties of neurons and have well-established regulated secretory pathways. LCB.1 did not bind in immunofluorescence staining of PC12 cells, therefore it was not possible to determine the localization pattern of the 50 kD protein. Since the LCB.1 binding site on LCb was known, it was of interest to determine if this sequence might also be found in a known protein of 50 kD (in an attempt to identify the LCB.1 50 kD antigen). A search in the PIR protein data base (Protein Information Resource) was performed by I. Näthke using the interface 'Eugene' to access and execute a similarity search program in the MBIR programs (Molecular Biology Information Resource, Dept. Cell Biology, Baylor College of Medicine). The closest match was identity of 9 residues out of a stretch of 14 found in the mouse protein SURF-1. This protein is predicted to be 34,798 daltons,

LCB.1 50 kD antigen

properties:

1) binds LCB.1 in Western blots
2) about 50 kD on SDS-PAGE
3) boiling sensitive

cellular location:

1) 100,000 x g pellet of 0.5% NP40 cellular lysate
2) cannot be seen by immunofluroescence in PC12 cells

present in*:

1) rat brain
2) mouse brain
3) human brain
4) PC12 lysate

not present in*:

1) rat liver
2) mouse liver
3) chicken brain
4) chicken liver

Figure 2.4 Characteristics of the 50 kD antigen of the LCB.1 mAb

*by Western blotting

however, and therefore is probably not the 50 kD protein that LCB.1 detects in immunoblots.

With the binding characteristics of the monoclonal LCB.1 determined, it was evident that LCB.1 could be used to quantitate LCb by immunoblotting. Thus both anti-LCa and anti-LCb antibodies were available to quantitate LCa and LCb in tissues and cells. Since an immunoblotting method was chosen for quantitation of the clathrin light chains, the binding of LCB.1 to the 50 kD protein did not interfere with analysis of LCb levels.

Light chain quantitation

Bovine tissue homogenates were electrophoresed on SDS polyacrylamide gels along with dilutions of known quantities of purified bovine adrenal LCa or LCb (Figure 2.5). Proteins were transferred to nitrocellulose which was then probed with anti-LCa (X16) or anti-LCb (LCB.1) mAb plus iodinated rabbit anti-mouse Ig. After exposure to film, densitometry was performed. A standard curve for the purified light chains was

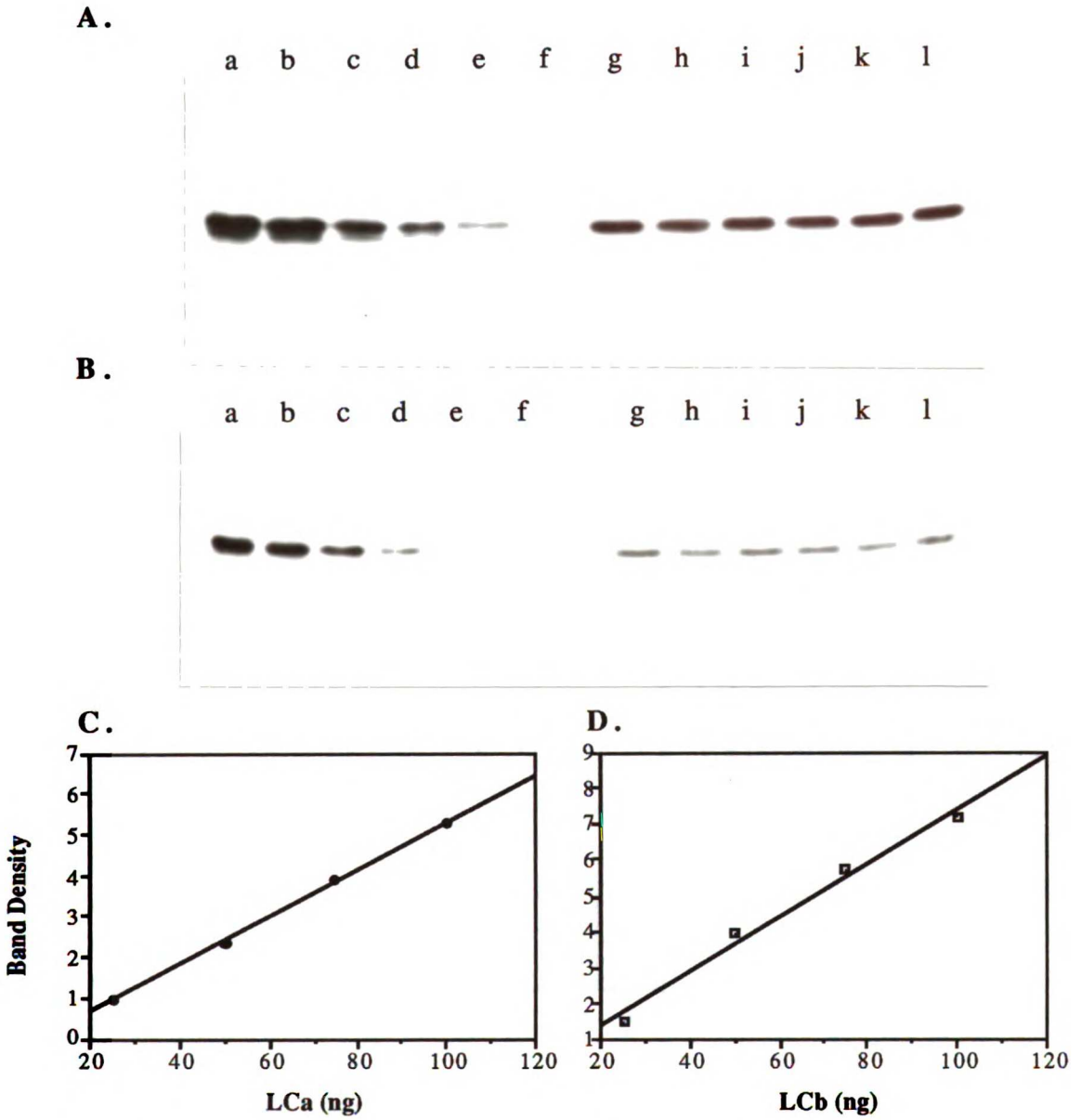


Fig. 2.5. Method for quantitation of clathrin light chain ratios.

Boiled post-nuclear supernatants of bovine kidney cortex and medulla were run on SDS polyacrylamide gels (10%) alongside purified adrenal LCa or LCb. Proteins were transferred to nitrocellulose and reacted with X16 (anti-LCa) or LCB.1 (anti-LCb). Bound antibody was detected with ^{125}I-rabbit anti-mouse Ig. The autoradiographs of the nitrocellulose blots are shown. Densitometry of the autoradiographs produced a linear standard curve (from four of the six quantities loaded) for each light chain. (A) X16 binding to LCa. Lanes a-f are purified adrenal LCa: a) 100 ng, b) 75 ng, c) 50 ng, d) 25 ng, e) 12.5 ng, f) 6.3 ng. Lanes g-i are kidney cortex samples prepared from three separate kidneys. Lanes j-l are kidney medulla samples from three separate kidneys. (B) LCB.1 binding to LCb. Lanes a-f contain purified adrenal LCb: a) 100 ng, b) 75 ng, c) 50 ng, d) 25 ng, e) 12.5 ng, f) 6.3 ng. Lanes g-l as in panel A. (C) standard curve from densitometry of purified LCa: $y = -0.4665 + 57.388x$ with a correlation coefficient of 0.999; (D) standard curve from densitometry of purified LCb: $y = -0.1365 + 75.332x$ with a correlation coefficient of 0.993. The standard curves were used to calculate the quantity of light chain in the unknown samples. Only points which could be interpolated on the curve were used.

Sample	LC_a/LC_b (ng/ng)*	
Adrenal cortex	1.01 ± 0.06	(3)
Adrenal medulla	0.72 ± 0.08	(3)
Kidney cortex	1.00 ± 0.07	(3)
Kidney medulla	0.97 ± 0.10	(3)
Spleen	1.24 ± 0.28	(3)
Primary lymphocytes	1.00 ± 0.11	(2)
Brain cortex	0.33 ± 0.01	(3)

Table 2.2. Clathrin light chain ratios in tissues

Light chain ratios were determined as in Figure 2.5. *The sample standard deviation of the ratios is given along with the number of samples tested (in parentheses).

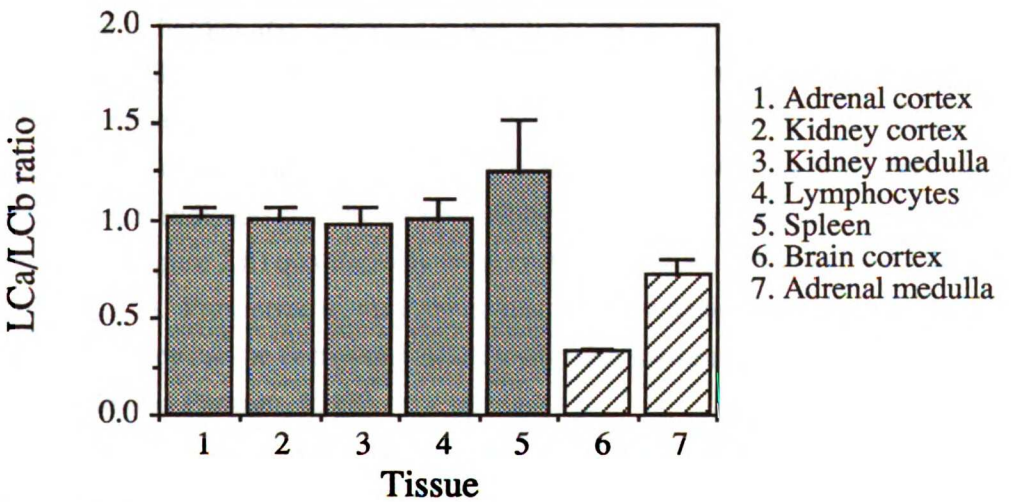


Figure 2.6 Light chain ratios in bovine tissues

Light chains were quantitated as described in Figure 2.5. **Hatched bars indicate tissues specializing in regulated secretion**; solid bars indicate tissues that do not **specialize in regulated secretion.**

constructed from densitometry of the gels and used **to determine the quantities of light chain in unknown samples.**

Light chain expression levels in tissues

Most of the tissues contained an LCa:LCb ratio of approximately 1:1 (Table 2.2; Figure 2.6). However, both brain cortex and adrenal medulla were found to have more LCb than LCa suggesting a correlation between regulated secretion and LCb expression.

Because tissues are generally a mixture of different cell types, it is possible that each tissue examined included cells with different light chain ratios. Since the ratios of light chains determined for these tissues represent the total LCa divided by total LCb, cellular variation in ratios is obscured in these numbers.

Light chain expression levels in cells

To examine the light chain ratios in individual cell types, cell lines with and without a regulated secretory pathway were analyzed by one of two methods. Light chains in bovine and human cells were quantitated by the immunoblotting method described above (Table 2.3; Figure 2.7). For rat and mouse cell lines, light chain ratios were estimated by immunoprecipitation of clathrin from ^{35}S-cysteine-labeled cells (Figure 2.8). This second approach was implemented because the anti-LCb antibody (LCB.1) used for immunoblotting analysis does not react with rat or mouse LCb. LCB.1 recognizes residues 9-22 (numbered using bovine LCa as a reference) at the amino terminus of LCb which are identical in human and bovine LCb and differ by one amino acid in rat LCb (Jackson and Parham, 1988). Absolute light chain ratios cannot be determined from immunoprecipitated clathrin because the light chains are turned over at different rates, albeit slowly (see below). However, a comparison of the light chain ratios determined by both methods for the human cell line LB indicates that the relative labeling of immunoprecipitated light chains with ^{35}S-cysteine (Figure 2.8) qualitatively reflects the light chain ratio measured by immunoblotting (Figure 2.7). Furthermore, rat light chains have the same cysteine content as human light chains so they can be compared directly with the immunoprecipitated LB light chains (Jackson and Parham, 1988).

Sample	LCa/LCb (ng/ng)*	
EBTr	2.02 ± 0.69	(2)
MDBK	5.22 ± 0.36	(3)
LB	4.97 ± 0.91	(7)
Supe T	0.83 ± 0.20	(4)

Table 2.3. Clathrin light chain ratios in cell lines

Light chain ratios were determined as described in Figure 2.5. *The sample standard deviation of the ratios is given along with the number of samples tested (in parentheses).

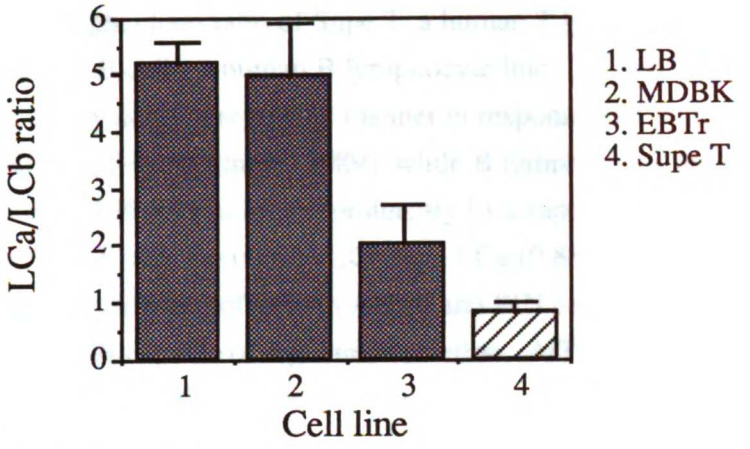


Figure 2.7 Light chain ratios in cell lines

Light chains were quantitated as described in Figure 2.5. Hatched bar indicates a cell line specializing in regulated secretion. LB, MDBK, EBTr, and Supe T are human lymphocytes, bovine kidney cells, bovine tracheal cells, and human T lymphocytes, respectively.

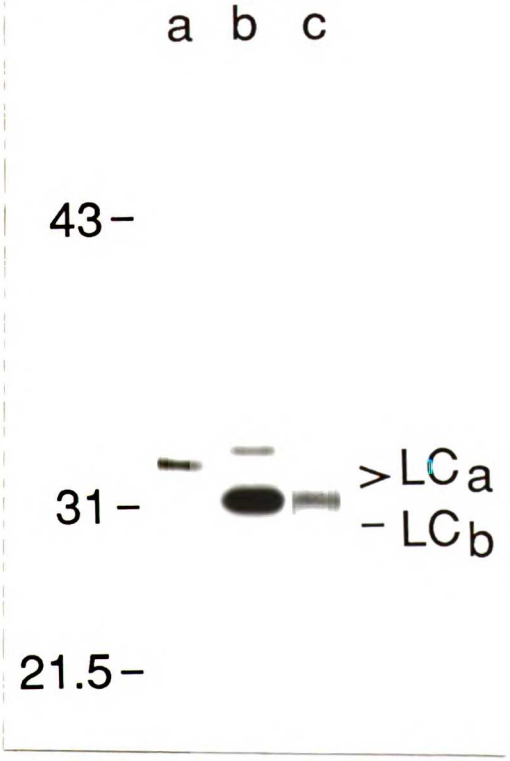


Fig. 2.8. Comparison of light chain ratios in LB, PC12 and AtT20 cells.

Cells were labeled for 10 hours with ^{35}S-cysteine and clathrin was immunoprecipitated from cell lysate with anti-clathrin heavy chain antibody (X22). Immunoprecipitated light chains were isolated by boiling and analyzed by SDS-polyacrylamide (10%) gel electrophoresis. (Lane a) LB cells (these cells have a 5:1 LCa to LCb ratio); (lane b) PC12 cells; (lane c) AtT20 cells. Molecular weight standards are indicated.

Cell lines with a regulated secretory pathway were found to have a higher proportion of LCb than other lines (Figure 2.7, Figure 2.8). Supe T, PC12, and AtT20 are lines which maintain a regulated secretory pathway, while MDBK, LB, and EBTr are lines which do not. The light chain ratio of Supe T, a human T lymphocyte cell line, can be compared to that of LB cells, a human B lymphocyte line. T lymphocytes secrete lymphokines or cytolysins in a regulated manner in response to T cell receptor interactions with a target cell (Kupfer and Singer, 1989), while B lymphocytes secrete antibodies in a constitutive manner. LB cells have predominantly LCa (approximately a 5:1 LCa:LCb ratio) while the Supe T cells have more LCb than LCa (0.83:1 LCa:LCb ratio). The intensely predominant labeling of LCb in AtT20 and PC12 cells also indicates increased LCb expression in cells capable of regulated secretion. AtT20 cells are mouse pituitary tumor cells which secrete ACTH in response to 8-bromo-cAMP (Moore et al., 1983). PC12 cells are rat adrenal pheochromocytoma cells which secrete norepinephrine in response to various secretagogues (Greene and Rein, 1977). In the cell lines which do not maintain a regulated secretory pathway (MDBK, LB and EBTr) LCa expression predominates. Of the thirteen tissues and cell lines tested for light chain levels, the five samples with predominant expression of LCb were those capable of regulated secretion.

Light chain ratios in assembled and unassembled clathrin

The ratios of light chains in assembled and unassembled clathrin were measured to determine whether the total cellular light chain ratio is reflected in assembled clathrin. Cells with high and low LCa:LCb ratios were chosen for analysis. Clathrin from a post-nuclear supernatant of tissue or cell homogenate was separated into assembled and unassembled fractions by centrifugation at 100,000 x g. The pellet (assembled clathrin) and supernatant (unassembled clathrin) were diluted to equal volumes and light chains were quantitated as above. Bovine brain cortex was used as a source of cells with a low LCa:LCb ratio. In this tissue, the same light chain ratio was found in the total, assembled, and unassembled clathrin fractions (Table 2.4; Figure 2.9) indicating no preferential incorporation of light chains into assembled structures. The percentage of assembled LCa and LCb was determined to be 88% and 87%, respectively. This is in agreement with the previous analysis of rat brain (Goud et al., 1985) showing that 86% of clathrin was assembled. LB cells were selected to represent cells containing predominantly LCa. Similar to brain, most of the light chains in these cells were in the assembled pool (70-79% assembled). Therefore it appears that the ratio of LCa to LCb in a cell does not confer a specific level of assembly. Much more of the clathrin was assembled in these

	Brain cortex		LB	
LCa/LCb (ng/ng)				
Total clathrin	0.33 ± 0.01	(3)	4.97 ± 0.91	(7)
Assembled	0.33 ± 0.01	(3)	3.95 ± 0.78	(4)
Unassembled	0.31 ± 0.01	(3)	6.65 ± 0.68	(4)
%LCa assembled**	88% ± 1%	(3)	70% ± 6%	(4)
%LCb assembled	87% ± 1%	(3)	79% ± 4%	(4)

Table 2.4. Ratios of light chains in assembled and unassembled clathrin

Postnuclear supernatants were centrifuged at 100,000 x g for 1 hr. The pellets (containing assembled clathrin) were resuspended into the same volume as the supernatants (containing unassembled clathrin) and both were electrophoresed by SDS-PAGE. Light chain ratios were determined as described in Figure 2.5.
*The sample standard deviation of the ratios is given along with the number of samples tested (in parentheses).
**Percentage of each light chain assembled was determined as: % assembled = assembled/(assembled + unassembled) where all values are in ng.

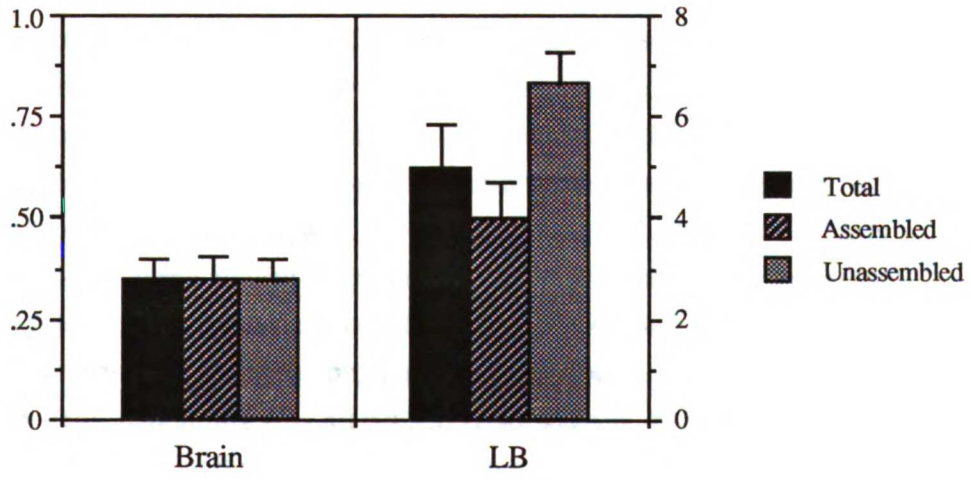


Figure 2.9 Light chain ratios in assembled and unassembled clathrin

Data plotted from Table 2.4.

B lymphoblastoid cells than in the primary lymphocytes studied previously (30% assembled) (Goud et al., 1985). However, since 85-95 % of the lymphocytes in blood are T lymphocytes, the difference in assembly states may be due to the difference in cell type. Alternatively, the rapidly dividing LB cells may require more endocytic activity and thus utilize more assembled clathrin than lymphocytes circulating in the blood. Unlike brain, LB cells consistently showed a small difference in the light chain ratio of the assembled and unassembled pools of clathrin, but the large predominance of LCa was maintained in both pools.

2.3 Discussion

Prevalence of LCb correlates with the regulated secretory pathway

Expression patterns of clathrin light chains, LCa and LCb, were investigated to determine their differential influence on clathrin function. Correlation between an increased proportion of LCb in a tissue or cell and the presence of a regulated secretory pathway was established. There are two stages in the regulated secretory pathway that might specifically require LCb. The first is in the formation of secretory granules where clathrin has been implicated in concentrating and packaging proteins (Tooze and Tooze, 1986; Orci et al., 1984). The second is the coordinated rapid retrieval of granule membrane after exocytosis. Coated pit number rises rapidly after adrenal medulla cells are stimulated to secrete catecholamines (Geisow and Childs, 1985), and after synaptic vesicles fuse with the presynaptic membrane in neuronal cells (Miller and Heuser, 1984).

Although regulated secretory cells have a predominance of LCb, all cells examined express both LCb and LCb and the intracellular distribution of LCb appears to be similar to that of LCa (Puszkin et al., 1989). LCb specific antibodies stain the Golgi and periphery of many different cell types (not shown). Given its ubiquitous distribution, LCb is probably not restricted to specialized functions. Rather its presence could confer the capability of providing a specialized clathrin function when needed. A likely possibility is an involvement in coated pit upregulation, a process which is also utilized to a small extent in non-secretory cells, explaining why all cells have some LCb. Rapid coated pit upregulation occurs not only after regulated secretion but also after treatment of some cells with growth factors such as epidermal growth factor and nerve growth factor (Connolly et al., 1984; Connolly et al., 1981).

A major difference between LCb and LCa is that LCb is readily phosphorylated *in vitro* (Usami et al., 1985) and *in vivo* (Bar-Zvi et al., 1988). Phosphorylation of LCb could provide a signal influencing regulation and recruitment of triskelions for coated pit

formation. Indeed, there is potential for coordinate phosphorylation and dephosphorylation of LCb and coated pit upregulation. Epidermal growth factor, which stimulates coated pit formation, also activates a casein kinase II (Sommercorn et al., 1987), possibly the same casein kinase II which has been shown to phosphorylate LCb (Bar-Zvi and Branton, 1986). The phosphorylation acceptor site (Hill et al., 1988) is located in the part of LCb most different from LCa and is included in the epitope recognized by monoclonal antibody LCB.1 (residues 9 to 22 using bovine brain LCa as a reference). This epitope on coated vesicles and triskelions was previously shown to be exposed to the cytoplasm and would therefore be accessible to activated kinases (Brodsky et al., 1987).

The presence of a phosphorylation site on LCb and the predominant expression of LCb in specialized secretory cells suggests that the LCb light chain may have evolved to fulfill regulatory functions different from those of LCa. Recent data demonstrating stimulation of the 70 kD uncoating protein preferentially by LCa as compared to LCb further indicates a divergence in function of the two light chains (DeLuca-Flaherty et al., 1990). Either the single light chain of yeast is able to perform the function of both LCa and LCb, or yeast does not have all the clathrin-utilizing functions of mammalian and avian cells. To our knowledge, no evidence for a regulated pathway of secretion in yeast exists (Kelly, 1985). Therefore yeast may not need more than one light chain since there appears

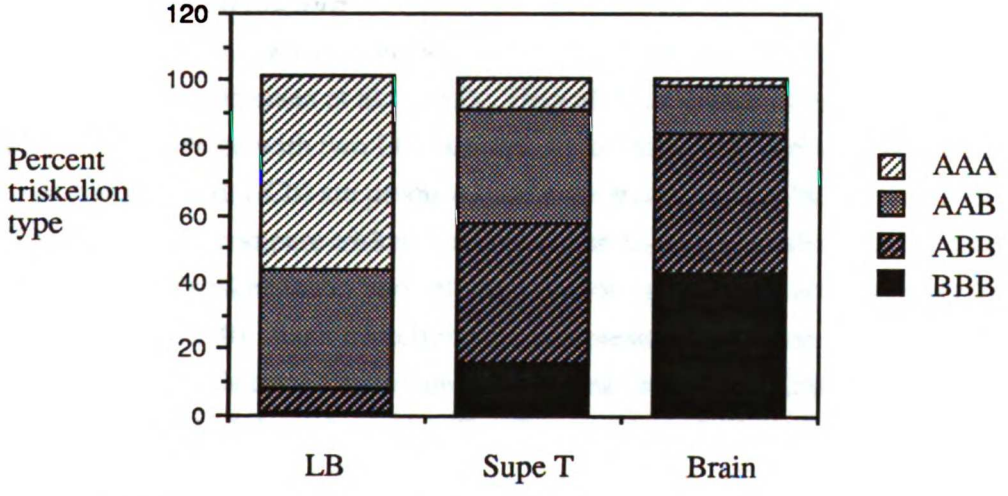


Figure 2.10 Theoretical ratios of triskelion types in different cells

The theoretical percentage of each type of triskelion has been calculated for brain, Supe T, and LB cells using the light chain ratio data from Tables 2.2 and 2.3. In these calculations it is assumed that the light chains are randomly distributed on the triskelions as has been indicated by previous work (Kirchhausen et al., 1983). The relative amounts of the different triskelion types are given by the binomial distribution $r^3 : 3r^2 : 3r : 1$ where r = LCa/LCb.

to be no need for regulated endocytosis. Selection for a second light chain may have occurred during the development of multicellular organisms when cell-cell communication (in the form of hormones and synapses) required a more complex method for clathrin regulation.

Assembled versus unassembled light chain ratios

The light chain ratio in assembled brain clathrin corresponds to the total light chain levels expressed in the tissue. Thus, there is no preferential incorporation of light chains into triskelions or assembled clathrin in brain. These measurements do not indicate whether coated vesicles are formed with a random distribution of light chains or with selected light chain ratios as suggested by Puzskin et al. (1989). In fact, the small but consistent difference in the light chain ratios of assembled and unassembled clathrin in LB cells could be explained by the formation of different types of coated vesicles with different light chain ratios. Conversely, the slightly higher LCa:LCb ratio in the unassembled pool may be due to the uncoating enzyme's preference for LCa. However, the light chain ratios observed in assembled clathrin still maintain a predominance of LCa close to that measured in total clathrin in LB cells. This demonstrates that all forms of triskelions are assembly competent.

Triskelion distribution

Previous work has indicated that clathrin light chains are randomly distributed on triskelions (Kirchhausen et al., 1983). Thus all four possible types of triskelions (those with all LCa, those with two LCa and one LCb, those with one LCa and two LCb, and triskelions with all LCb) are produced and their frequency can be estimated by a binomial distribution. To compare triskelions in cells and tissues, we calculated the theoretical distribution of triskelions in two cell lines and one tissue with very different light chain ratios (Figure 2.10). Each triskelion type is represented by three capital letters indicating the number of LCa and LCb polypeptides on that triskelion. Cells with a light chain ratio of approximately 1:1, such as Supe T cells, would produce all four types of triskelions in significant quantities and the majority would contain both LCa and LCb. In contrast, cells with very high or very low light chain ratios would have predominantly only three of the four types of triskelions. For example, less than 1% of the LB triskelions would be of the BBB type and only 2% of the brain triskelions would be of the AAA type. Thus almost all of LB triskelions will have at least one LCa polypeptide and almost all of the brain triskelions will have at least one LCb. Since regulated secretory cells have a predominance

of LCb, most of the triskelions in these cells could potentially be regulated by the phosphorylation and dephosphorylation of LCb.

Chapter 3: Clathrin biosynthesis and turnover

3.1 Introduction

In vivo radioactive labeling studies have demonstrated that clathrin exists in several states (Fig 3.1). Brodsky (1985b) showed that triskelions can be found unassembled in the cytoplasm or in an assembled fraction of LB cells, a human B lymphoblastoid cell line. In addition, at least one light chain, LCa, can be found in a pool which is unassociated with heavy chain. This 'free' pool of LCa has a half-life of approximately 30 minutes (Brodsky, 1985). Once bound to heavy chain, light chains do not alternate between a bound and free state.

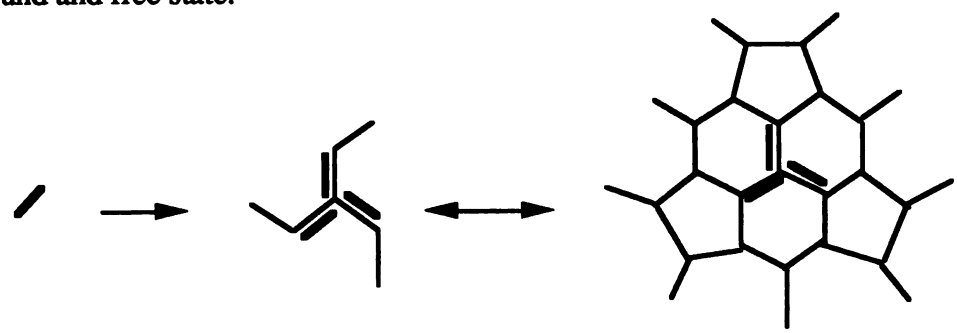


Figure 3.1 Schematic of the *in vivo* forms of clathrin in LB cells.
Single and double arrowheads indicate the irreversibility and reversibility of the reaction, respectively.

The initial studies concerning the *in vivo* forms of clathrin raised several additional questions. In particular: How stable is the triskelion as a complex? Is endocytosis regulated by the rates of turnover or availability of the light chain subunits? What is the function, if any, of the free pool of LCa? Is there a free pool of LCb as well? These questions were subsequently addressed experimentally and the results are reported in this chapter. These studies revealed that the free pool of LCa is most likely present as a result of a lack of available heavy chain binding sites. Thus, free LCa is probably not functional and instead simply represents LCa in excess of heavy chain binding sites. These studies also revealed that LCa is turned over at a faster rate than both LCb and the heavy chain, but all of the clathrin subunits have half-lives sufficiently long to undergo many rounds of endocytosis before degradation. Therefore, it is unlikely that endocytosis is regulated by the rates of turnover or availability of the light chain subunits. Since endocytosis might be regulated by the post-translational modification of the clathrin subunits or of the assembly

complexes, modification of these proteins by fatty acids and phosphorylation was examined. No fatty acid acylations were found; however, several of the subunits were found to be modified by phosphorylation in PC12 cells.

3.2 Results

Pool of heavy chain-unassociated LCa is transient

To determine the nature of the free pool of LCa, BL3 cells (bovine B lymphocytes) were metabolically labeled with ^{35}S-protein labeling mix (NEN) for 4 hours (Figure 3.2).

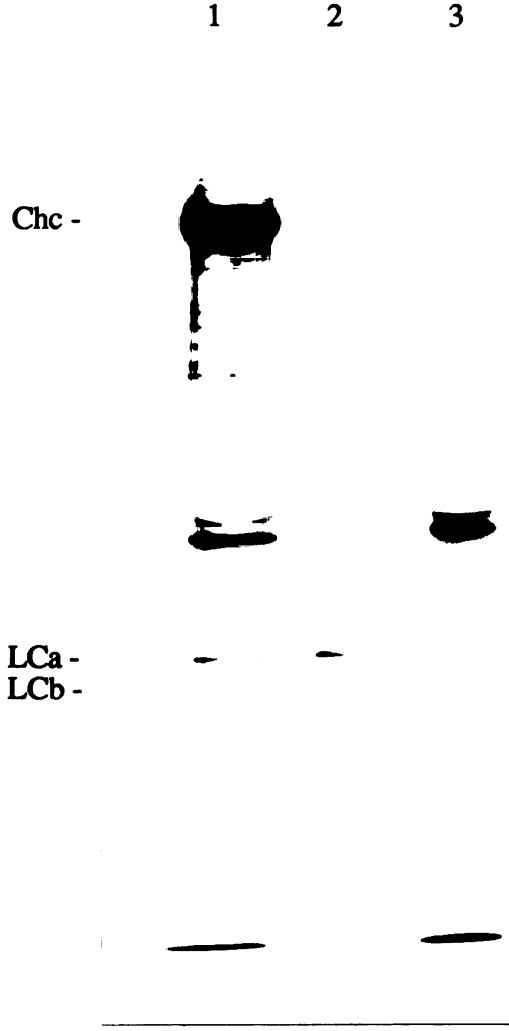


Figure 3.2 Biosynthetic labeling of heavy chain-associated and -unassociated LCa

BL3 cells were labeled for 4 hrs with ^{35}S-protein labeling mix (NEN). Lane 1- heavy chain-associated LCa immunoprecipitated with X22; lane 2- total LCa immunoprecipitated from boiled lysates with X16; lane 3- 'free' pool of LCa immunoprecipitated from unboiled lysates with X16. Chc represents clathrin heavy chain.

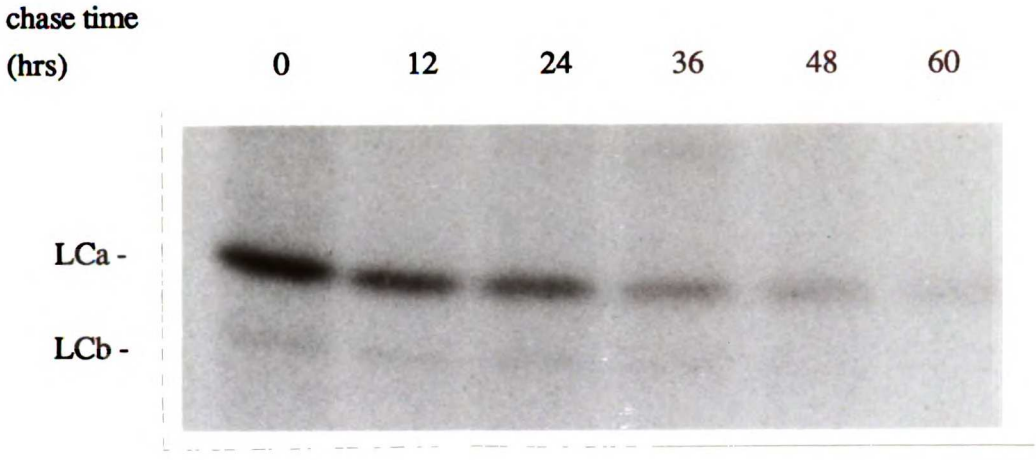


Figure 3.3 Pulse chase of clathrin light chains associated with heavy chain

LB cells were pulsed for 10 minutes with ^{35}S-methionine and chased with unlabeled methionine for the indicated times. Heavy chain-associated light chains were immunoprecipitated with antibody against the heavy chain (X32). The immunoprecipitates were then electrophoresed by Laemmli SDS-PAGE, and the gel was dried and placed against film. The resulting autoradiogram of the light chains is shown.

Heavy chain-associated LCa was immunoprecipitated with mAb X22 which recognizes a site on the clathrin heavy chain. Free LCa was immunoprecipitated with the monoclonal antibody X16. This antibody recognizes only free LCa since the epitope of this mAb is in the heavy chain binding region of LCa (Brodsky et al., 1987). Radiolabeled proteins were visualized by autoradiography (see Chapter 7, Materials and Methods).

In this and most subsequent studies, no free LCa (unassociated with heavy chain) could be detected. In some experiments, however, free LCa was present. Thus the free pool of LCa is transient. As a control, lysates were boiled (which dissociates LCa from heavy chain (Brodsky et al., 1983)) and immunoprecipitated with X16 showing that the antibody could immunoprecipitate free LCa when it was present. No correlation could be made between the presence of free LCa and cell growth conditions (serum lot, medium source, and pH of the medium were tested).

The finding that the free pool of LCa is transient can be explained by additional data from pulse-chase studies. The pulse-chase experiment by Brodsky (1985) showed that newly synthesized LCa did not maximally bind heavy chain until well into the chase period (4 hrs). Concurrently, the newly synthesized free pool of LCa disappeared. Thus the free pool of newly synthesized LCa appears to become heavy chain-associated over a period of time. During those periods when no free LCa could be detected, newly synthesized LCa maximally bound heavy chain at the zero time point after the chase (Figure 3.3). Thus, all newly synthesized LCa was able to bind to a site on the heavy chain. Together these

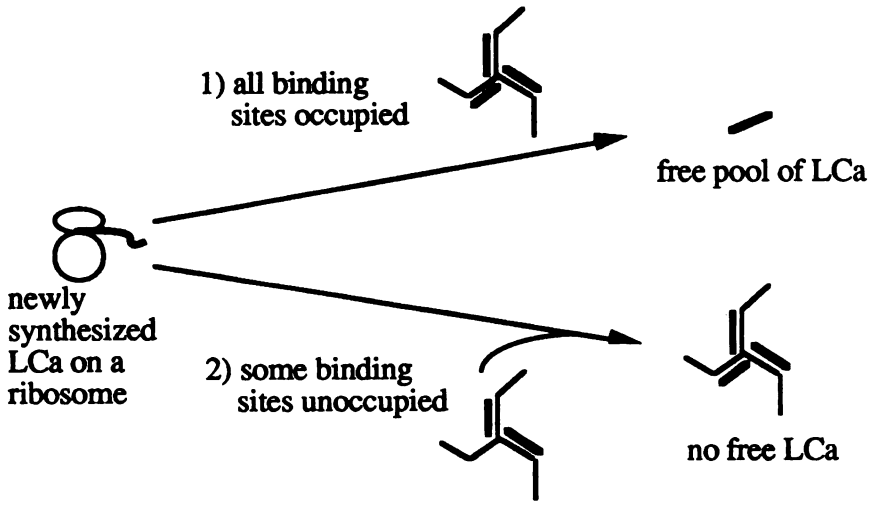


Figure 3.4 Proposed scenarios for the fate of newly synthesized LCa.

After synthesis on a ribosome LCa can either 1) remain free due to a lack of available heavy chain sites, or 2) find a site on the heavy chain to which to bind.

experiments indicate that the amount of LCa synthesized is occasionally in excess of available heavy chain binding sites. When this occurs, the light chain remains in a free pool until a site becomes available (Figure 3.4).

Attempts to find a free pool of LCb were hindered by two problems. First, LB cells have very little LCb compared to LCa (see Chapter 2) and therefore it is harder to detect. Second, the antibody that binds in the heavy chain-binding site of LCb is weak compared to the antibody used for the free LCa experiments. However, it is likely that there is a small pool of free LCb present with free LCa since the two light chains compete for binding at the same heavy chain site (Winkler and Stanley, 1983; Brodsky et al., 1987).

Turnover of clathrin subunits

To determine if the clathrin subunits have different fates after synthesis and if clathrin might be subject to regulation by rapid changes in its subunit levels, the half-lives of LC_a, LC_b, and the heavy chain were determined. LB cells were pulse-labeled for 10 minutes with ^{35}S-methionine, followed by a chase with medium containing excess unlabeled methionine plus $NaSeO_4$ and ascorbic acid. The addition of $NaSeO_4$ and ascorbic acid was found to be particularly crucial to the success of these experiments. Both compounds help protect the cell from the harmful effects of the radiolabel. Selenium is a cofactor for the enzyme glutathione reductase (Lehninger, 1982), which maintains the proper reducing environment in the cell. Ascorbic acid (vitamin C) is a free radical

scavenger which limits the damage done to the cells by free radicals generated by exposure to radioactivity. Addition of $NaSeO_4$ and ascorbic acid dramatically improved the condition of the cells, as determined morphologically, and the cells appeared healthy for radiolabel exposure times of up to 72 hours. Therefore, all pulse chase experiments included these two compounds in the medium.

Total cellular LCa and LCb were quantitatively isolated at various times after chase medium was added by immunoprecipitation with a combination of mAbs X16 and X43 from the supernatants of lysates that were boiled for 10 minutes and centrifuged. The lysates were boiled for two reasons. Boiling precipitates many cellular proteins, but leaves the light chains in solution thus making the immunoprecipitate cleaner. In addition, the antibodies which are the most effective at precipitating the light chains, X16 and X43, recognize an epitope which is hidden when the light chains are bound to heavy chain (see Fig 1.10). Boiling releases the light chains from the heavy chain and makes available the binding sites of these antibodies (Brodsky et al., 1983). Preliminary studies were performed to ensure that the immunoprecipitations were quantitative. This was done by completing a second round of immunoprecipitation. Any light chains not precipitated in the first round would be immunoprecipitated in the second. mAbs X16 and X43 immunoprecipitated 100% of their antigens in the first round. Samples were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography to determine the levels of labeled light chain remaining at each time point during the cold chase (Figure 3.5).

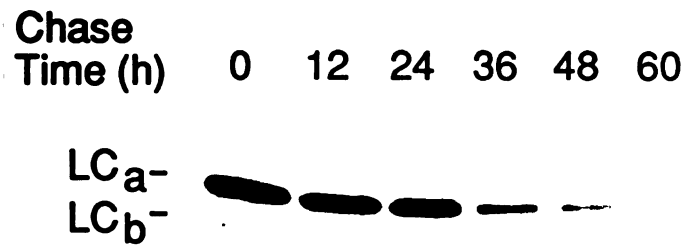


Fig. 3.5 Degradation of clathrin light chains.
Autoradiogram of light chains labeled with ^{35}S-methionine for 10 minutes and chased with cold methionine for the indicated time periods. Lysates were boiled and light chains were quantitatively immunoprecipitated from the soluble proteins with a mixture of anti-LC_a and anti-LC_b antibodies (X16/X43). Immunoprecipitates were electrophoresed on 10% SDS-PAGE, the gel was dried and exposed to film.

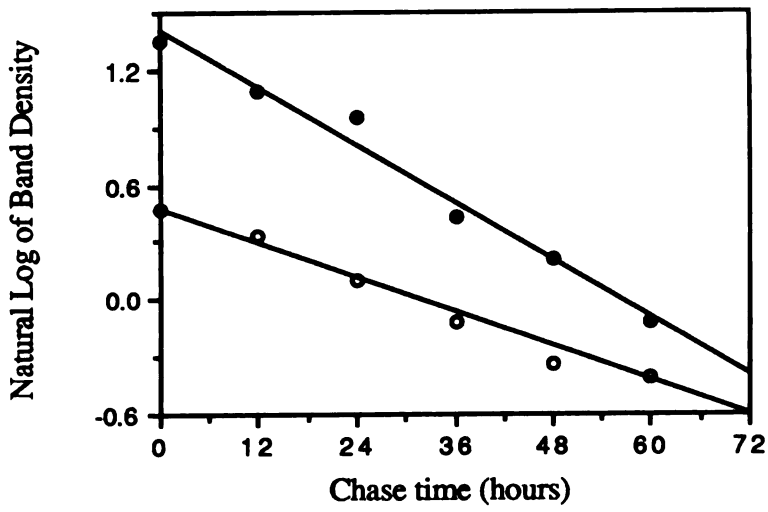


Figure 3.6 Half-life determination of the clathrin light chains

Natural log of the light chain band density versus chase time. (•) LCa degradation where the line can be defined as y = 1.402 - 0.025x with a correlation coefficient of 0.999. This line gives a half-life of 27 hours for LCa. (o) LCb degradation where the line can be defined as y = 0.574 - 0.016x with a correlation coefficient of 0.993. This line gives a half-life of 45 hours for LCb.

Plotting the natural log of the densities of the bands in Figure 3.5 versus time yielded straight lines (Figure 3.6). These lines can be described by the equation:

$$(1) \qquad \ln\{[P]_t/[P]_0\} = -k_d t$$

where $[P]_t$ represents protein concentration at time t, $[P]_0$ is protein concentration at time zero, and k_d is the degradation constant. This equation is derived from the equation which describes the relationship between protein synthesis and degradation:

$$d[P]/dt = k_s - k_d[P] \qquad \text{(Dice and Goldberg, 1974)}$$

k_s (rate of synthesis) is equivalent to zero in a pulse-chase experiment since theoretically no protein is being labeled after the pulse. Therefore this equation becomes:

$$d[P]/dt = - k_d[P]$$

This can be rearranged to:

$$d[P]/[P] = -k_d dt$$

Integrating this gives equation (1) above.

From these lines the half-lives ($t_{1/2}$) can be determined since $t_{1/2} = \ln 2/k_d$. In the above experiment, half-lives of LCa and LCb were calculated to be 27 and 44 hours, respectively. Additional experiments yielded an average half-live of 24 hours for LCa and 45 hours for LCb.

The half-life of the heavy chain was established from separate quantitative immunoprecipitations using the X32 monoclonal antibody, specific for clathrin heavy chain (Fig. 3.7). The zero time point in the heavy chain half-life experiment does not fall on the curve and was not used to calculate the half-life. This was a consistent finding and is either the result of inefficient chase with unlabeled methionine or incomplete translation or folding of the heavy chain in the ten minute pulse label period. The densitometry data were plotted as before yielding a straight line for time points 12 hours through 60 hours (Figure 3.8).

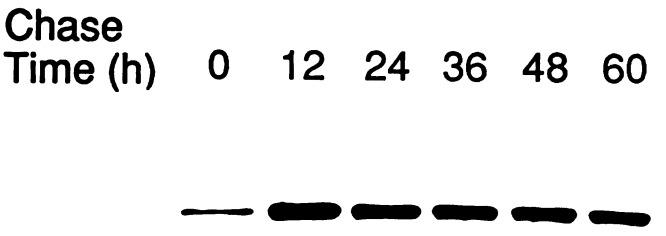


Fig. 3.7. Degradation of clathrin heavy chain.

Autoradiogram of clathrin heavy chain labeled with ^{35}S-methionine for 10 min, chased with cold methionine for the indicated time periods, and quantitatively immunoprecipitated with X32 mAb.

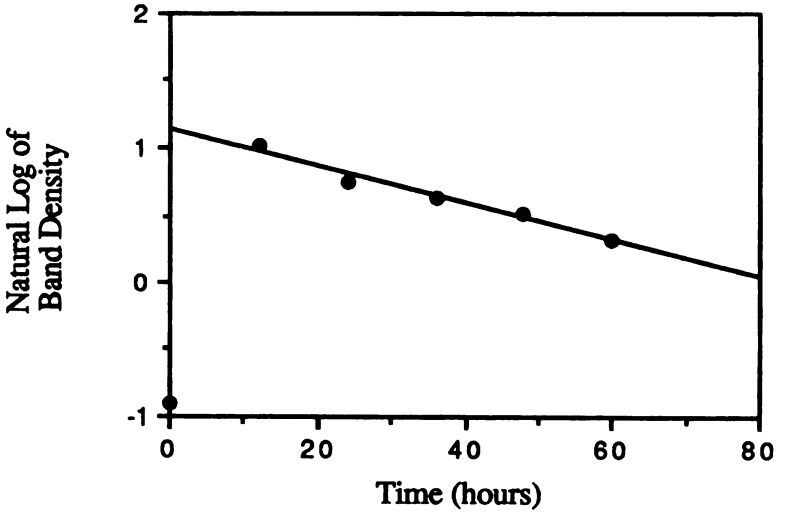


Figure 3.8 Half-life determination of the clathrin heavy chain.

Natural log of the light chain band density versus chase time. The half-life was calculated as described in figure 3. The obtained line can be defined as y = 1.1344 - 0.0137x with a correlation coefficient of 0.986. This line gives a half-life of 50 hours for the heavy chain. The zero time point falls well below the line and was not used to determine the half-life (see text).

This yielded a half-life of 50 hours for the clathrin heavy chain. These results indicate that the triskelion is a stable complex that likely undergoes many rounds of endocytosis before any of its subunits are degraded.

Protein modifications

Since clathrin is evidently not regulated by the turnover or availability of its subunits, endocytosis must be regulated by other means. Post-translational modification of clathrin or the assembly proteins is a likely means of regulating the process of endocytosis. Two types of protein modifications were examined: acylation with fatty acids and phosphorylation.

Since clathrin and the assembly complexes are associated with membranes, it was of interest to determine if they are modified by fatty acids. Two fatty acid modificationswere examined: palmitylation and myristylation. Palmitylation, which occurs at cysteine residues, turns over at a more rapid rate than the protein to which it is attached indicating that it could serve as a regulatory modification (Sefton and Buss, 1987). Myristylation, a modification on glycine residues at the amino terminus, tends to turn over only as rapidly as the protein suggesting that this modification may not serve a regulatory role (Sefton and Buss, 1987). However, since the clathrin light chains have blocked amino-termini (F. Brodsky, personal communication), they were examined for myristylation as well as palmitylation.

To determine if either clathrin or the AP2 complex are modified with fatty acids, cells were incubated with ^{3}H-palmitate or ^{3}H-myristate in medium with fatty acid-free serum. Cells were then lysed and clathrin and AP2 complexes were immunoisolated with specific antibodies X22 and AP.6, respectively. After separation on SDS-PAGE, the gels were exposed to film. These studies indicated that none of the clathrin or AP2 subunits is modified by palmitate or myristylate (Figure 3.9, Figure 3.10). Transferrin receptor was used as a positive control to show that palmitylation was occuring under these conditions. Apparently, endocytosis is not regulated by fatty acylation of either clathrin or AP2.

Since some of the clathrin and AP2 subunits are known to be phosphorylated (Keen and Black, 1986, Bar-Zvi et al., 1988), it is possible that phosphorylation serves as a regulatory modification for endocytosis. However, there continues to be disagreement on the *in vivo* phosphorylation state of the subunits of AP2. Keen and Black (1986) report that the 100 kD AP proteins are phosphorylated *in vivo*, however the 50 kD AP2 protein is not. In contrast, Bar-Zvi and coworkers (1988) report that both the 100 kD AP proteins and the 50 kD protein are phosphorylated *in vivo*. They argue that Keen and Black did not

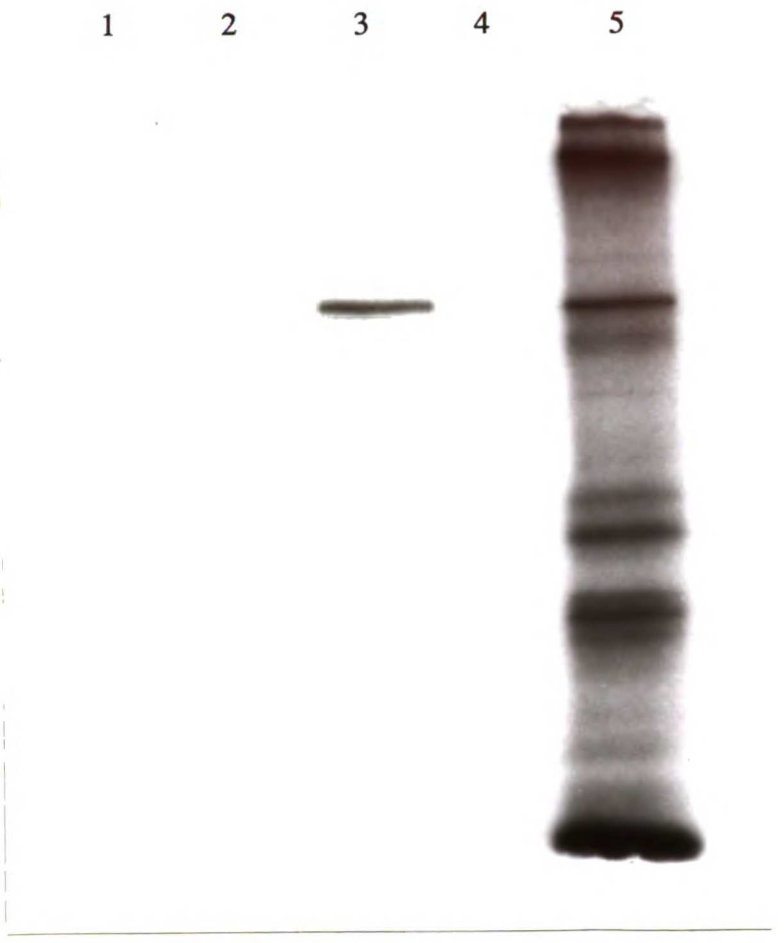


Figure 3.9 Lack of palmitylation of clathrin or AP2

LB Cells were biosynthetically labeled with ^{3}H-palmitic acid for 4 hours. Clathrin and AP2 were immunoprecipitated with X22 and AP.6 antibodies, respectively, and electrophoresed on SDS-PAGE. The autoradiogram is shown: Lane 1: X22 immunoprecipitate; lane 2: AP.6 immunoprecipitate; lane 3: anti-transferrin receptor immunoprecipitate; lane 4: 29B5 immunoprecipitate (negative control); lane 5: total cell lysate.

use a sufficient variety of kinase and phosphatase inhibitors to prevent post-lysis phosphorylation and dephosphorylation. To resolve this discrepancy, phosphorylation studies were performed in PC12 cells. PC12 cells were labeled with inorganic phosphate for 2 hours. Lysates were then made in buffer containing numerous kinase and phosphatase inhibitors (EDTA, EGTA, NaF, sodium tartrate, vanadium pentoxide, sodium pyrophosphate) to maintain the *in vivo* phosphorylation state. Clathrin and AP2 were immunoisolated as described for the fatty acid labeling experiments and electrophoresed on

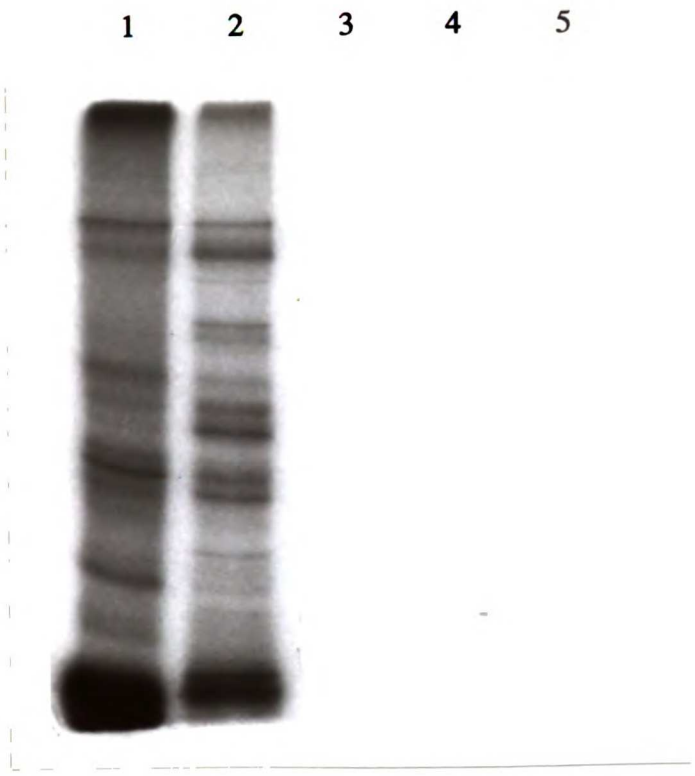


Figure 3.10 Lack of myristylation of clathrin or AP2

LB Cells were biosynthetically labeled with ^{3}H-myristic acid for 4 hours. Clathrin and AP2 were immunoprecipitated with X22 and AP.6 antibodies, respectively, and electrophoresed on SDS-PAGE. The autoradiogram is shown: Lane 1: total cell lysate from ^{3}H-palmitic acid label; lane 2: total cell lysate from ^{3}H-myristic acid label ; lane 3: 29B5 immunoprecipitate (negative control); lane 4: AP.6 immunoprecipitate; lane 5: X22 immunoprecipitate.

SDS-PAGE. Proteins that were radiolabeled with ^{32}PO4 were detected by exposure of the gel to film (Figure 3.11). These studies revealed that both the 100 kD and 50 kD AP2 proteins are phosphorylated *in vivo*. Thus Keen and Black, who used only NaF, EGTA, and EDTA as kinase/phosphatase inhibitors, probably did not sufficiently prevent post-lysis changes in the phosphorylation states of these proteins.

Bar-Zvi and coworkers (1988) found that the apparent phosphorylation specific activities of LCb and the AP2 subunits were different in the assembled and unassembled fractions of cell lysates. The unassembled fractions of LCb and the 50 kD protein were more highly phosphorylated than the assembled fractions. Conversely, the 100 kD proteins were more highly phosphorylated in the assembled fractions. These results suggest that phosphorylation might be a mechanism for the regulation of the assembly of

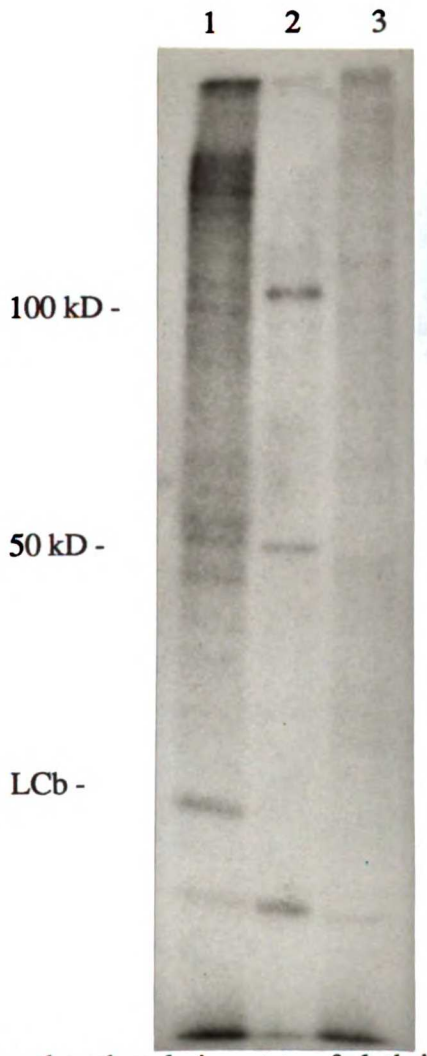


Figure 3.11 *In vivo* phosphorylation state of clathrin and AP2 subunits

PC12 cells (1 x 10^7) were trypsinized, washed and resuspended in medium containing 1 mCi $^{32}PO_4$ for 2 hours, 37°C. Cells were washed, lysed in buffer containing phosphatase/kinase and protease inhibitors, and immunoprecipitated as described (Chapter 7). Lane 1: X22 immunoprecipitate; lane 2: AP.6 immunoprecipitate; lane 3: antiLeu10 (control) immunoprecipitate.

clathrin and AP2 complex. Since secretion has been shown to precede an increase in clathrin assembly (Geisow and Childs, 1985; Miller and Heuser, 1984), it is appropriate to compare the phosphorylation states of these proteins before and after stimulation of secretion. To determine if phosphorylation states change during stimulation of secretion PC12 cells were labeled in suspension with ^{32}P-phosphate for 1 hour and then stimulated with 55 mM KCl. At intervals of two minutes cells were put on ice, washed, and lysed. Total clathrin and the AP2 complex were immunoprecipitated with X22 and AP.6 mAbs. Immunoprecipitates were electrophoresed on SDS-PAGE and the gel was exposed to film

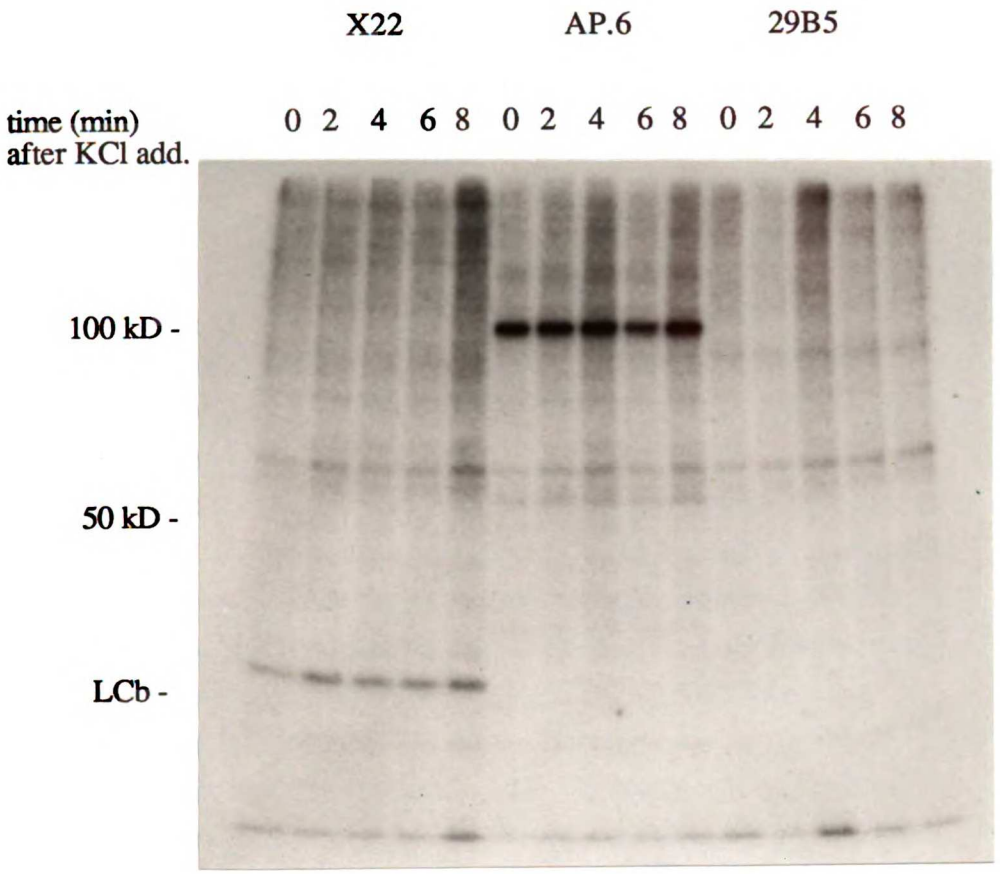


Figure 3.12 *In vivo* **phosphorylation of clathrin and AP2 after stimulation with KCl.**

PC12 cells were labeled in suspension with ^{32}P-phosphate for 1 hour and then stimulated with 55 mM KCl. At intervals of two minutes cells were put on ice, washed, and lysed. Total clathrin and the AP2 complex were immunoprecipitated with X22 and AP.6 mAbs. Immunoprecipitates were electrophoresed on SDS-PAGE and the gel was exposed to film

(Figure 3.12). There were no obvious changes in the phosphorylation levels of the proteins after stimulation with 55 mM KCl. Although a small variation in label intensity was apparent, these variations could be attributed to slight differences in amounts of protein immunoprecipitated (as determined by Coomassie staining of the gel). Thus, this study revealed no change in the phosphorylation pattern of LCb or the 100 kD and 50 kD subunits of the AP2 complex. However, it is not clear whether clathrin assembly was actually altered in these cells as a result of KCl stimulation.

Numerous attempts were made to develop assays to measure changes in clathrin assembly due to stimulation of secretion. One of these assays was a nitrocellulose ripping assay wherein cells were grown on nitrocellulose (HATF membranes from millipore), stimulated with 55 mM KCl (or unstimulated for control) and ripped open with another HATF membrane. Although X22 binding indicated that clathrin was present on the upper

and lower membranes, there was no apparent change in clathrin levels. Another assay was attempted in which the cells were stimulated with KCl in suspension and then homogenized, and the post-nuclear supernatants were spun at 100,000 xg to separate assembled and unassembled clathrin. Again there was no apparent change in clathrin assembly. These assays were also unsuccessful when attempted with primary adrenal chromaffin cells. (If further attempts are made to develop this type of assay, it is recommended that the cells are allowed to grow at least 4-5 days on a substrate before stimulating with 55 mM KCl.) Thus these studies were unable to determine whether phosphorylation plays a role in regulating assembly or disassembly of clathrin or the assembly complexes.

It should be noted that the relative labeling intensities of LCb and the 50 kD and 100 kD subunits of AP2 are different in the two phosphorylation experiments (Figures 3.11 and 3.12). This possibly could be the result of different labeling times (one hour in Figure 3.12 versus two hours in Figure 3.11). If the difference in labeling patterns is due to a difference in length of labelling time, that would suggest that the phosphorylation/dephosphorylation rates of the proteins are different. For example, at one hour of labeling time with $^{32}PO_4$ the 50 kD subunit is not nearly as strongly labeled as the 100 kD subunit. However, at two hours of labeling the 50 kD subunit is as strongly labeled as the 100 kD. Thus one possible explanation is that the rate of phosphorylation of the 50 kD may be slower than the 100 kD subunit.

3.3 Discussion

The half-life of LCa was calculated to be 24 hours. A free pool of LC_a (unassociated with heavy chain) in these cells was previously shown to have a half-life of approximately 30 minutes (Brodsky, 1985b). Further studies on this free pool revealed that it is sometimes, but not always, present and indicated that it probably represents light chain made in excess of available heavy chain binding sites. The lack of a biphasic curve in the light chain half-life experiments indicates that the free pool was either relatively small or was not present in this particular study. Thus, the half-life of 24 hours is that of heavy chain-associated LCa. The half-life of LCb was determined to be approximately 45 hours, which is close to twice that of LCa. Thus LCb must be less susceptible to degradation *in vivo*. Purified LCb also degrades more slowly than purified LCa *in vitro* after long term storage (>2 months at 4°C) and upon incubation with proteases (not shown). An additional factor which may contribute to the longer half-life of LCb is its apparent higher affinity for the heavy chain. Clathrin extracted with thiocyanate retains LCb almost exclusively (Schmid et al., 1984). In addition, LCb competes more effectively with iodinated light

chains for binding sites on heavy chain than does LCa (Brodsky et al., 1987). Binding to the heavy chain may stabilize the light chains and reduce their susceptibility to proteolytic enzymes. Clathrin heavy chain was found to have a half-life of 50 hours, sufficiently long to keep the light chains bound until they are degraded. This finding correlates with previous data which showed that the light chains do not alternate between a bound and free state (Brodsky, 1985b). Assuming one round of endocytosis takes one minute (Pearse and Bretscher, 1981), half of the heavy chains will undergo at least 3000 rounds of endocytosis before they are degraded. These data explain earlier results which showed that endocytosis continues despite protein synthesis inhibition (Goldstein et al., 1979).

The half-lives for the clathrin subunits are of about average length for cellular proteins (Goldberg and Dice, 1974) and indicate that clathrin triskelions are passed on to daughter cells during mitosis. The activity of proteins with very short half-lives (<30 minutes) is frequently regulated by rapid changes in levels of available protein (Goldberg and Dice, 1974). Since all three clathrin subunits have half-lives on the order of 1-2 days, this is probably not a mechanism for the regulation of endocytosis. Thus endocytosis must be regulated by other means, possible by phosphorylation or binding of calcium by the light chains (Mooibroek et al., 1987).

Neither clathrin nor AP2 appears to be modified by myristic acid or palmytic acid. Thus any interaction with the cellular membranes must be regulated by other modifications or properties of these proteins. Phosphorylation/dephosphorylation is a likely method for regulating endocytosis. Although it has been clearly demonstrated that LCb and the 50/100 kD proteins of the AP2 complex are phosphorylated *in vivo*, it has not been shown how these modifications might play a role in regulating the assembly or disassembly of the assembly proteins and clathrin. Clearly, an important step in understanding the events in the endocytic process will involve the elucidation of the role of phosphorylation.

Chapter 4: Manipulation of light chain ratios in cells

4.1 Introduction

To study further the role of the two light chains of clathrin, it was of interest to obtain cells that could be altered in their LCa:LCb ratios. The approach taken was to introduce the cDNA for one of the light chains behind an inducible promoter into a cell line to obtain stably transfected cells. The level of introduced light chain could then be increased by inducing its production. Since the light chains compete for heavy chain binding, and since LCa has a short half-life when not bound to heavy chain (Brodsky, 1985b; Chapter 3), it seemed plausible that overexpression of one of the light chains would compete out the other. A similar overexpression method has been previously used to demonstrate competition between subunits of cAMP-dependent protein kinase (Otten and McKnight, 1989). Light chain levels could potentially be changed by other methods (e.g. antisense mRNA expression, antisense oligodeoxynucleotides), however, these methods may lead to clathrin heavy chains devoid of light chain. This might cause major disturbances in clathrin function. Therefore, a better approach would be to swamp out one light chain by overexpressing the other. In collaboration with A. Jackson (University of Cambridge, England), light chain transfectants of C1R cells and PC12 cells were developed. The major proportion of the work on C1R cells was done by A. Jackson. Described here is the development and study of the mutant PC12 cells along with pertinent data from the C1R cell line.

C1R cells were chosen because they are a fast growing line that express substantially more LCa than LCb (A. Jackson, personal communication). PC12 cells were chosen for the presence of their regulated secretory pathway and because they have approximately twice as much LCb as LCa. Since the studies reported in Chapter 2 indicate that LCb may play a specialized role in regulated secretion, it was of interest to determine what would happen to the regulated secretory pathway if LCb levels were reduced by overexpressing LCa. The studies reported in this chapter show that LCa:LCb ratios on clathrin heavy chain can be dramatically reduced in C1R cells by this method and it appears to be more difficult deplete the cells of LCb than LCa. Attempts to reduce dramatically the levels of LCb in PC12 cells were unsuccessful; however, from these studies was obtained a PC12 clone that produced no LCa at all. The production of transfectants and initial characterization of the LCa negative PC12 clone is described in this chapter.

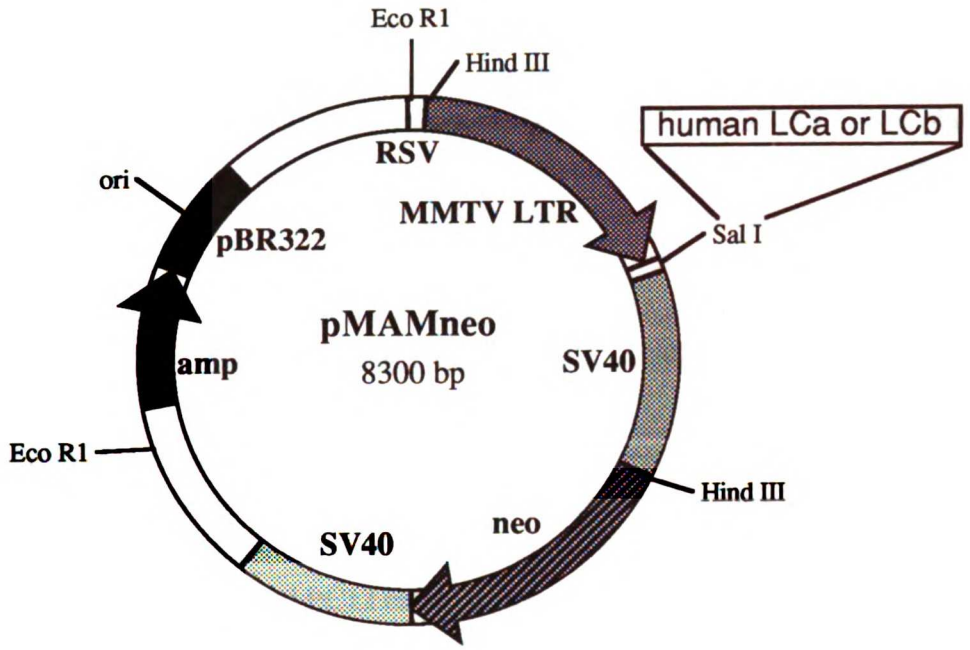


Figure 4.1 pMAMneo vector for expression of human clathrin light chains (Clonetech)

4.2 Results

Transfection of cells with light chain cDNA

The plasmid construct used, pMAMneo (Clonetech), contains all the necessary components for selection and expression of human LCa in eukaryotic cells (Figure 4.1). Two selection markers are present on this plasmid. The ampicillin resistance gene is present for selection of successfully transformed *E. coli*, and the neomycin resistance gene enables selection of successfully transfected mammalian cells (Sambrook et al., 1989). Those mammalian cells containing functional plasmid will be resistant to geneticin (G418) (Katoh et al., 1987). The plasmid also contains the PBR322 origin of replication enabling replication in *E. coli*. The cDNA for human LCa or LCb was inserted behind the mouse mammary tumour virus (MMTV) promoter. This promoter can be induced by treatment with dexamethasone (DEX) and various other steroids (Otten et al., 1988).

C1R cells were transfected by electroporation with pMAMneo, pMAMneo-LCa, and pMAMneo-LCb (Sambrook et al., 1989). $PC12_k$ (R. Kelly) cells were transfected with pMAMneo and pMAMneo-LCa by lipofection. Lipofectin (BRL) is a positively charged solution of liposomes which bind to DNA and are thought to deliver the DNA into cells by membrane-lipid fusion (Innes et al., 1990). After incubation of the PC12 cells with the lipofectin/DNA mixture, the cells were incubated in medium containing G418 (see

Chapter 7 for methodology). Those cells which have taken up the DNA should be resistant to G418. After several lipofection attempts with PC12 cells, resistant colonies were obtained. These colonies were picked with sterile toothpicks and transferred to 96-well plates. The colonies were then expanded successively to 24-and 6-well plates. After transfer to a 100 mm plate, the cells were frozen in liquid nitrogen in two 1-ml aliquots. A total of fifty-nine pMAMneo-LCa PC12 clones were isolated. Twenty-two control clones which were transfected with empty vector alone (pMAMneo) were also isolated and frozen in liquid nitrogen. Those clones containing pMAMneo and pMAMneo-LCa were identified as V1-V59 (V referring to empty vector) and A1-A59, respectively.

Antiserum production

In order to determine rapidly and reliably the relative levels of expression of LCa and LCb in PC12 cells, a new antibody was needed for quantitating relative levels of LCa and LCb in transfected PC12 cells. Jackson found that antiserum raised against the consensus sequence (identical in both LCa and LCb) is a useful tool for estimating LCa:LCb ratios (personal communication). This antiserum was found to immunoblot LCa and LCb equally well. The advantage of this antiserum is that levels of LCa and LCb can be compared directly in the same lane on one immunoblot. In addition, standard curves, such as those used in Chapter 2, are unnecessary since the antiserum recognizes both light chains equally well.

To prepare the anticonsensus antiserum, the peptide from the consensus region (a.a. 23-44) was synthesized by the Biomolecular Resource Center at UCSF both with and without an amino-terminal cysteine (Table 4.1). The cysteine was added to enable conjugation of the peptide to a carrier protein via the sulfhydryl moiety. The conjugation of peptides (and other small molecules) to an immunogenic protein usually results in a stronger immune response (Weir, 1986). Purity was determined by HPLC at the Biomolecular Resource Center. The peptide containing the N-terminal cysteine was coupled via MBS (Figure 4.2) to keyhole limpet hemocyanin (KLH), a protein widely used to generate immune responses to small molecular weight compounds including peptides (Weir, 1986).

(C)EEDPAAAFLAQQESEIAGIEND

	quantity	purity
no cys:	65 mg	95% pure
w/cys:	150 mg	83% pure

Table 4.1 Consensus peptide.

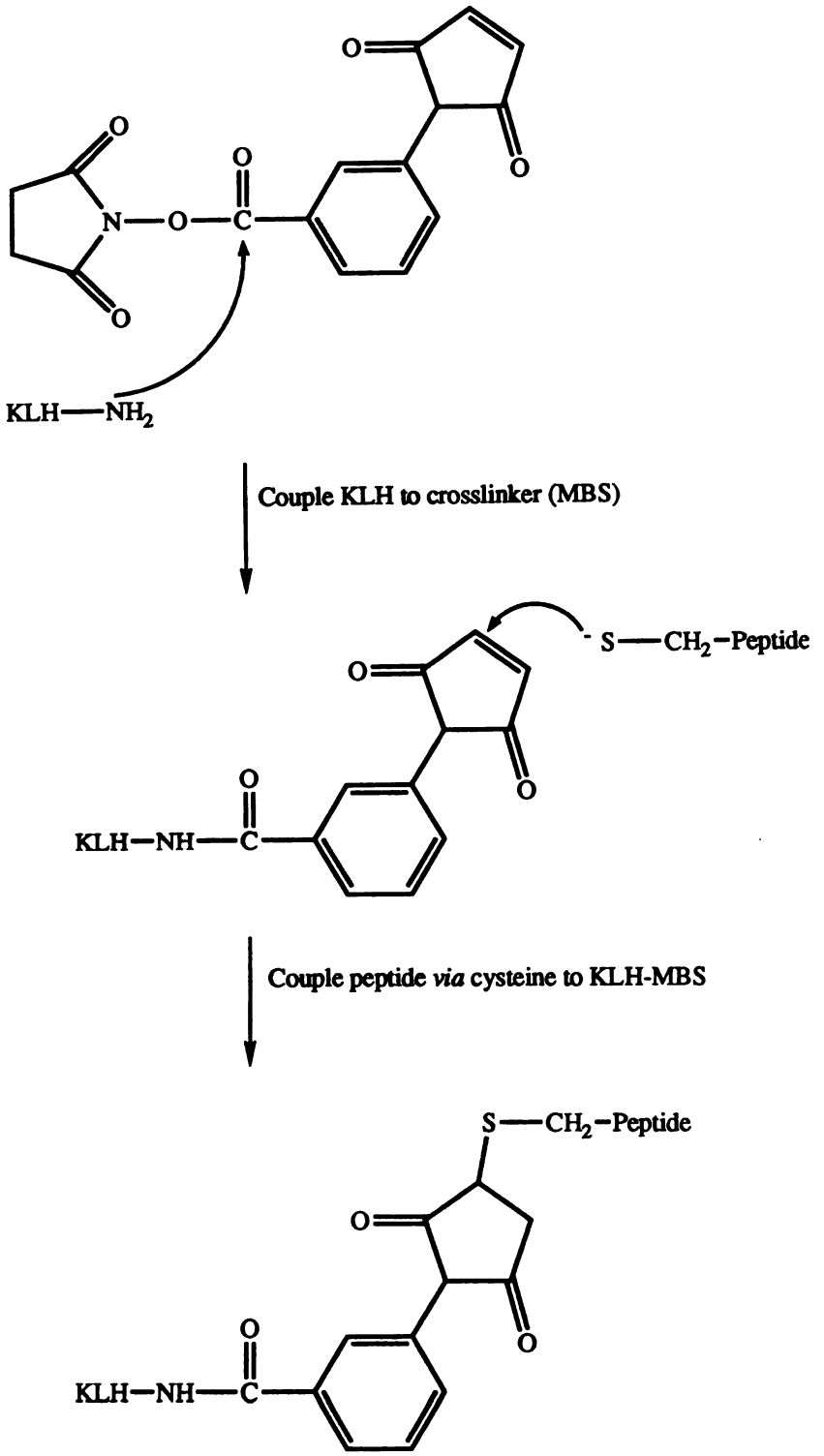


Figure 4.2 Coupling of the consensus peptide to keyhole limpet hemocyanin.
Details of the method can be found in section 7.4

day	date	procedure
Day 0	3/16/90	Prebleed - 10 mls
		Initial immunization with 0.5 mg/rabbit - PLN (peripheral lymph node)
Day 21	4/6/90	Boost with 0.25 mg/rabbit - IM (intramuscular)
Day 31	4/16/90	Bleed I - 50 mls
Day 42	4/27/90	Boost with 0.25 mg/rabbit - IM
Day 52	5/7/90	Bleed II - 50 mls
Day 63	5/18/90	Boost with 0.25 mg/rabbit - IM
Day 74	5/29/90	Bleed III - 50 mls
Day 84	6/8/90	Boost with 0.25 mg/rabbit - IM
Day 94	6/18/90	Bleed IV - 50 mls

Table 4.2 Protocol for antiserum production against the light chain consensus sequence.

Antiserum to the conjugate mixture was raised in two rabbits by the Berkeley Antibody Company (Table 4.2). Each bleed was checked for immunoblotting ability against cell lysates (Figure 4.3). Rabbit #1 gave a strong immune response which was high in all bleeds. Rabbit #2 did not mount as strong an immune response, however bleed 5/29/90 is strong enough to be used as antiserum in immunoblotting of lysates. The

	1	2	3	4	5	6
Rabbit #	1	1	2	2	1	1
Bleed	PB	B1	PB	B1	B1	B1

Figure 4.3 Binding of anticonsensus serum to crude cell lysates

Lysates of Supe T cells and purified light chains were used to determine the ability of the antiserum to specifically recognize light chains. Immunoblots were performed as described (Chapter 7). Lanes 1-4: Supe T lysates; Lane 5: purified LCa; Lane 6: purified LCb. PB refers to prebleed serum. B1 refers to bleed 1 serum.

antiserum from rabbit #1 was tested against purified LCa and LCb and was shown to bind both equally well (not shown). Therefore, this anticonsensus antiserum was used to measure the LCa:LCb ratio in cell lysates.

Induction of exogenous light chain production

PC12 cells transfected with human LCa were tested for LCa expression. Cells were induced with dexamethasone for 3 days, cell lysates were prepared, and electrophoresed on SDS-PAGE. After blotting onto nitrocellulose, the membranes were probed with anticonsensus serum, enzyme-coupled second antibody and substrate. Human LCa could be detected as a band of slightly lower molecular weight than rat LCa (Fig. 4.4). Of the fifty-nine G418^r LCa transfected clones, only 11 expressed the transfected LCa in detectable levels (Table 4.3). The best exogenous LCa expressor, A6, was chosen for further experiments. Attempts were made to increase the exogenous LCa production (in order to reduce LCb expression), however, the best change in LCa:LCb ratio obtained was approximately 1:2 to 1:1. Altering the Dex concentration had no effect above 10 μM. In one experiment where length of DEX treatment was extended out to three weeks, no further change in light chain ratio was seen beyond the 1:1 ratio.

Apparently there are limits on high expression levels of LCa with this expression system in PC12 cells. However, this expression system can be used successfully to change dramatically the light chain ratios in C1R cells by high expression of LCb. The light chain ratios have been successfully altered in C1R cells where the LCa:LCb ratio can be changed from 5:1 to 1:7 (A. Jackson, personal communication). This change in ratios is not only found in the total light chain pool, but is also found to occur on the heavy chain.

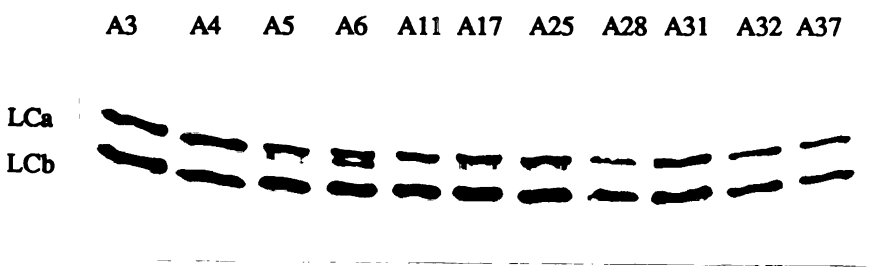


Figure 4.4 Detection of exogenous human LCa in PC12 cells

Anticonsensus serum was used to immunoblot lysates of PC12 cells that were treated with 1 μM dexamethasone for 3 days to induce expression of exogenous human LCa. Human LCa runs slightly lower than rat LCa in SDS-PAGE.

Clone	exogenous LCa production	Clone	exogenous LCa production
A1	-	A28	-
A2	-	A29	-
A3	-	A30	-
A4	(+)	A31	-
A5	+	A32	+
A6	++	A33	-
A7	-	A34	-
A8	-	A35	has all LCb
A9	-	A36	-
A10	-	A37	-
A11	(+)	A38	-
A12	-	A39	-
A13	-	A40	-
A14	-	A41	-
A15	-	A42	-
A16	(+)	A43	-
A17	+	A49	(+)
A18	-	A50	-
A19	-	A51	-
A20	-	A52	-
A21	-	A53	-
A22	-	A54	-
A23	-	A55	+
A24	-	A56	-
A25	+	A57	-
A26	-	A58	+
A27	-	A59	-

Table 4.3 Isolated clones of PC12 cells transfected with cDNA for human LCa.
A plus in parentheses indicates a very low level of inducible LCa expression.

Not only can the LCa:LCb ratio be altered, but the high level of LCb appears to reduce the amount of LCa present in these cells. This is not entirely surprising since the light chains compete for binding on the heavy chain (Winkler and Stanley, 1983; Brodsky et al., 1987) and since LCa has a short half-life when not bound to heavy chain (Brodsky, 1985; see Chapter 3). Thus it appears that high levels of expression of one light chain can compete out the other light chain. Attempts to reduce dramatically the LCb levels in C1R cells and PC12 cells failed, however.

Isolation of an LCa negative PC12 clone

Of the fifty-nine PC12 clones isolated after LCa transfection, one clone (A35) expressed no exogenous or endogenous LCa as determined by immunoblotting with X16

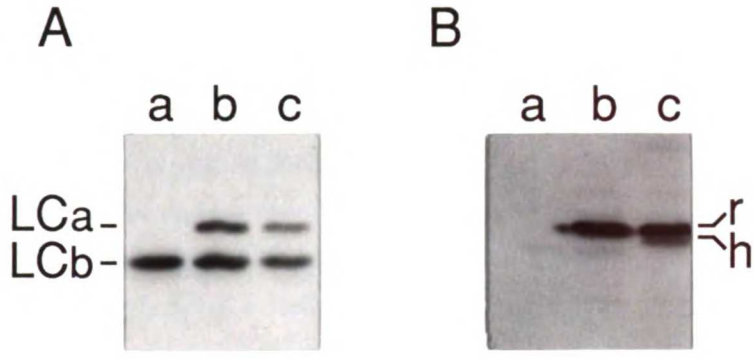


Figure 4.5 Immunoblot of light chains in LCa negative and control PC12 cells

Immunoblots were performed as described (Chapter 7). Anticonsensus serum (panel A) and the LCa specific mAb X16 (panel B) were used to detect light chains. Lane a: A35 lysate; lane b: V6 (control); lane c: A6. r and h indicate rat and human LCa, respectively.

cell	LCa/HC	LCb/HC	(LCa + LCb)/HC
wild-type	0.21	0.79	1.00
V5	0.22	0.79	1.02
A35 (LCa^-)	0.0	1.04	1.04

Table 4.4 Relative levels of clathrin light and heavy chains in LCa negative and control PC12 cells

Cell lysates were electrophoresed by SDS-PAGE and transferred to nitrocellulose. The low molecular weight region of the blot was probed with antipeptide 2 serum, biotinylated goat-antirabbit, and ^{125}I-streptavidin. The high molecular weight region of the blot was probed with TD.1 (anticlathrin heavy chain monoclonal), biotinylated horse-antimouse, and ^{125}I-streptavidin. Blots were exposed to film and densitometry was performed on the resulting autoradiograms. Numbers listed are arbitrary units and indicate relative levels of light chain versus heavy chain in the different cells. All numbers were normalized so that (LCa + LCb)/HC equaled 1.00 for wild-type.

and anticonsensus serum (Figure 4.5). These two antibodies bind in distinct locations on the light chains (see Chapters 2,3) indicating that LCa is not expressed in this clone. At the time of the writing of this thesis, one southern blot analysis had been performed on the LCa-negative and control clones and this preliminary study (by A. Jackson) revealed no differences in the restriction patterns (using Sal I and Hind III) between wild-type and A35.

To determine if the cells compensate for the loss of LCa with higher levels of LCb, the cells were examined for their relative levels of light chains and heavy chain (Table 4.4). Clathrin light chains and heavy chains were quantitated by immunoblotting as described previously (Chapter 2). These studies indicate that the LCa-negative cells do maintain a higher level of LCb relative to heavy chain to replace the missing LCa.

4.3 Discussion

In collaboration with A. Jackson (University of Cambridge, England) a system has been developed for expression of exogenous light chains in C1R and PC12 cells. A significant change in light chain ratio was obtained after expression of LCb in C1R cells. These cells could be shown to change their light chain ratios from 5:1 to 1:7 as a result of LCb expression. This indicates that the pMAMneo vector system is acting as a high level expression system in C1R cells. These results also support the idea that LCa and LCb compete for the same heavy chain binding sites *in vivo* since the level of LCa dropped after high levels of exogenous LCb were expressed.

Although the C1R cells could be induced to express high levels of LCb thereby reducing LCa levels, the reverse could not be done. Of the ten C1R clones transfected with LCa, none showed a significant change in light chain ratio. It is possible that this could be due to a lack of protein production since with PC12 cells only 19% of the clones resistant to G418 actually produced exogenous light chain. However, the PC12 cells that produced exogenous LCa were resistant to LCb elimination. It therefore seems likely that both C1R and PC12 cells show a resistance to LCb elimination in this system. This could be due to the higher affinity of LCb (compared to LCa) for the heavy chain, or it may be due to the relatively longer half-life of LCb than LCa. Whatever the case may be, we were unsuccessful in our attempts to eliminate the LCb in PC12 or C1R cells. Success was obtained in eliminating the cells of LCa, however, and the remainder of this thesis describes the experiments done on these cells.

Chapter 5: Clathrin function in cells with reduced or abolished LCa expression

5.1 Introduction

C1R and PC12 cells have been obtained with reduced or abolished LCa expression, respectively. These cells provide an ideal opportunity for determining the role of LCa in clathrin function. It is already apparent from the isolation of a LCa negative cell that the presence of LCa is not necessary for cell survival or growth. To delineate the role of LCa, experiments on clathrin function were undertaken. The results obtained indicate that clathrin assembly, endocytosis, regulated secretion, clathrin localization, and sorting by clathrin all appear to occur normally in cells lacking LCa. Thus it appears that LCb can substitute for LCa. If LCa does play a specialized role in clathrin function, it is probably much more subtle than originally thought.

5.2 Results

Assembled versus unassembled clathrin levels

Since LCa has been suggested to be a target for disassembly of coated vesicles (DeLuca-Flaherty et al., 1990) the assembly state of clathrin was examined in the LCa negative clone and in control lines by immunoblotting of cell fractions containing assembled and unassembled clathrin (Figure 5.1). Cell lysates were fractionated by centrifugation at 100,000 x g. These fractions were run on SDS-PAGE alongside known quantities of purified bovine clathrin and blotted to nitrocellulose. The blots were probed with monoclonal TD.1 and biotinylated horse antimouse and ^{125}I-streptavidin. After exposure of the blots to film, the density of resulting bands was determined on a DU-64 spectrophotometer. The signal of TD.1 binding to purified clathrin was used to determine the linearity of autoradiogram exposure. No difference in the percentage of clathrin heavy chain assembled was found between the control clones and the LCa negative clone (Table 5.1). In addition, no difference was found in the assembly state of clathrin in C1R cells with very different light chain ratios (not shown). Since C1R and PC12 cells have 87% (A. Jackson, personal communication) and 35% of their clathrin assembled, respectively, a change in the light chain ratio does not appear to change the assembly state of clathrin regardless of the normal state for the cell.

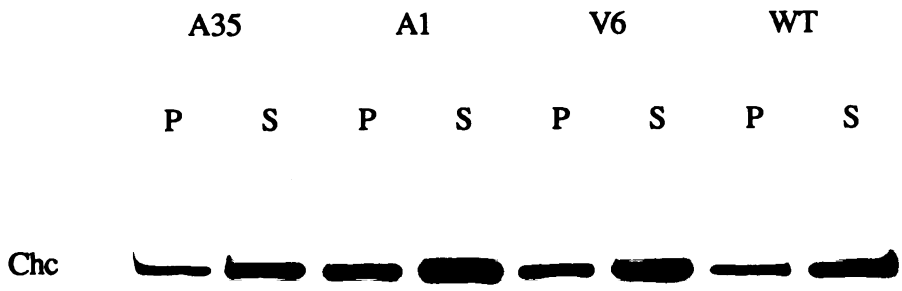


Figure 5.1 Immunoblot of clathrin heavy chain in assembled and unassembled fractions

Lysates of PC12 cells were spun at 100,000 x g to separate assembled (pellet) and unassembled (supernatant) clathrin. Samples were electrophoresed on SDS-PAGE, transferred to nitrocellulose and immunoblotted with monoclonal TD.1 (anti-clathrin heavy chain). Biotinylated horse-anti-mouse and 125I-streptavidin were used to detect the TD.1. The autoradiogram is shown. P and S refer to pellet and supernatant, respectively. Chc refers to clathrin heavy chain.

cell	% heavy chain assembled
Wild type	33 ± 2 (3)
V6 (vector)	38 ± 5 (3)
A1 (A transfected, nonproducer)	39 ± 2 (2)
all controls	36 ± 4 (8)
A35 (LCa^-)	35 ± 8 (3)

Table 5.1 Heavy chain assembly state in LCa negative and control PC12 cells.

Percentage of clathrin heavy chain assembled was determined as shown in Figure 5.1

Endocytic rates

To determine if the rate of endocytosis might be altered in cells with reduced LCa, transferrin endocytosis and recycling was measured in C1R cells (Figure 5.2) by a modification of the method of Hopkins and Trowbridge (1983). Cells were preincubated in binding buffer (PBS, 1mg/ml BSA, 10 mM PIPES pH 7.4) for 4 hrs, rinsed and incubated in ^{125}I-transferrin for 1.5 hrs at 4°C. Cells were rinsed and allowed to internalize and recycle transferrin at 37°C for the indicated time period. The medium was saved and counted and represented recycled transferrin. The cells were stripped of surface transferrin with low pH buffer and the level of stripped transferrin was counted. Remaining counts

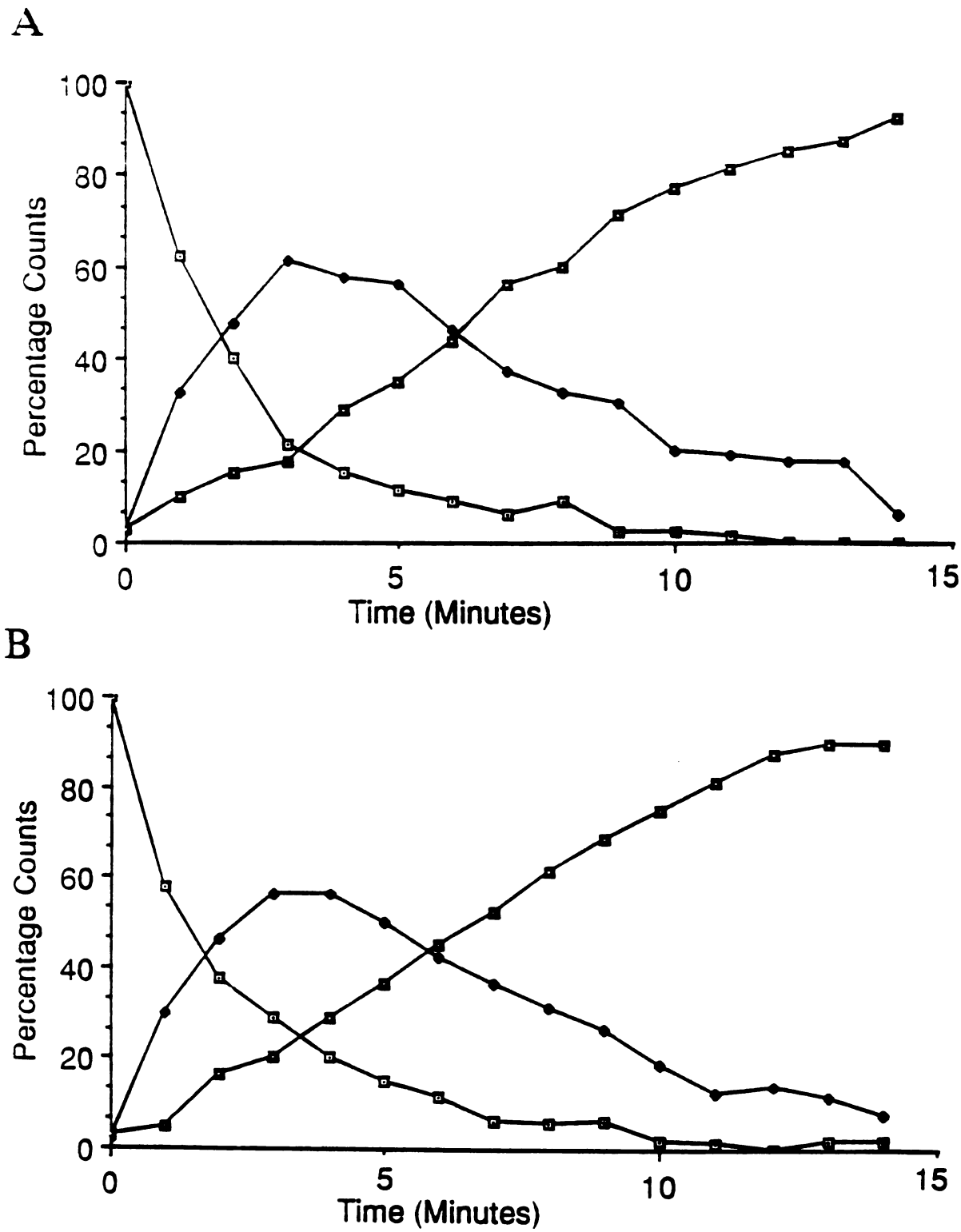


Figure 5.2 Endocytosis and recycling of transferrin in control and LCa reduced C1R cells

Cells were treated with 1 μM dexamethasone for 48 hours and then assayed for endocytosis. Iodinated transferrin was prebound at 4°C for 1 hr and excess rinsed away. The cells were shifted to 37°C and at intervals of one minute were put on ice. After spinning at 1000 x g, the supernatant (closed squares) was counted (recycled transferrin). Cell-surface transferrin (open squares) was measured as counts stripped from the cells with a 5 minute wash in acetic acid buffer pH 4.0. Counts remaining with the cells after this treatment (diamonds) represent internalized transferrin. Panel A: C1R transfected with pMAMneo; Panel B: C1R transfected with pMAMneo-LCa.

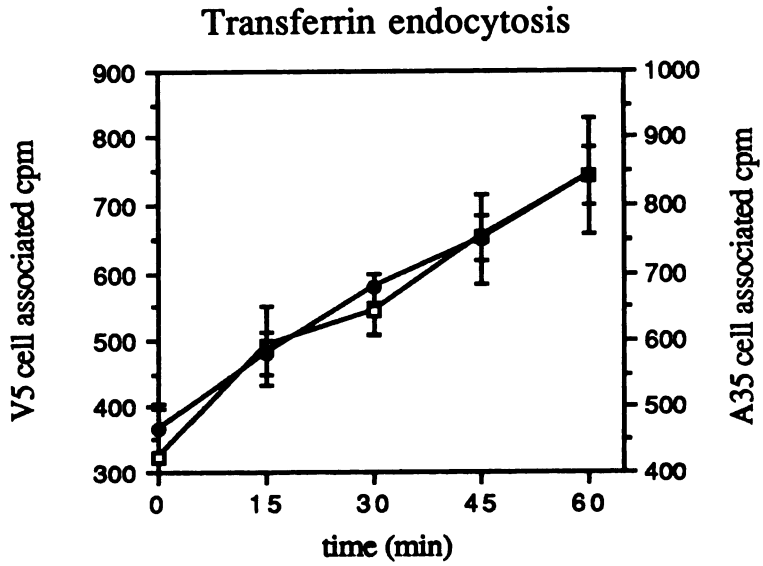


Figure 5.3 Endocytosis of transferrin in control and LCa negative PC12 cells

Cells were preincubated for 4 hrs at 37°C in binding buffer. After washing, ^{125}I-transferrin was prebound at 4°C for 90 min, and cells were shifted to 16°C for the indicated times. The cells were washed three times in PBS and dissolved in 1 M NaOH for counting. Closed circles represent A35 cells, open squares are control V5 cells.

represent internalized transferrin. No significant difference in endocytic or recycling rates was found between cells with a low LCa:LCb ratio and control lines with a high LCa:LCb ratio. Some variation in rates were observed depending on the growth state of the cells, with exponentially growing cells showing the fastest rates. However, provided comparisons were all done together with cells in the same growth state, the control and LCa reduced cells gave identical curves.

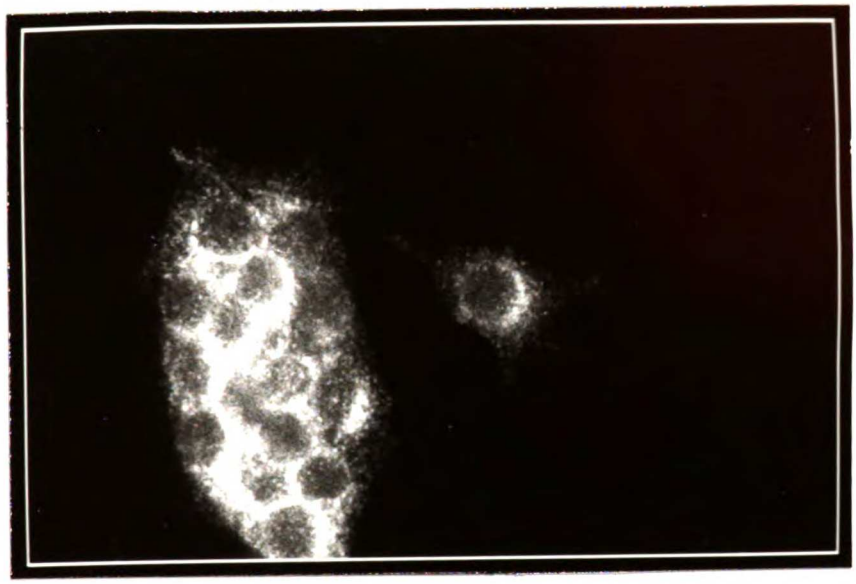
To determine if cells completely lacking LCa endocytose at normal rates, the LCa negative PC12 clone, A35, was tested for endocytosis. It was not possible to use the same methodology in PC12 cells since only 40% of the cell-surface counts could be removed after 15 minutes in low pH stripping buffer. In addition, the rate of transferrin endocytosis is very low in this cell line so that, in general, rates of uptake were difficult to measure. Therefore, endocytosis was measured in the absence of recycling by performing the experiments at 16°C (Hopkins and Trowbridge, 1983). After prebinding transferrin at 4°C, the rate of ^{125}I-transferrin accumulation was measured and compared in the LCa negative and control lines (Figure 5.3). There is no significant difference in the rate of endocytosis of the LCa negative and control clones.

A

B

Figure 5.4 Localization of clathrin heavy chain in LCa negative and control cells
Monoclonal antibody X22 was used to detect clathrin heavy chain in fixed and permeabilized PC12 cells. Rhodamine conjugated goat-anti-mouse was used to detect the anti-clathrin mAb. See Chapter 7 for details of the methodology. Panel A: V5 (control PC12 clone); Panel B: A35 (LCa negative PC12). Magnification is 1400X.

A



B

Figure 5.5 Localization of clathrin LCa

Antipeptide 1a serum (DeLuca-Flaherty et al., 1990) was used to detect clathrin LCa in fixed and permeabilized PC12 cells. Rhodamine conjugated goat-anti-rabbit IgG was used to detect the antiserum. See Chapter 7 for details of the methodology. Panel A: V5 (control PC12 clone); Panel B: A35 (LCa negative PC12). Magnification is 1400X.

A

B

Figure 5.6 Localization of secretogranin I in LCa negative and control cells

Anti-secretogranin I monoclonal antibody (Boehringer Mannheim) was used to detect secretogranin I in fixed and permeabilized PC12 cells. Rhodamine conjugated goat-anti-mouse IgG was used to detect the anti-secretogranin I mAb. See Chapter 7 for details of the methodology. Panel A: V5 (control PC12 clone); Panel B: A35 (LCa negative PC12). Magnification is 1400X.

Localization of clathrin and proteins sorted by clathrin

Clathrin is localized at both the Golgi region and plasma membrane of cells. Previous work has indicated that LCa and LCb are found in both regions of the cell (Puszkin et al., 1989). However, it was not known whether clathrin triskelions with only one type of light chain would be found in both regions of the cell. To address this question, immunofluorescence with anticlathrin antibodies was performed on the LCa negative clone, A35 (Figure 5.4). These studies indicate that clathrin heavy chain containing only light chain b can localize efficiently to both the plasma membrane and the Golgi region. Anti-LCa serum (antipeptide 1a serum) (DeLuca-Flaherty et al., 1990) stains both the Golgi and plasma membrane in the wild type cells, but as expected does not stain the LCa negative clone, thus confirming that the clone is not producing LCa (Figure 5.5).

To determine if the absence of LCa might affect the trafficking of other proteins by clathrin, we examined the immunofluorescence staining pattern of secretogranin I (Figure 5.6) and mannose-6-phosphate receptor (not shown). Comparison of the LCa negative clone with a control clone revealed that the localization pattern of the proteins were unaffected by the absence of LCa. Although this method can measure only gross differences, there appear to be no major disturbances in the trafficking of these proteins.

Secretion

Since clathrin is involved in the formation of secretory granules (Tooze and Tooze, 1986; Orci et al., 1984), it was of interest to determine whether cells lacking LCa could be induced to secrete normally (Figure 5.7). Cells were plated on poly-D-lysine for 4-5 days and then incubated with ^{3}H-norepinephrine for 2 hours (Lowe et al., 1988). After extensive washing the cells were incubated with 55 mM KCl. Both control cells and the LCa negative clone were induced to secrete ^{3}H-norepinephrine upon stimulation by 55 mM KCl. No difference in levels or rate of norepinephrine secretion was found between the LCa negative and control cells.

It is not clear whether norepinephrine is restricted to dense-core secretory granules (it may also be secreted from synaptic vesicles), therefore it was of interest to examine secretion that is selective for the dense-core granules. This was accomplished by examining the secretion of two dense-core proteins, secretogranins I and II (Figure 5.8). PC12 cells were labeled for 6 hours with ^{35}S-SO_4 which labels primarily the secretogranins and some proteoglycans (Tooze and Huttner, 1990; M. Grimes, personal communication). The cells were then washed and incubated in DME H21 with serum for

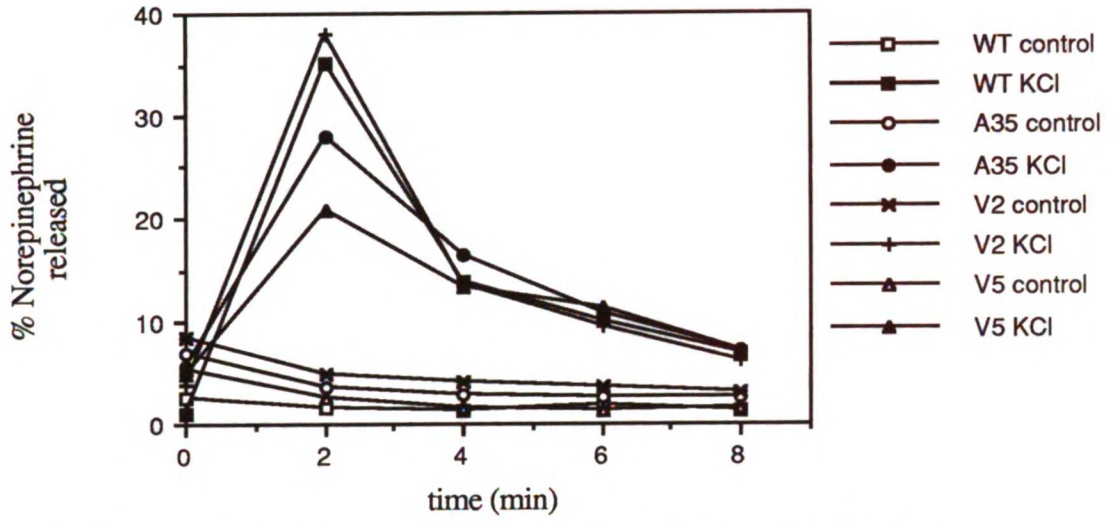


Figure 5.7 Norepinephrine secretion in LCa negative and control PC12 cells

A35 is the LCa negative clone. V2 and V5 are clones transfected with pMAMneo vector. WT represents wild type PC12 cells. KCl and control indicates cells incubated with and without 55 mM KCl, respectively.

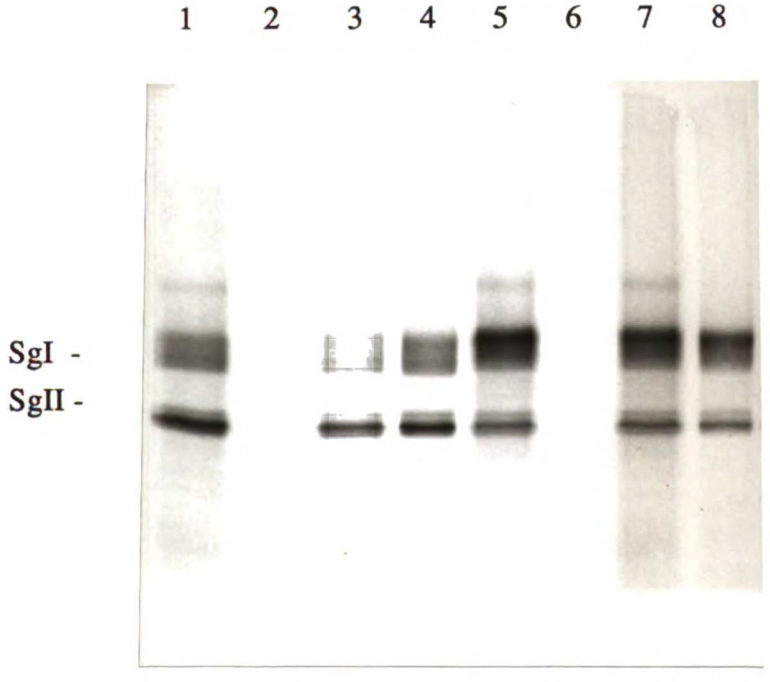


Figure 5.8 Secretion of secretogranins in LCa negative and control PC12 cells

Cells were labeled with ^{35}S-SO_4 for 6 hrs, chased in normal medium for 15 hours, and then incubated with or without 55 mM KCl. Medium was collected and TCA precipitated, solubilized in urea and subjected to electrophoresis. Unsecreted secretogranins were visualized concurrently by electrophoresis of cell lysates. **Lanes 1-4: Wild type PC12; Lanes 5-8: A35 (LCa negative); Lanes 1,2,5,6: no KCl stimulation; Lanes 3,4,7,8: stimulation with KCl; odd numbered lanes: cell lysates; even numbered lanes: medium.**

15 hours to allow secretion of all sulfated proteins in the constituitive secretory pathway. The cells were then washed very extensively and induced to secrete with 55 mM KCl. As in the norepinephrine assay, the LCa negative PC12 clone secreted as well as the control clone. Thus LCa is not necessary for the formation or secretion of dense-core granules. These studies also indicate that sulfation, a modification which occurs in the trans-Golgi (Baeuerle and Huttner, 1987), is unaffected by the absence of clathrin LCa.

5.3 Discussion

C1R clones with reduced LCa and an LCa-negative PC12 clone were tested for a variety of clathrin functions and were found to mediate these functions with the same kinetics and efficiency as wild-type cells. These results suggest that for many clathrin functions, LCb can substitute for LCa in LCa-depleted cells. It is possible, of course, that the clathrin functions tested are indifferent to changes in light chain ratios because neither light chain is required for these functions. However, the phenotype of a yeast mutant with a complete deletion of its single clathrin light chain gene has revealed that the light chain plays a critical role in clathrin's contribution to maintenance of growth rate and mating function, which depend on endocytosis and secretion (Silveira et al., 1990). Therefore, it is expected that in a more complex cell, at least one of the clathrin light chains should be required for these critical clathrin functions. The possibilities that the mammalian LCa is a highly specialized or even vestigial product of a duplicated light chain gene should also be considered as an explanation for the dispensability of LCa. However, the high degree of conservation of LCa between rat, human and bovine clathrin (Jackson et al., 1988) and the fact that some cell types, such as reticulocytes and B lymphocytes express LCa in vast excess over LCb (Bar Zvi et al., 1988; Acton and Brodsky, 1990) suggest that LCa must be maintained and expressed to perform a function. If the LCa-specific function is highly specialized, then the tests of common clathrin functions carried out in these studies may have overlooked its specialized role. However, in blood cells, where LCa expression predominates, it must also mediate common clathrin functions.

The lack of effect of LCa depletion on the intracellular distribution of clathrin confirms that the light chain polymorphism does not influence clathrin localization. In conjunction with this finding, LCa depletion does not selectively affect the sorting function of clathrin in the trans-Golgi, as measured by localization of mannose-6-phosphate receptor and secretogranins, compared to the sorting function at the plasma membrane, as measured by endocytosis of transferrin. At least in these basic clathrin functions, LCb and LCa must play an equivalent role.

One function that was expected to be affected by depletion of LCa was the overall level of assembly of clathrin. *In vitro*, LCa is the favored substrate for stimulation of hsc 70, the uncoating ATPase, so it was predicted that coated vesicles with all LCb or even reduced LCa might be uncoated less efficiently. There are several explanations for the lack of change in clathrin assembly levels in LCa-depleted cells. Although hsc 70 can disassemble clathrin-coated vesicles *in vitro*, its role *in vivo* has not yet been confirmed. The uncoating activity might simply be a result of the fortuitous presence of a recognition site for hsc 70 on the clathrin light chains and result from conformational changes imparted to the light chains after binding and ATP hydrolysis by this protein,without reflecting a process that happens *in vivo*. Particular antibodies which bind to assembled clathrin near the light chain binding site also have depolymerizing activity (Blank and Brodsky, 1986). Alternatively, hsc 70 may initiate uncoating *in vivo* by binding to the light chains but the initiation of uncoating may not be the rate limiting step in coated vesicle disassembly, such that more efficient stimulation of this process by LCa would not be reflected in overall levels of clathrin assembly. Finally, *in vivo*, LCb might stimulate uncoating activity as efficiently as LCa. For example, its phosphorylation state might improve its recognition as a substrate for the uncoating reaction. In this regard, it should be noted that in a study of LCb phosphorylation in reticulocytes, Bar Zvi and Branton (1988) observed that the LCb in unassembled clathrin was phosphorylated to a greater extent than that associated with assembled clathrin.

In the C1R transfectants, overexpressed LCb competed efficiently for LCa binding sites on the clathrin heavy chain, demonstrating that the relative amounts of LCa and LCb present in the cell influence the light chain composition of triskelions. It would appear, however, from preliminary attempts to overexpress LCa and eliminate LCb, the reverse competition is less efficient, suggesting that if LCb is present, it has preferential association with the heavy chain. The longer half-life of heavy chain-associated LCb (45 hrs) compared to that of heavy chain-associated LCa (24 hrs) is further evidence for preferential binding of LCb (Acton and Brodsky, 1990). Thus, while light chain distribution on triskelions is random (Kirchhausen et al., 1983), the binding of LCb seems to be favored. Because all cells have triskelions with four different light chain compositions (Acton and Brodsky, 1990), the different types of triskelions could potentially have specialized functions. Analysis of clathrin functions in PC12 cells with only LCb suggests this is probably not the case, since a single type of clathrin triskelion can perform many clathrin functions. However, triskelions with all LCb, may be more versatile than triskelions with all LCa. Previous studies demonstrated a predominance of LCb in cells with a regulated secretory pathway (Acton and Brodsky, 1990), suggesting a specialized role for LCb in the

secretory process. In the LCa-negative PC12 cells LCb could substitute for LCa in normal clathrin functions, as shown in C1R cells, and still play a specialized role in secretion. While secretory activities were not exhaustively analyzed in the LCa-negative cells, it might be expected that they would only be affected by elimination of the LCb light chain, an experimental challenge for the future.

If cells with only LCb can perform clathrin functions normally, why are there two types of light chains? One possibility is that LCa and LCb perform the same functions but LCb is more highly regulated. Cells which are undergoing rapid and continuous (non-regulated) endocytosis such as reticulocytes or activated B lymphocytes have much more LCa than LCb (Bar-Zvi et al., 1988; Acton and Brodsky, 1990). Those cells which require clathrin functions to be more highly regulated, such as neurons, which need to coordinate rapid clathrin assembly for reuptake of membrane after secretion, have predominantly LCb. If the function of LCb can be regulated, perhaps using LCb costs more energy than using LCa. For example, phosphorylation of LCb may be required for some clathrin functions whereas LCa might be always 'turned on'. Thus it might be less expensive of energy for the cell to have LCa if it does not require clathrin to be highly regulated by LCb phosphorylation or dephosphorylation.

Chapter 6: Discussion and Conclusions

The experiments reported in this thesis were designed to deepen our understanding of the role of the light chains, LCa and LCb, in clathrin function. Much information has been gathered about LCa and LCb, and from these investigations we have many more pieces of the puzzle with which to try to construct a picture of their roles. Although the exact functions of the light chains are still not known, these studies have eliminated many possible functions for LCa and LCb while suggesting others. This chapter summarizes the results presented in this thesis as well as attempts to present our current understanding of the possible roles of the clathrin light chains.

Pulse-chase studies of clathrin indicate that the triskelion is a fairly long-lived multimeric complex. Once bound, the light chains do not alternate between a bound and free state. Since LCa is turned over approximately twice as fast as the other subunits (24 hours versus 45 hours and 50 hours for LCb and the heavy chain, respectively), LCa must come off a triskelion and be rapidly degraded. It is possible that a triskelion that loses its LCa obtains another light chain to fill the unoccupied space. It seems likely that the selective degradation of LCa serves no function and simply reflects the susceptibility of the protein to proteases *in vivo* since LCa is more susceptible than the other clathrin subunits to proteases *in vitro*. The free pool of LCa (heavy chain-unassociated) has characteristics that suggest it is a pool of recently synthesized LCa that is unable to find a spot on heavy chain on which to bind. It apparently serves no function since it is only intermittently present in very small amounts and appears to simply be excess light chain.

Since one round of endocytosis takes only a minute or less, a triskelion can undergo many rounds of endocytosis before it is degraded. Therefore, regulation of endocytosis cannot occur by turnover of clathrin subunits but must be by some other means, possibly by post-translational modifications. None of the clathrin subunits or AP2 subunits appears to be modified by fatty acids. Therefore association of these proteins with the membrane and with membrane proteins must be regulated by other means. Phosphorylation is a likely candidate for regulation of endocytosis since LCb, AP50, and the AP100 kD proteins are phosphorylated at different levels in the assembled and unassembled fractions. In addition, incubation of phosphorylated LCb with phosphorylated AP50 causes rapid dephosphorylation of AP50 (Hanson et al., 1990). It seems highly likely that phosphorylation/dephosphorylation will be found to play a major role in the regulation of clathrin and AP function.

Initial studies indicated that the relative amounts of LCa and LCb present in clathrin depended on the tissue source, therefore it was of interest to determine if a pattern in light chain expression could be found. An assay was developed for quantitation of the light chains in cells and tissues, and from these studies a correlation was found between the presence of a regulated secretory pathway and the predominance of the LCb form of light chain. This finding suggests that some part of the regulated secretory pathway requires (or prefers) LCb. Clathrin is thought to serve two roles in the regulated secretory pathway: formation of dense-core granules and regulated reuptake of membrane after secretion. It is not known if LCb plays a specialized role in one or both of these processes. Regulated secretory cells devoid of LCb will be necessary to answer this question.

One longstanding question is whether a cell uses (assembles) the same light chain ratio that it produces. To address this question, relative light chain levels were quantitated in assembled and unassembled clathrin. These studies revealed that the cells use the same ratio that they produce. Thus selection for use of a particular light ratio must occur at the level of synthesis and not at the protein level. This suggests that there may not be any selection for a particular type of light chain (or triskelion) to be present in a particular spot in a coated lattice or vesicle. Rather, it seems more likely that any light chain or triskelion may fit in any spot of a clathrin lattice.

What then, is the purpose of the two light chains? To answer this question more directly, a system was devised in which light chain ratios could be altered *in vivo*. From these studies C1R cells were obtained that could be induced to change their LCa:LCb ratio from 5:1 to 1:7. For unknown reasons, no C1R cells were obtained that could be induced to increase the LCa:LCb ratio higher than 5:1. A PC12 LCa -negative clone was also isolated in these studies. These LCa-negative PC12 cells and the C1R cells with highly reduced LCa levels were then used to investigate the role of LCa in clathrin function. Several clathrin functions and properties (assembly, endocytosis, clathrin localization, sorting by clathrin, and regulated secretion) were shown to be unaffected by an absence of clathrin LCa.

Clearly, LCa is not required for survival or normal cell growth, although we know that yeast growth is severely impaired in cells lacking its light chain (Silveira et al., 1990). Thus it is likely that the main function of the light chains are shared by LCa and LCb and that LCb can substitute for LCa when necessary. Since LCa is found to predominate in cells which are endocytosing rapidly (e.g. activated lymphocytes, reticulocytes (Bar-Zvi et al., 1988)) and LCb is found to predominate in cells which display a substantial amount of regulated endocytosis, it is possible that LCa is always 'turned on' whereas LCb is regulated and can be 'turned on' or 'turned off'. Together with previous phosphorylation

studies of LCb, these data suggest that LCa and LCb perform similar functions, and these functions may be more highly regulated in LCb than LCa. Thus, the key to solving the mystery of clathrin light chain function may be in understanding the role of phosphorylation of LCb and the assembly proteins in clathrin regulation.

Chapter 7: Materials and methods

Procedures described in this chapter are those which were commonly used during the duration of this thesis project. Details concerning the experimental procedures of specific experiments are described along with the results in chapters 2 - 5. This chapter is organized so that any of the methods may be reproduced easily. For each procedure, the materials needed are listed first, followed by a step-by-step protocol, and finally any 'notes' or helpful hints.

7.1 Cell lines and source

Line	species	cell type	source
LB	human	EBV-transformed B lymphocyte	V. Engelhard
IM9	human	B lymphoblastoid	ATCC
Supe T	human	T lymphocyte	C. G. Davis
PC12	rat	adrenal pheochromocytoma	L. Reichardt
$PC12_k$	rat	adrenal pheochromocytoma	R. B. Kelly
AtT20	mouse	pituitary	H-P. Moore
BL3	bovine	B lymphocyte	G. Thielen
MDBK	bovine	kidney	ATCC
Fibroblast	human	primary fibroblast	UCSF Cell Cult. Fac.
EBTr	bovine	embryonic trachea fibroblast	ATCC

7.2 Cell growth conditions

cell line	medium	FCS,HS	splitting conditions
LB	RPMI 1640	10%,0%	1:25 every 2-3 days
IM9	RPMI 1640	10%, 0%	1:25 every 2-3 days
Supe T	RPMI 1640	0%, 10%	1:10 every 3 days
PC12*	DME H21	7%, 7%	1:3 every 2-3 days
$PC12_k$*	DME H21	5%, 10%	1:2 every 2-3 days
AtT20*	DME H21	7%, 7%	1:3 every 2-3 days
BL3	RPMI 1640	0%, 10%	1:20 every 2-3 days
MDBK*	RPMI 1640	0%, 10%	1:10 every 3 days
Human Fibroblasts*	DME H21	0%, 10%	1:10 every 3-4 days
EBTr*	RPMI 1640	0%, 10%	1:2 to 1:4 per week

*Adherent cells

materials and methods

1. flasks and plates- cells were generally grown on Falcon or Nunc tissue culture plasticware with no additional extracellular matrix treatment unless indicated.
2. pipets- sterile plastic pipets were used to transfer and feed cells.
3. sterile hood- all tissue culture work was performed in a sterile laminar flow hood.
4. incubators- set at 37°C, 5-7% CO_2 (none of the lines showed any apparent preference for either concentration). Humidity was maintained with water trays.
5. Saline-trypsin-versene (STV) (0.25% trypsin, 0.2 g/L EDTA, 1.0 g/L glucose, 0.4 g/l KCl, 0.004 g/L phenol red, 8.0 g/l NaCl, 0.58 g/l $NaHCO_3$). STV was generally used to remove attached cells from cultureware (1ml/80 cm^2 flask or 3ml/175 cm^2 flask). In some instances, as noted in the text, cells were removed with PBS containing 5 mM EDTA.
6. poly-D-lysine treatment- tissue culture plates were incubated for 1 hr with enough solution of poly-D-lysine hydrobromide MW > 300,000 (P7505 Sigma) to cover the bottom of the plate. The solution then was removed and saved for use again (this solution was reused up to five times). The plates were then washed four times with PBS and allowed to dry.

7.3 Antibody specificities

antibody	mAb/as	made in	specificity	protein specificity	epitope
X22	mAb	m	m, h, r, d, b, mk	clathrin heavy chain	proximal arm
X32	mAb	m	h, b	clathrin heavy chain	unknown
X16	mAb	m	m, h, r, d, b, mk	clathrin LCa	a.a. 125-144
X43	mAb	r	h, b	clathrin LCb > LCa	a.a. 93-106
LCB.1	mAb	m	h, b, c	clathrin LCb	a.a. 9-22
AP.6	mAb	m	b, h, r	α adaptin	N-term. 30kD
AP.7	mAb	m	b, h	α adaptin	N-term. 30kD
29B5*	mAb	m		none	p-nitrophenol
α-Leu10**	mAb	m	h	HLA-DQ1,3	unknown
consensus	as	rb	b, h, m, r	clathrin LCa = LCb	residues 23-44
peptide 1a#	as	rb	h, r	LCa	residues 1-22

note: light chain residues are numbered using bovine LCa as a reference.

mAb = monoclonal antibody, as = antiserum

b = bovine, c = chicken, d = dog, h = human, m = mouse, mk = monkey, r = rat

All antibodies were produced in the laboratory of F. Brodsky except as noted:

*obtained from L. Herzenberg

**obtained from Becton Dickinson Immunocytometry Systems

#obtained from A. Jackson (University of Cambridge, England)

7.4 Antiserum production

The cross-linking method was adapted from Pierce instructions for MBS and from a method obtained from D. Simonetti (Genentech). The immunization protocol used by the Berkeley antibody company (BAbCO) is listed below.

materials

1. Keyhole limpet hemocyanin (Calbiochem #374811 in $(NH_4)_2SO_4$))
2. peptide synthesized with a cysteine at the amino terminus for coupling
3. PBS (no azide)
4. 50 mM $NaPO_4$ buffer, pH 7.5
5. 50 mM $NaPO_4$ buffer pH 6.5
6. MBS (m-Maleimidobenzoic acid N-hydroxysuccinimide ester: Sigma)
7. dimethylformamide (DMF)
8. BioRad econo-pac 10DG column

method

1. Synthesize peptide with an amino-terminal cysteine.
2. Couple the peptide to keyhole limpet hemocyanin (KLH)
   a. Dialyze 30 mg KLH against PBS (no azide) for 48 hours, 3 changes of buffer to remove the ammonium sulfate. Then dialyze against 50 mM $NaPO_4$ buffer, pH 7.5 once overnight.
   b. Dissolve MBS in dimethylformamide (DMF) to 15 mg/ml (dissolves readily).
   c. For each 5 mg KLH add 63 µl MBS (in DMF) slowly with stirring. Shake 30 min., r.t.
   d. Desalt KLH-MBS mixture on BioRad econo-pac 10DG column equilibrated in 50 mM $NaPO_4$ buffer pH 6.5. Collect 1 ml fractions. Do quick Bradford protein assay on the fractions. Pool fractions containing protein.
   e. Dissolve peptide at 5 mg/ml in 50 mM $NaPO_4$ pH 6.5.
   f. Add KLH-MBS dropwise to peptide solution. Shake 1 hr, r.t.
   g. Concentrate solution with preparative Centricon-30KD. This mixture is ready for use as an antigen in immunization protocols.
3. Give this mixture to Berkeley antibody company (BAbCO) to raise antiserum in two rabbits. Their protocol for antiserum production is as follows:

Day 0	Prebleed - 10 mls
	Initial immunization with 0.5 mg/rabbit - PLN
Day 21	Boost with 0.25 mg/rabbit - IM (intramuscular)
Day 31	Bleed I - 30 mls
Day 42	Boost with 0.25 mg/rabbit - IM
Day 52	Bleed II - 30 mls
Day 63	Boost with 0.25 mg/rabbit - IM
Day 74	Bleed III - 30 mls
Day 84	Boost with 0.25 mg/rabbit - IM
Day 94	Bleed IV - 30 mls

7.5 SDS polyacrylamide gel electrophoresis

This protocol was adapted from Laemmli (1970).

materials

1. Upper acrylamide (30% acrylamide,1.6% methyl-bisacrylamide)
2. 4X Upper Tris (0.5 M Tris, pH 6.8, 0.4% SDS)
3. Lower acrylamide (30% acrylamide, 0.8% methyl-bisacrylamide)
4. 4X Lower Tris (1.5 M Tris, pH 8.8, 0.4% SDS)
5. Ammonium persulfate (10% in water)
6. TEMED
7. Laemmli running buffer
8. 2X Reducing Sample buffer (8% SDS, 10% BME, 10% Upper Tris, 0.002% Bromophenol blue, 25% glycerol)
9. Coomassie Blue stain (10% HOAc, 45% MeOH, 0.2% Coomassie Blue R)
10. Destain (40% MeOH, 10% HOAc)
11. Agarose (1% agarose in dH_2O)
12. Adjustable slab gel unit, plates, combs, spacers (C.B.S. Scientific)

method

1. Boil samples in reducing sample buffer for 10' at 100°C.
2. Melt agarose.
3. Assemble glass plates with spacers on bottom and sides. Clip together.
4. Pipet melted agarose around sides to seal spacers and plates.
5. Mix lower acrylamide as indicated below.
6. Pour into assembled gel plates.
7. Pipet a thin layer of water sat'd butanol over lower acrylamide (to form flat interface).
8. When gel is polymerized, pour off butanol and rinse gel with water several times. Remove excess water.
9. Mix upper acrylamide as indicated below.
10. Pour on top of lower gel. Put comb in immediately.
11. When acrylamide is polymerized, remove bottom spacer and clip plates into gel box containing running buffer. Avoid getting bubbles under gel plates.
12. Remove comb from upper gel.
13. Load gel with syringe or thin-tipped pipets.

13. Stack gel: 1.5 mm thick - 20 mAmps
    0.75 mm thick - 15 mAmps
    Run gel: 1.5 mm thick - 40 mAmps
    0.75 mm thick - 30 mAmps
14. When dye front reaches bottom of gel. Turn off the power and dump the buffer. Remove the plates from the gel box and remove spacers from plates. Pry plates apart with scooper spatula and place gel into Coomassie stain. Alternatively, if transferring proteins to nitrocellulose, place gel into a solution of running buffer without SDS and follow immunoblotting protocol.

Thin gels (0.75mm x 15 x 15):

	10%		12%		15%	
Separating gel	1 gel	(2 gels)	1 gel	(2 gels)	1 gel	(2 gels)
Lower acrylamide (ml)	4.0	(8.0)	4.8	(9.6)	6.0	(12.0)
Lower Tris (4x) (ml)	3.0	(6.0)	3.0	(6.0)	3.0	(6.0)
Water (ml)	5.0	(10.0)	4.2	(8.4)	3.0	(6.0)
APS (µl)	100	(200)	100	(200)	100	(200)
TEMED (µl)	20	(40)	20	(40)	20	(40)

4.5 % acrylamide/0.12% bis

Stacking gel	1 gel	(2 gels)
Upper acrylamide (ml)	0.75	(1.5)
Upper Tris (4x) (ml)	1.25	(2.5)
Water (ml)	3.00	(6.0)
APS (µl)	50	(100)
TEMED (µl)	10	(20)

Thick gels (1.5mm x 15 x 15) = 4 thin minigels:

	10%		12%		7%	5%
Lower gel	1 gel	(2 gels)	1 gel	(2 gels)	1 gel	1 gel
Lower acrylamide (ml)	8.0	(15.0)	12.0	(18.0)	7.0	5.0
Lower Tris (4x) (ml)	6.0	(11.25)	7.5	(11.25)	7.5	7.5
Water (ml)	10.0	(18.75)	10.5	(15.75)	15.5	17.5
APS (µl)	150	(300)	150	(300)	150	150
TEMED (µl)	20	(40)	20	(40)	20	20

Upper gel	1 gel	(2 gels)
Upper acrylamide	1.5	(3.0)
Upper Tris (4x)	2.5	(5.0)
Water	6.0	(12.0)
APS	50	(100)
TEMED	10	(20)

7.6 Immunoblotting

materials

1. 1X Laemmli buffer, no SDS
2. Nitrocellulose (0.45 μm pore size)
3. Transfer buffer (1 X Laemmli running buffer, 20% MeOH)
4. Amido black stain (0.1% Amido Black, 40% MeOH, 10% HOAc)
5. Destain (40% MeOH, 10% HOAc)
6. TBS (10 mM Tris-HCl, pH 7.4, 0.9 % NaCl)
7. TBS-NaCl (1X TBS, 0.5 M NaCl)
8. Blocking solution (1% non-fat dry milk in TBS or PBS or 1% BSA in TBS or PBS)
9. 1°mAb-
    a) monoclonals: 5-20 μg/ml
    b) antiserum: 1:100 - 1:1000 dilution
10. 2°mAb
    a) ^{125}I-Rabbit antimouse- 1 x 10^6 cpm/ml
    b) biotinylated horse antimouse or goat antirabbit at 2 ug/ml; wash; then ^{125}I-Streptavidin at 1 x 10^6 cpm/ml
    c) goat antimouse (or antirabbit) horseradish-peroxidase- recommended dilution by manufacturer (Biorad)
    d) goat antimouse (or antirabbit) alkaline phosphatase at manufacturers (Biorad) recommended dilution.
11. Substrates:
    HRP substrates:
    a) Diaminobenzidine (5 mg DAB in 100 ml TBS, then add 10μl hydrogen peroxide) or
    b) 4-chloronapthol (10-30 mg Chlor-Napthol, dissolve in 10 ml EtOH first, take up to 100 ml with TBS, then add 10μl hydrogen peroxide)
    Alk. Phos. substrates:
    Vector (Burlingame, CA) alkaline phosphatase stain kit

method

1. Remove gel from gel plates and put into 1X Laemmli buffer no SDS. Leave on shaker for approx. 10 min.
2. Soak sponges in transfer buffer
3. Put genie blot apparatus together (Idea Scientific):

plastic screen
metal plate
plastic screen
2 sponges
1 piece of filter paper
nitrocellulose (prewet with transfer buffer)
gel
1 piece of filter paper
2 sponges
plastic screen
metal plate
plastic screen
plastic lid

4. Run at constant voltage at 12 V for 1.5-2 hours. (This may vary depending on the protein and % acrylamide of the gel).
5. Remove filter paper and stain with amido black. Save amido black and rinse blot with destain and water. Xerox blot. (After xeroxing, remove amido black thoroughly with destain if you are using a color development assay. Rinse away excess destain with water.)
6. Block with blocking solution > 2 hr on shaker at r.t.
7. Remove blocking solution and add primary antibody solution. Shake for > 2 hr at r.t.
   note: some 1° antibodies work better if given a longer time to incubate (e.g. 24 hours for TD.1)
8. Rinse blot: 2X in TBS (or PBS) 5 min each
   1X in TBS-NaCl 5 min
   1X in TBS (or PBS) 5 min
9. Add secondary antibody in blocking solution. Shake for > 2 hr at r.t.
10. Rinse as in step 8.

(11) For enzymatic reactions: add substrate to paper, swirl dish to develop.

note: Azide <u>cannot</u> be used in buffers if HRP is used as the enzyme!!
0.05% Tween-20 can be added in all TBS buffers, however, its use depends on the 1° antibody chosen:
LCB.1 - do not use Tween
anticonsensus serum - do not use Tween
TD.1 - can use Tween, incubate overnight - 24 hrs for best signal

7.7 Agarose Gel Electrophoresis

This method was adapted from Sambrook et al., (1989).

materials

1. 1 X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA)
2. 1% agarose in TAE buffer
3. 6 X sample buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol, 1% SDS, 50 mM EDTA)

method

1. Melt agarose. Let cool to around 50°C. Pour into mold and place comb into agarose.
2. Transfer gel to electrophoresis apparatus. Remove comb. Add 1X TAE to cover gel by 1-2 mm.
3. Load samples into gel.
4. Run gel at 1 V/cm

7.8 Biosynthetic labeling of proteins (Methionine labeling is used as an example)

This method was adapted from Brodsky (1985b).

materials

1. Lysis buffer
   a. Trimer Lysis Buffer (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP40)
   b. Standard Lysis Buffer (10 mM Tris-HCl, pH 7.3, 1 mM MgCl2, 0.5% NP40)
2. ^{35}S-methionine (New England Nuclear; NEG 009T)
3. Methionine-free medium (Gibco select-amine kit or made in UCSF TC facility) with 5% dialyzed FCS.
4. PBS
5. Protease inhibitors- 1000 X PMSF (3.8 % in ethanol)
   1000 X TAME (11.4 % in ethanol)
   1000 X pepstatin (0.07% in ethanol)

method

1. Wash 2 x 10^7 cells in 5 mls methionine-free medium with 5% dFCS.
2. Resuspend cells at 2 x 10^6/ml (10 ml) in methionine-free medium with 5% dFCS and leave 30-45 minutes, 37°C.
3. Add 1 mCi ^{35}S-methionine (New England Nuclear NEG-009T) and leave culture 4 hours (or chosen length of time), 37°C.
4. Centrifuge to pellet cells and wash cells in 5 mls PBS.
5. Resuspend cells in 1 ml lysis buffer, leave on ice 30 min, spin out nuclei in microfuge (1000 x g) and store supernatant (lysate) at -70°C.

Notes: Variations on this theme can be used to label proteins with other radioactive molecules. The lysis buffer used depends on whether clathrin is to remain in its *in vivo* assembly state or unassembled. Phosphate-free medium was prepared by the UCSF Cell Culture Facility and 1 X HEPES was used to buffer the medium. For fatty acid labeling studies, delipidated serum was obtained from G. Davis and used in normal medium.

7.9 Pulse chase studies

This method was adapted from Brodsky (1985b).

materials

1. Methionine-free medium (Gibco select-amine kit or made in UCSF TC facility) with 5% dFCS, 1X pyruvate, 1X glutamine
2. Media with 10% FCS + 10 X methionine, 1 X pyruvate, 1 X glutamine
3. Cold serum-free medium, 1 X pyruvate, 1 X glutamine
4. Lysis buffer
   a. Trimer Lysis Buffer (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP40)
   b. Standard Lysis Buffer (10 mM Tris-HCl, pH 7.3, 1 mM MgCl2, 0.5% NP40)
5. ^{35}S-methionine (New England Nuclear; NEG 009T)
6. 15 ml tubes with 4 mls cold serum-free medium (one per time point)
7. eppendorf tubes containing cold lysis buffer with protease inhibitors
8. Protease inhibitors - 1000 X PMSF (3.8 % in ethanol)
   1000 X TAME (11.4 % in ethanol)
   1000 X pepstatin (0.07% in ethanol)
9. Timer

method

1. Warm methionine-free and 10X methionine medium.
2. Count cells (2 x 10^6 cells/time point; three immunoprecipitation/time point)
3. Wash in 10 mls methionine-free medium
4. Resuspend in warm methionine-free medium (4 x 10^6 cells/ml). Leave 45 min -1 hr at 37°C.
5. Add ^{35}S-methionine (1 mCi/1.2 x 10^7 cells). Incubate 10 min, 37°C.
6. Add warm chase medium (10 X methionine medium) for a concentration of 8 x 10^5 cells/ml.
7. For time points:
   a. take 2 x 10^6 cells and add to equal volume cold serum-free medium (after 0' time point, spin remaining cells, resuspend in 10X methionine medium at 4 x 10^5 cells/ml to get rid of excess label)
   b. spin at 4°C 5 min, 2300 rpm in RT-6000.
   c. wash cells in 10 ml cold serum-free medium

d. solubilize pellet in 150 µl lysis buffer with protease inhibitors. Leave on ice, 30 min.
e. spin 10 min, at 1000 x g, 4°C to remove nuclei. Recover supernatant (cell lysate) and freeze at -80°C.

8. Supernatants are ready for immunoprecipitation.

notes: Ascorbic acid (0.2 mM) and Na_2SeO_4 (1 nM) were added to the labeling and chase medium to increase the scavenging of free radicals produced by the radioactive label in order to maintain the viability of the cells for up to 60 hours.

7.10 In vivo phosphorylation-secretion studies

materials

1. Buffer X (10 mM HEPES pH 7.4, 20 mM EDTA, 20 mM EGTA, 100 mM NaF, 10 mM Natartrate, 50 µM vanadium pentoxide, 12.5 mM Napyrophosphate, 0.02% NaN_3)
2. Buffer Y (10 mM Glycine pH 9.0, 20 mM EDTA, 20 mM EGTA, 100 mM NaF, 10 mM Natartrate, 50 uM vanadium pentoxide, 12.5 mM Napyrophosphate, 0.5% NP40, 0.02% NaN_3)
3. Buffer Z (10 mM Tris pH 8.0, 20 mM EDTA, 20 mM EGTA, 100 mM NaF, 10 mM Natartrate, 50 uM vanadium pentoxide, 12.5 mM Napyrophosphate, 0.5% NP40, 0.02% NaN_3)
4. DME H21 (no phosphate) + 1X HEPES
5. DME H21 (no phosphate) + 1X HEPES + 10% dFCS + 0.2 mM ascorbic acid
6. 2.5 M KCl (sterile)
7. PMSF
8. Aprotinin
9. STV
10. ^{32}P-potassium monohydrogen phosphate in water (New England Nuclear, Cat #NEX055)
11. sterile pipets
12. plexiglass box
13. 15 ml sterile tubes

method

1 Trypsinize and count cells. Spin 2.5 x 10^7 (5 x 10^6/time point) in 50 ml tube.
2. Rinse in warm DME H21 (no phosphate) + HEPES twice.
3. Resuspend cells in warm DME H21 (no phosphate)+ HEPES + 10% dFCS + 0.2 mM ascorbic acid 26 ml total (5ml/plate).
4. Add 5 mCi $^{32}PO_4$ (dipotassium) (1 mCi/time point).
5. Aliquot 5 ml into 5 separate 15 ml tubes. Incubate 1 hour, in 37°C water bath.
6. Do one tube at a time:

time point 16' 8' 4' 2' 0'
KCl add

a. add 100 µl of 2.5 M KCl to tube, vortex (gives final KCl concentration of 50 mM). Put back into water bath.

b. wait specified time period (0, 2, 4, 8, 16 min)
d. Spin at 4°C. Remove liquid.
e. Wash cells once in cold 5 ml Buffer X
g. Resuspend pellet in Buffer Y (0.5 ml) + 0.005% PMSF + Aprotinin and put into screw-cap eppendorf.
h. Let sit 30 min 4°C.
i. Spin out nuclei 10' 1000 x g. Pipet supe (lysates) into screw cap containing 29B5-Sepharose G.

notes: The amount of ^{32}P-PO_4 used can probably be reduced to 0.25 mCi/time point.

7.11 Formation of immune complexes

This method was adapted from Brodsky (1985b).

materials

1. Sheep antimouse IgG (SAM) (heavy and light chain specific) (no cross reaction to human IgG) 18.7 mg/ml total protein, 7 mg/ml antibody)
2. Buffer E (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% NP40, 0.02% NaN_3)
3. Monoclonal antibodies

method

1. Reconstitute a vial of SAM with 5 mls glass distilled water or thaw an aliquot of reconstituted SAM stored at -70°C.
2. Microfuge amount of SAM and monclonal antibodies needed, 15 min.
3. Combine 300 µl SAM with 200 µg of monoclonal antibody into a total volume of 300 µl with buffer E. Freeze freshly reconstituted SAM. Keep thawed aliquots at 4°C.
4. Leave complexes to form overnight at 4°C.
5. Remove liquid from above settled immune complexes.
6. Resuspend settled complexes in 2 ml cold buffer E in a 15 ml Falcon tube.
7. Centrifuge 5 min, 1000 x g, 4°C.
8. Remove supernatant and wash in 2 mls cold buffer E.
9. Centrifuge, remove supernatant and resuspend complexes in 500 µl of buffer E. 100 µl of this suspension will be used for each immune precipitate. 250 µl will be used to preclear 1 ml of lysate in 2 lots of 125 µl. Store in fresh Eppendorf tubes at 4°C until use. Complexes up to 1 month old are effective.

7.12 Immunoprecipitation with immune complexes

This method was adapted from Brodsky (1985b).

materials

1. Labeled cell lysate
2. Washed immune complexes- specific & nonspecific
3. Tube rotator at 4°C
4. Buffer E (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% NP40, 0.02% NaN_3)
5. Screw-cap eppendorf tubes

method

1. Thaw lysates. Add PMSF to 0.005% as soon as thawed.
2. First preclear: Add 125 µl of control immune complex to each ml of lysate. Leave on rotator, 30 min, 4°C. Microfuge 10 minutes at 1000 x g. Save supernatant.
3. Second preclear: Add 125 µl of control immune complex to each precleared ml of lysate. Leave on rotator, 30 min, 4°C. Microfuge 10 minutes at 1000 x g. Save supernatant.
4. Specific immune precipitation: Divide precleared lysate into aliquots, one for each specific antibody. Add 100 µl of specific immune complexes. Leave on rotator at least 1 hour, 4°C. Microfuge 10 min. Discard supernatant.
5. Wash pellet:
   1 X in buffer E
   1 X in buffer E + 0.5 M NaCl
   1 X in buffer E
6. Resuspend pellet in 100 µl reducing sample buffer. Be sure pellet is well dispersed. Boil 10 minutes and store samples at -70°C.
7. One half of a sample from 100 µl of complex will not overload one well in a 10-well gel (0.75 mm thick). For smaller wells, use less sample or bands will be distorted.

note: The amount of complex used for each immune precipitation can sometimes be reduced to avoid band distortion. Appropriate amounts should be determined empirically. For quantitative immunoprecipitations: 100 µl of X32 can immunoprecipitate clathrin heavy chain from 2.8 x 10^6 cells. 100 µl of an

X16/X43 mix can quantitatively immunoprecipitate both light chains from 4.5 x 10^6 cells.

7.13 Autoradiography

For details concerning this method see 'Gel electrophoresis of proteins: a practical approach' (eds. Hames & Rickwood).

materials

1. Kodak X-OMAT AR diagnostic film (8" x 10")
2. Intensifying screen
3. Film holder
4. Enlightening (for gels containing ^{35}S or ^{3}H samples) - note: light sensitive
5. Freezer at -80°C
6. Gel dryer
7. Automated X-ray film developer

method

1. After gel is fixed with destain, soak gel in Enlightening 15-30 minutes (in dark).
2. Soak a piece of filter paper in liquid the gel is floating in and place gel on top. Cover gel with saran wrap.
3. Put on gel dryer and dry for 2 hours.
4. Remove saran wrap and place dried gel against film. Place film against intensifying screen. Load into film holder.
5. Place in -80°C freezer.
6. Remove from freezer and let warm up. Develop film in automated developer.

7.14 Densitometry

For details concerning this method see 'Gel electrophoresis of proteins: a practical approach' (eds. Hames & Rickwood).

materials

1. Exposed film
2. Beckman DU-64 Spectrophotometer
3. Scanning accessories/area integration software

method

1. Load exposed film into the scanning accessory.
2. Scan the film using 595 nm. Pick peaks manually and determine the area integration using the software package. This area is the band density. When bands were not of equal length, the length was then multiplied against the density to give a total value for labeling.

notes: It is important to make sure that exposure is linear with radioactivity. This can be done by preflashing the film or by running a standard curve of protein.

7.15 Brain clathrin light chain preparation

The method described here was adapted from Ungewickell (1983).

materials:

1. Bovine brains- processed within 2 hours of slaughter, transported on ice
2. Buffer D (10 mM HEPES, pH 7.2, 150 mM NaCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% NaN_3)
3. Buffer A (100 mM MES, 0.2 mM EGTA, 0.2 mM $MgCl_2$, 0.02% NaN_3, pH 7.0)
3. Buffer I (10 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 0.02% NaN_3)
4. Buffer I + salt
5. Protease inhibitors
    a. PMSF - 1000 X PMSF (3.8 % in ethanol)
    b. Aprotinin - 1000 X (2.5 mg/ml in water, stored at -20°C)
6. DE52 resin

method

Clathrin light chains were prepared by purification from a boiling resistant fraction of isolated coated vesicles:

1. Put 1 brain in blender and add Buffer D to 600 ml total volume. Add 800 µl aprotinin and 800 µl PMSF. Blend 2 x 30 seconds on high, then add Buffer D to 800 mls.
2. Centrifuge at 5000 x g for 30 minutes at 4°C.
3. Pour off and save supernatant. Resuspend each pellet in 166 ml buffer D and shake hard. Recentrifuge, saving supernatants.
4. Measure volume of each supernatant as it is decanted. Stir at room temperature and add 1/20 the volume of buffer D containing 20 % Triton X-100 (buffer at room temp). Add 1/1000 the volume of aprotinin and PMSF. Stir for 10 minutes.
5. Centrifuge at 150,000 x g, 1 hr, 4°C. (Extracted supernatants may be kept at 4°C for up to 48 hrs.) Discard supernatants, add additional extracts and re-centrifuge. Repeat until all extracts have been centrifuged (pellets may be kept at 4°C for several days).
6. Remove high speed pellets and homogenize in buffer D with 1% Triton X-100. Total homogenate volume should be about 35 ml/brain. After homogenization and 1/1000 protease inhibitors.
7. Add 10-15 ml to top of sucrose gradient:

5 ml 5% sucrose in Buffer A w/1% Triton
10 ml 10% " "
5 ml 25% " "
Centrifuge in SW 28 rotor at 28,500 rpm (45,000 xg) for 45 minutes, setting the acceleration and deceleration at '1'.

8. Four layers are seen-
top a clear red layer
a yellowish cloudy layer
a soft gray layer above pellet
a creamy colored hard pellet
Remove and save cloudy yellowish layer being careful not to take any gray layer. Dilute the cloudy layer with 2 volumes of Buffer ? (no triton). Add 1/1000 protease inhibitors.
9. Centrifuge in Ti45 at max rotor setting, 1 hr, 4°C. Discard supernatants, add additional extracts and repeat. These pellets are 'coated vesicles' which may be stored at 4C for up to one week.
10. Homogenize CV pellets in buffer I for DEAE column. Volume after homogenization = 50 mls.
11. Place homogenized CV pellets (leave in homogenizer tube) into boiling water bath (100 ml beaker), cover homogenizer and bath with aluminum foil and boil 15 minutes.
12. Cool on ice 5-10 minutes, then centrifuge in SS-34 rotor, 20,000 rpm for 40 minutes without brake on. Collect supernatant for loading on DEAE column.
13. Set up DEAE column as follows:
a. Pour 10 ml pipet to about 11 ml with extender using 50% slurry of DEAE (DE52 Sepharose).
b. Let settle some under gravity flow then transfer to cold room and pump at 150 until bed stabilizes.
c. Remove extender and extra gel, pump directly to top of bed at 250 for 2.5 hrs to wash (0.10 ml/m tubing)
14. Load about 35 ml sample onto column by pumping at setting 350. Collect 100 drops per fraction during loading and washing. Wash at setting 150. Collect 50 fractions for load and wash or until absorbance is back to baseline.
15. Set up gradient former--100 ml each of 40 mM and 300 mM NaCl in buffer I
0.5 AUFS
0.2 mm/min chart

collect 80 fractions
Elute at pump setting 200.

7.16 Adrenal clathrin light chain preparation

The protocol for brain clathrin light chains was used with the following changes (Parham et al., 1989; Ungewickell, 1983):

step 1. 1 1/2 lbs of adrenals were cleaned and cut into small pieces. 6 500-ml GS3 bottles were filled with 1.5 lbs adrenal homogenate.

step 8. 8 layers are seen in sucrose gradients after spinning:

top- cloudy
clear
red cloudy
light yellow
light gray soft
clear
soft gray pellet
dark hard pellet

remove and save red and light yellow layers.

step 15. Use a lower NaCl gradient in the DE52 column: 20 mM-260 mM.

7.17 Protein Determination

materials

Protein determination kit (United States Biochemical Corporation). This assay is based on Coomassie dye-binding (Bradford, 1976).

method

Kit instructions were followed.

7.18 Immunofluorescence

From Wong et al. (1990).

materials

1. Chamber slides (Lab-Tek from Nunc, Inc.)
2. 3.7% paraformaldehyde (3.7% paraformaldehyde in 1X PBS)
3. 0.04% saponin (0.04% saponin in dH_2O)
4. blocking solution (5%FCS, 1X PBS, 0.1% TX100, 0.02% SDS, 0.02% NaN_3)
5. mounting medium (Fluromount G (Fisher), with phenylenediamine)

method

1. Seed cells on 2 chambered slides the day before experiment
   a. Put 1 ml of medium in each chamber
   b. Put 1 drop of cells taken from a confluent flask (75 cm2) after trypsinization into each chamber (cells suspended in 10 mls).
   c. Incubate overnight. Results in 75-80% confluency.
2. Wash each chamber 1X quickly with 0.5 ml PBS.
3. Fix cells with 3.7% paraformaldehyde, 0.5 ml/chamber for 10 min, r.t.
4. Remove paraformaldehyde (aspirate) and add 0.5 ml/chamber of 0.04%saponin to permeabilize the cells. Leave 10 min., r.t.
5. Wash each chamber quickly 1X with about 0.5 ml PBS.
6. Add blocking solution, 0.5 ml/chamber, leave 1 hr. R.T.
7. Remove blocking solution. Add 1^o Ab, 1 ml/chamber in blocking solution:
   purified Ab's: 20 µg/ml
   supernatants: neat
   Ab-conjugates: 20µg/ml
   Ascites: 1/50 dilution
   Incubate 1 hr, r.t.
8. Wash each chamber 4 x 5 minutes with 1 ml PBS
9. Add 2^oAb (FITC-GAM, Rh-GAM) at recommended dilution in 0.5 ml/chamber of blocking solution. Incubate 1 hr, r.t.
10. Wash 4 x 5 minutes each as before.
11. Wash 1 X 5 minutes each with dH_2O, air dry
12. Mount with 1 drop mounting medium.

notes: 8-well chamber slides can be replaced with 2-well slides to make manipulations easier and to decrease antibody consumption. PC12 cells were seeded on poly D-lysine coated slides and induced to differentiate with NGF for 1-2 days (to flatten the cells) before fixing.

7.19 ELISA

materials

1. 96 well Immulon 1 (or 2) plates (mfr: Dynatech, vendor: Fisher)
2. Antigen in PBS + 0.02% NaN_3 (5-20 ug/well should be more than sufficient)
3. Blocking solution:
   A. 1% milk in PBS + 0.02% NaN_3
   or
   B. 0.5% BSA (bovine serum albumin) in PBS + 0.02% NaN_3
4. Primary antibody - serial dilutions in blocking solution
   A. purified monoclonal - 5 ug/ml should be more than sufficient to start with
   B. antiserum - start with it undiluted
5. Secondary antibody (at recommended dilution in blocking solution)
   A. Goat anti-mouse Alkaline phosphatase (Biorad)
   B. Goat anti-rabbit Alkaline phosphatase
6. Substrate - to make 10 mls
   2 tablets (5 mg each) p-Nitrophenlyphosphate (Sigma 104 tablets)
   2 mls 0.5 M glycine pH 10.5
   0.15 mls 0.1 M $MgCl_2$
   add water to 10 mls
7. PBS + 0.02% NaN_3
8. PBS + 0.5 M NaCl + 0.02% NaN_3

method

1. Put 50 µl of antigen in PBS into each well of 96-well immulon plate. Wait 1 hour, r.t. (To determine the minimum amount of antigen needed, do serial dilutions and use with constant antibody concentration)
2. Remove liquid. Fill wells with blocking solution. Wait 1 hour, r.t.
3. Remove liquid. Add 50 µl of primary antibody/well. Wait 1 hour, r.t. (to determine antibody titer, do serial dilutions in blocking solution).
4. Remove liquid. Wash 4X as follows (fill wells):
   2 X with PBS
   1 X with PBS + 0.5 M NaCl
   1 X with PBS
5. Add 50 µl of secondary antibody/well (use recommended dilution). Wait 1 hour, r.t.
6. Remove liquid. Wash as in step 4.

7. Add 100 µl of substrate. Wait for color (yellow) to develop at r.t. (generally takes 20 min - 1 hour).
8. Read at 410 nm on ELISA plate reader.

notes: Horse radish peroxidase can be used as the secondary antibody conjugate however do not use any NaN_3 in the buffers and use the appropriate substrates.

7.20 RIA

Adapted from Brodsky (1985a).

materials

1. Microtest III flexible 96-well assay plates (Falcon)
2. Antigen in PBS + 0.02% NaN_3 (5-20 ug/well should be more than sufficient)
3. Blocking solution:
   A. 1% milk in PBS + 0.02% NaN_3
   or
   B. 0.5% BSA (bovine serum albumin) in PBS + 0.02% NaN_3
4. Primary antibody - serial dilutions in blocking solution
   A. purified monoclonal - 5 ug/ml should be more than sufficient to start with
   B. antiserum - start with it undiluted
5. ^{125}I-rabbit antimouse
6. PBS + 0.02% NaN_3
7. PBS + 0.5 M NaCl + 0.02% NaN_3
8. 96-well plate cutter

method

The method is identical as for the ELISA assay except that after the final wash (step 6) the dry wells are cut off of the plate and counted in a gamma counter.

7.21 Titration of Geneticin (G418):

materials

1. Geneticin (G418)
2. 6-well tissue culture plates
3. poly-D-lysine (Sigma: use per instructions)

method

1. Poly-lysine treat 4 6-well tissue culture plates.
2. Dissolve 0.1 grams Geneticin in 1 ml DME H21 + 10%FCS + 5%HS and filter with 0.2 μm filter.
3. Put 1 ml DME H21 + 10%FCS + 5%HS in each well. Add volume of G418 (50 mg/ml stock) indicated below.

Plate 1 (control): all wells contain no G418

		50 mg/ml stock
Plate 2:	well 1- 100 μg/ml	4 μl
	2- "	"
	3- 200 μg/ml	8 μl
	4- "	"
	5- 300 μg/ml	12 μl
	6- "	"
Plate 3:	well 1- 400 μg/ml	16 μl
	2- "	"
	3- 500 μg/ml	20 μl
	4- "	"
	5- 600 μg/ml	24 μl
	6- "	"
Plate 4:	well 1- 700 μg/ml	28 μl
	2- "	"
	3- 800 μg/ml	32 μl
	4- "	"
	5- 900 μg/ml	36 μl
	6- 1000 μg/ml	40 μl

4. Count cells and dilute to 400 cells/ml in DME H21 + 10%FCS + 5%HS. Add 1 ml of the cell suspension to each well. Incubate the plates.

5. After 10-14 days aspirate the supernatant and wash the cells with PBS and stain with 0.5% methylene blue in 50% methanol for 20 minutes. (Alternatively count by eye under scope)
6. Score the plates by calculating the percentage of survival of individual colonies or percent confluency. The percentage of survival in the presence of each dilution of G418 is calculated versus the percentage of survival in the absence of G418. The lowest concentration at which all cells die after 10-14 days is the optimal concentration to use for selection.

7.22 Transfection of PC12 cells and isolation of clones

This method was adapted from the lipofectin instructions (BRL) and from a protocol of the R. Kelly laboratory (University of California, San Francisco).

materials

1. pMAMneo
   pMAMneo-LCa
   These vectors were obtained from A. Jackson, who prepared the pMAMneo-LCa from pMAMneo (Clonetech) and cDNA for human LCa.
2. Lipofectin (BRL)
3. 100 mm tissue culture dishes
4. sterile polystyrene tubes
5. sterile yellow tips
6. sterile water
7. DME H21, serum-free
8. G418 (or appropriate selection agent)

method

1. Plate 3 x 106 cells/100 mm petri dish and allow to attach overnight.

# plates	contents
3	vector + lipofectin
3	LCa-vector + lipofectin
1	lipofectin alone

2. Prepare DNA-Lipofectin solution (add DNA to water first, then add Lipofectin; do these steps in polystyrene tubes, not polypropylene!):

DNA	water to final vol of	Lipofect soln (1 mg/ml)
15 ug vector	150 µl	150 µl
15 ug LCa-vector	150 µl	150 µl
	50 µl	50 µl

   Mix gently. Do not vortex. Let stand 15 minutes at room temperature.
3. Wash the cells twice with warm DME H21 (serum free). Add 4 mls warm DME H21 (serum free).
4. While gently swirling the plate, add dropwise 100 µl of DNA-Lipofection sol'n as uniformly as possible.
5. Incubate at 37C 10% CO_2.

6. 20 hours after lipofection, add HS and FCS to normal growth concentrations of 10% HS, 5% FCS (this length of time may be necessary for sufficient transfection).
7. Begin selection with G418 in 72 hours (0.5 mg/ml was found to be optimal concentration for PC12 cells)
   (Make a 10X solution of G418 in DME H21 + serum, sterile filter and freeze at -20C, use within 2 months)
8. Replace medium every 4-5 days.
9. When all cells are dead in control (no DNA) plate, reduce the G418 concentration to 0.25 mg/ml. This usually takes 10-14 days.
10. When colonies can be seen by the naked eye on the plate, they are ready to be picked.
11. Select 10-20 well-separated colonies to be picked and circle with marking pen on bottom of plate.
12. Remove medium
13. Wet sterile toothpick in medium (flat end) and gently scrape a cell colony.
14. Twirl toothpick in medium in a well of a 96-well plate.
15. Repeat steps 14-15 until all colonies are picked.
16. Let cells grow until dense in 96-well plate.
17. Transfer to 24-well plate, then when dense to a 6-well plate, and then to a small flask.

7.23 Iodination of Proteins

materials

1. PBS, pH 7.4 + 0.02% NaN_3
2. Prepacked Biorad econo-pac 10DG column
3. 0.3 M phosphate buffer, pH 7.4
4. 0.5% bovine serum albumin in PBS
5. Iodobead (Pierce Chemicals #28666)
6. Saturated L-tyrosine dissolved in 0.3M phosphate buffer pH 7.4
7. 25 µg of protein to be iodinated at > 0.5 mg/ml
8. 1 mCi ^{125}I-NaI (Amersham IMS.30)

method

1. Wash column with bed volume of PBS/BSA
2. Prepare protein for iodination (25 µg purified protein brought up to 50 µl with PBS. Then add 50 µl 0.3 M phosphate buffer pH 7.4.)
3. Iodination
   In labeling room, working in the fume hood--add 1 iodobead to protein in eppendorf 1.5 ml tube. Then add 1 mCi ^{125}I. Let react 5 minutes, mixing occasionally. Then add saturated 25 µl L-tyrosine to quench reaction for 5 min, with occasional mixing.
4. Separate iodinated protein and iodine
   Load onto the column and collect 1 ml fractions. Count 1 µl of each fraction to determine which fractions contain the iodinated protein (should be in fractions 3-4, sometimes 5)

7.24 Norepinephrine Secretion Assay

Adapted from Lowe et al., (1988).

materials

1. ^{3}H-Norepinephrine (Amersham)-10 μCi/plate
2. poly-D-lysine
3. DME H21 + 0.1 mg/ml pargyline (made fresh)
4. 10 cm tissue culture dishes
5. DME H21, serum-free
6. secretagogue (55 mM KCl in medium, no serum)

method

1. Treat 10 cm dishes with poly-lysine.
2. Grow PC12 cells to confluence on 10 cm poly-lysine treated dishes at least 4 days.
3. Load cells with ^{3}H-norephinephrine as follows:
   a. incubate cells at 37°C, 1-2 hr, with 10 μCi ^{3}H-NE per dish in medium containing 0.1 mg/ml pargyline.
   b. Wash plates five times with 4 ml fresh medium without serum.
4. Stimulate to secrete:
   a. immediately prior to incubating cells with medium + secretagogue, leave cells in 5 ml medium (no serum) for two minutes, remove and save as 0' time point.
   b. Incubate cells with warm medium containing appropriate factor(s).
   c. At intervals of 2 minutes remove medium (save) and replace with new medium.
   d. After removal of medium for fifth time point (8'), trypsinize cells off of plates with 1 ml trypsin. Add 4 ml NP40 lysis buffer (or use 5 ml 1M NaOH to strip cells off plate) This fraction contains unsecreted counts.
5. Count fractions

(note: it appears to be vital that the cells have been plated down for 4-5 days and are dense)

7.25 Sulfate labeling for secretion studies

This method was adapted from Tooze and Huttner (1990) and from Grimes and Kelly (submitted) with helpful advice from M. Grimes (University of California, San Francisco).

materials

1. PC12 cells confluent (4-5 days) on poly-D-lysine coated 6-well plates
2. ^{35}S-SO_4
3. SO_4-free DME H21
4. 5X DME H21
5. Chase medium: DME H21 medium + 5% FCS + 10% HS
6. Screw-cap eppendorfs
7. Stimulation medium: 55 mM KCl in serum-free medium
8. Lysis buffer
9. 1 mg/ml deoxycholate in 100% TCA (kept at 4°C)
10. Acetone at -20°C
11. Gel solutions: stack- 4.5% acrylamide + 0.3% Bis
    lower- 8% acrylamide + 0.4% Bis

    to make lower: 1.6 ml 30% acrylamide-8% bis
    + 6.4 ml 30% acrylamide-3% bis
    = 8.0 ml 30% acrylamide-4% bis
    add 7.5 ml lower tris buffer
    add 14.5 ml water
    = 30 ml solution of 8% acrylamide-4% bisacrylamide
11. 6M Urea

method

1. Check PC12 cells grown on poly-D-lysine 4-5 days to see that they are confluent
2. Remove medium and rinse in PBS once.
3. Add medium containing 0.5 mCi/ml ^{35}S-SO_4 to each well (1ml/well):
   1.24 ml ^{35}S-SO4
   2.76 ml DME H21 no sulfate
   0.18 ml 5X DME H21 + 5X Hepes
   4.18 ml total

   note: it had to be done this way because the ^{35}S-SO_4 was so dilute

4. Incubate 6 hrs, 37°C.
5. Remove medium. Rinse each well 2X in 2ml warm PBS.
6. Add chase medium (1ml/well). Incubate 15-18 hours, 37°C.
7. Add 1 ml stimulation medium. Wait 5 min, 37°C.
8. Collect medium and save in 1.5 ml tube.
9. Lyse cells left on plate in 500µl of lysis buffer, spin out nuclei, transfer to clean tube.
10. TCA precipitate the medium samples by adding 100µl TCA solution per 1ml sample. The medium samples should turn yellow, possibly turbid. Incubate TCA ppt on ice for one hour. Spin out TCA ppt in eppendorf centrifuge (you may not be able to see the ppt), then wash pellets 1X in -20°C acetone. Spin again. Let acetone evap. Add 50µl 6M urea, incubate 40°C, 5 min. Add 50µl 2X RSB. Incubate 5 min, 40°C.
11. For cell lysates, add 4X sample buffer, boil, load directly on gel. Do not TCA ppt since it is too difficult to redissolve the protein-TCA pellets.
11. Analyze on 8% acrylamide + 0.4% Bis gel (see materials). Stain/destain (you may be able to see secreted proteins). Add Enlightening 20 min, dry on gel dryer. Expose 2-5 days.

7.26 Transferrin endocytosis in PC12 cells

Adapted from Hopkins and Trowbridge (1983).

materials

1. poly-D-lysine coated 6-well tissue culture plates
2. DME H21 serum-free
3. PBS-4°C
4. Binding buffer:
5. refrig unit at 16°C
6. ^{125}I-Transferrin (rat)

method

1. Plate PC12 cells on poly-D-lysine coated 6-well plates. Incubate 2 days.
2. Remove medium. Preincubate cells in binding buffer, 4 hr, 37°C.
3. Rinse 3X in PBS (4°C).
4. Add 1 ml binding buffer containing ^{125}I-Transferrin (0.5 x 10^6 cpm/ml) at 4°C. Incubate at 4°C for 1.5 hours with periodic agitation.
5. Rinse zero time point 3X in PBS (4°C). Digest in 1 ml 1M NaOH.
6. Put rest of plates at 16°C. At 30',45', 60', 90', 120' remove plate and rinse 3X in PBS (4°C). Digest each well in 1 ml 1M NaOH. Count in liquid scintillation counter.

7.27 Plasmid midi-prep

This protocol was obtained from L. Chen (University of California, San Francisco).

(all items should be sterile)

solutions

LB medium (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, pH 7.0)
50 mM $CaCl_2$
500 X ampicillin (stock 25 mg/ml)
agar LB plates with antibiotic
glycerol (100%)
Solution I (50 mM Glucose, 25 mM Tris, 10 mM EDTA pH 8.0)
Solution II (0.2 N NaOH, 1% SDS)
Solution III (3 M KOAc pH 4.8)
isopropanol (@ -20°C)
TE buffer pH 7.5
5 M ammonium acetate
ethanol (100%, @ -20°C)
ethanol (70%)
RNAse (boiled, 10 mg/ml in 10 mM Tris pH 7.5, 10 mM NaCl)
buffered phenol (see protocol)
chloroform/isoamyl alcohol (24:1)
butanol
3M NaOAc

materials

E. coli
corex centrif. tubes (SS34 rotor)
vector
incubator set at 37°C
15 ml tubes
pipetman tips (blue and yellow)
eppendorf tubes (1.5 ml)

method

1. a. Take stab of E. coli glycerol stock (or 50 µl)
   b. Grow up in 5 ml LB medium overnight in 15 ml tube.

2. a. Take 1 ml E.coli grown overnight and put in 50 ml LB medium.
   b. Start checking O.D. after 1 hr at 600 nm (want O.D. between 0.3 and 0.5)

3. Spin cells at 2500 x g (3600 rpm in RT6000) for 5 min at r.t.

4. a. Gently resuspend pellet of cells in 2-4 ml of 50 mM $CaCl_2$.

b. Bring up to one-half of original volume (e.g. 25 ml) with 50 mM $CaCl_2$. Cells are now very fragile.

5. Incubate for 30 min on ice. Cells are now called competent.

6. a. Centrifuge cells for 5 min at 2500 x g at 4°C.
   b. Resuspend in one-tenth of the original volume (5 ml) of ice cold 50 mM $CaCl_2$. For increased competence, cells can be stored overnight at 4°C before use. Cell viability will decrease after 2 days.
7. a. In a sterile tube, mix about 0.1 µg of plasmid vector DNA or ligated plasmid vector with 200 µl of competent cells from step 6.
   b. Incubate on ice for 30 min.

8. Heat shock cells by transferring tube to a 42°C water bath for 2 min. Alternatively, a 37°C bath for 5 min can be used.

9. Add 1 ml of LB medium and grow for 45 min at 37°C with shaking or in a rotary drum, to allow expression of the antibiotic resistance gene.

10. a. Use bent-end glass rod (sterilized with 70% ethanol and flaming) to spread cells over surface of LB/agar/antibiotic plates. Spread several different amounts (e.g. 50, 100, and 200 µl) on separate plates to determine empirically the amount needed to generate adequate numbers of well-separated colonies.
    b. Store remaining cells at 4°C for further plating if necessary.
    c. Add 100 µl of untransformed bacterial cells from step 8 to another plate (with antibiotic) as a negative control.
    d. Place plates in a 37°C incubator, agar side down, for 20 min to allow medium to dry.
    e. Invert plates (agar on top) and grow overnight at 37°C. Colonies will become apparent at 12-16 hours.

11. Colonies are now ready for colony hybridization, mini-prep DNA analysis of plasmids, preparative plasmid growth or storage.

For storage at this stage:

A. Remove single colonies of cells from plate with sterile transfer loop and add to individual tubes containing 2 ml of LB medium supplemented with the appropriate antibiotic. Grow, with shaking, until culture becomes cloudy.

B. Take a 0.8 ml aliquot of cells from each tube and place in small sterile tubes. Add 0.2 ml of sterile glycerol to each tube. Mix. Freeze cells at -70°C. The bacterial transformants may be recovered by streaking the glycerol stock onto an appropriate LB agar-antibiotic plate to recover colonies.

12. a. Remove single colonies of cells from plate with sterile transfer loop and add to individual tubes containing 5 ml of LB medium supplemented with the appropriate antibiotic.
    b. Grow, with shaking, 5 hours or until culture becomes cloudy.

13. a. Add this starter culture to 100 mls LB medium (including antibiotic if appropriate).
    b. Grow overnight at 37°C until 'overgrown state'.

14. a. Pellet cultures in sterile 30 ml corex centrifuge tubes in SS-34 rotor for 5 minutes at 6,500 rpm (5096 x g).
    b. Decant, bleach, and discard supernatant.

15. a. Resuspend the cell pellets in 5 mls of Solution I.
    b. Incubate the suspension for 5 min at r.t.

16. a. Add 10 mls of Solution II and mix gently by inverting the tube.
    b. Incubate the preparation on ice 5 minutes (less than 10 minutes!!!!)

17. a. Add 10 mls of ice cold Solution III and mix gently by inverting the tube.
    b. Incubate the preparation on ice 15 minutes.

18. a. Centrifuge the preparation for 15 minutes in an SS-34 rotor at 6500 rpm (5096).
    b. Transfer the supernatant to a fresh tube by filtering through cheesecloth if necessary (optional step- may lose DNA in this step)

19. a. Precipitate the DNA by adding an equal volume of isopropanol.
    b. Incubate the preparation at ambient temperature for 20 min.
    c. Centrifuge the preparation in an SS-34 rotor for 15 min at 6,500 rpm.

d. Decant and discard the supernatant.

20. a. Resuspend the pellet in 8 mls of TE.
    b. Add 4mls of 7.5 M ammonium acetate.
    c. Incubate on ice 20 min.
    d. Pellet protein by centrifuging 15 min at 6500 rpm in SS-34 rotor

21. a. Transfer the supernatant to a fresh tube.
    b. Add two volumes of -20°C ethanol.
    c. Incubate on ice 20 min
    d. Pellet the plasmid DNA by centrifuging 15 min at 6500 rpm in SS-34 rotor.
    e. Decant and discard the supernatant.

22. a. Resuspend the pellet in 5 mls of TE containing 75 µg of boiled RNAse
    b. Incubate the preparation at r.t. for 30 min.

23. Phenol/chloroform extract the solution of DNA as follows:
    a. Add 5 mls of buffered phenol (see protocol below for preparation) and vortex to homogeneity. Save aq. phase (top). Discard rest.
    b. Add 5 mls of chloroform:isoamyl alcohol (24:1) and vortex to homogeneity.
    c. Centrifuge the extraction to speed the separation of phases and pack debris at the interface of phases.
    d. Put the upper aqueous layer in a fresh tube.
    e. Repeat steps a-d until the interface is clean.

24. Reduce the volume of the aqueous phase by extracting with butanol as follows:
    a. Add an equal volume of butanol and vortex to homogeneity.
    b. Centrifuge in a table-top clinical centrifuge at maximum speed for 5 min.
    c. Aspirate and discard the upper, saturated butanol layer.
    d. Repeat steps a-c to a final volume of 300 µl in a 1.5 ml eppendorf tube.

25. Chloroform extract the solution of DNA as in step 27 above except centrifuge 30 sec.

26. Ethanol precipitate the DNA as follows:
    a. Add 30 µl of 3 M NaOAc (0.1 volumes)
    b. Add 670 µls of -20 100% ethanol (2.5 volumes)

c. Mix and incubate the preparation on ice 10 min.
d. Microfuge 30 min at max. speed.
e. Aspirate and discard the supernatant.
f. Rinse the pellet with 100 µls of -20°C 70% ethanol.

27. Dry the DNA in a speed vac.

28. Resuspend the resulting pellet in 500 µl TE.

29. Analyze a 2 µl sample on a 1% agarose gel (TAE).

notes:

a. Agar plates with stock bacterial cells can be stored at 4°C for about 1 month. Seal edges of dish with parafilm.
b. Alternatively, frozen competent cells (e.g. HB101) can be purchased from either BRL or Vector Cloning Systems. See suppliers' protocol for methods to be used with these cells.
c. Follow sterile procedure for all steps in this method. All transfers must be kept sterile. Autoclave all tubes before using.
d. It is very important that cells grow logarithmically to achieve optimal transfection efficiency.

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Appendix 1: 100-kDa polypeptides in peripheral clathrin-coated vesicles are required for receptor-mediated endocytosis

Introduction

This appendix consists of an article published in the Proceedings of the National Academy of Sciences, USA. This study describes the effects of anti-adaptor antibodies injected *in vivo* on receptor-mediated endocytosis. The contribution of S. Acton to this paper included characterization of the antibody by immunofluorescence, immunoprecipitation (not shown) and by immunoblotting.

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100-kDa polypeptides in peripheral clathrin-coated vesicles are required for receptor-mediated endocytosis

(adaptors/assembly polypeptides/transferrin/liposomes)

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ABSTRACT **The role of the 100-kDa polypeptide components of clathrin-coated vesicles in endocytosis was investigated by microinjection of specific monoclonal antibodies. Receptor-mediated uptake of transferrin and liposomes was quantitatively inhibited. These results show that the 100-kDa polypeptides are directly involved in localized clathrin assembly at the cell periphery and are markers for the endocytic pathway. This demonstrates an *in situ* function of these polypeptides and the protein complexes in which they are found.**

Clathrin-coated vesicles are involved in endocytosis and the intracellular membrane trafficking of receptors (1). It has been proposed that, within the cell, localized control of clathrin assembly is provided by protein complexes that have been called adaptors (2–4) or assembly polypeptides (5). These are composed of the 100-, 50-, and 17-kDa polypeptide components of clathrin-coated vesicles (5–9). In this report, these multisubunit complexes will be referred to as adaptors (4). Adaptors copolymerize with clathrin *in vitro* (5–9), and in a coated vesicle they are situated between the clathrin coat and the membrane (10). Two forms of adaptors have been identified (5–9); one is associated with clathrin-coated vesicles of the Golgi, and the other is associated with clathrin-coated vesicles at the cell periphery (2, 3, 9). Both adaptors have been shown to bind purified cytoplasmic tails of cell surface receptors (11). Taken together, these data suggest that the adaptors perform multiple functions, including recognizing receptor tails, facilitating clathrin polymerization, and selecting the site of clathrin polymerization. Therefore, the peripheral adaptors should be essential to endocytic function and provide markers for the endocytic pathway. The experiments described below were designed to test this hypothesis.

MATERIALS AND METHODS

Protein and Antibody Preparation. Clathrin was prepared by the method of Pearse and Robinson (6). Peripheral adaptors were prepared by chromatography on HA-Ultrogel (Pharmacia) (7). Detection of AP.6 binding after sodium dodecyl sulfate/polyacrylamide (9%) gel electrophoresis (12) and Western blotting was by horseradish peroxidase coupled to goat anti-mouse IgG (Bio-Rad) by using 3′,3′-diaminobenzidine tetrahydrochloride (Sigma) as a substrate (13). The AP.6 and AP.7 monoclonal antibodies (mAbs) were produced from hybridomas derived from BALB/c mice immunized with adaptor proteins, which were stripped from purified clathrin-coated vesicles and eluted from a Sepharose CL-4B column (Pharmacia) (5). Immunization, fusion procedures, and subsequent antibody purification were identical to those used to produce X19, an anti-clathrin mAb, described previously (14). The 29B5 mAb, which is specific for dinitrophenol, was a gift of L. Herzenberg (Stanford University). Protein was assayed according to Bradford (15) by using bovine serum albumin as a standard.

Microinjection. For transferrin endocytosis experiments, antibodies were concentrated to 20 mg/ml in 50 mM Hepes, pH 7.3/80 mM KCl, and fluorescein conjugated to dextran (1 mg/ml) was added as a coinjection marker (Molecular Probes). Within a 20-min period, each antibody was injected into a line of 50–150 cells on a single, marked coverslip of HeLa cells that was grown 2 days in 5% calf serum in Dulbecco's modified Eagle's medium (Cell Culture Facility, University of California, San Francisco). The coverslip was mounted on a temperature-controlled block (35°C) on a modified inverted microscope with a ×40 phase lens and variable ×2 zoom lens attached to the camera port (Nikon) through which a video camera was used to monitor injections. Injection volumes were controlled by a pneumatic air system (Eppendorf). After postinjection incubations (as described in the figure legends), the cells were washed in serum-free medium and incubated 15 min at 37°C with 30 nM human transferrin labeled with Texas Red (2.6 mol of dye per mol of transferrin) (16). To stop endocytosis, coverslips were immersed in complete medium at 4°C, washed in cold medium at pH 6.2 for 5 min, fixed with 3.7% formaldehyde in phosphate-buffered saline for 20 min, washed with 25 mM glycine in 0.1 M Tris·HCl (pH 7.5), and mounted on a glass slide with 0.1% phenylenediamine (Sigma) in 90% (vol/vol) glycerol/20 mM Tris·HCl, pH 8.5. For liposome endocytosis, antibodies were injected into CV-1 monkey kidney cells with Texas Red-labeled transferrin as a coinjection marker. After postinjection incubations (as specified in the figure legends and text), cells were washed with serum-free Dulbecco's modified Eagle's medium and incubated 15 min with 3.8 μM liposomes (phosphatidylethanolamine/phosphatidylserine/cholesterol in a 2:1:1 molar ratio in 5 mM Hepes·HCl, pH 7.3/150 mM NaCl) containing 35 mM pyranine (Molecular Probes) in serum-free medium. The cells were then washed and returned to complete medium for 90 min at 35°C prior to video-enhanced microscopy (VEM) analysis.

We calculated the number of pyranine molecules per liposome on the basis of the liposome diameter (0.1 μm), the concentration of free pyranine in the encapsulation buffer, and a bilayer thickness of 40 Å (with 70 Å^2 per headgroup). We estimated that ≈3 μl of buffer was encapsulated in the 7 × 10^{12} vesicles formed per μmol of lipid; thus each vesicle contained 10^4 pyranine molecules.

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Abbreviations: mAb, monoclonal antibody; VEM, video-enhanced microscopy.

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Microscopy. For transferrin endocytosis, the coinjection marker was visualized with standard fluorescein optics with the addition of a dual bandpass barrier filter optimized for both fluorescein and Texas Red emission (528 ± 11 nm and 620 ± 15 nm, Omega Optical, Brattleboro, VT). Endocytosed transferrin was visualized with a 580-nm dichroic mirror with a rhodamine excitation filter (Nikon) and the dual fluorescein/Texas Red emission filter. The pyranine-loaded liposomes were viewed with a 400- to 455-nm dichroic mirror with either a 400 ± 40 nm or 450 ± 25 nm bandpass excitation filter (Corion, Holliston, MA) and a 520- to 550-nm bandpass emission filter. Eleven video frames of each image were averaged, and the background was subtracted to correct shading (17). To analyze the endocytic uptake, injected cells were located by the diffuse cytoplasmic coinjection marker and separate, averaged images of both the injection marker and ligand were collected (*i*) in real time and stored on a hard disk and (*ii*) from videotape by a second observer (F.M.B.). Images were collected from a silicon-intensified target tube camera (Dage MTI 65 SIT, Dage, Michigan City, IN) by a Nuvision image analysis system (Perceptics, Knoxville, TN). Cells were illuminated with mercury epi-illumination through a ×60 PlanApo 1.4 numerical aperture lens (Nikon). Most images were collected with the excitation beam attenuated through a neutral density 1.5 filter (Oriel, Stratford, CN).

Image Analysis. A series of neutral density filters was used to establish the linear range of the camera output by using a standard solution of Texas Red-labeled transferrin or pyranine. Several neutral density filters were also used to determine that the ligand uptake in noninjected cells was within this range. Thus, relative cellular fluorescence from liposome uptake was quantified, where a unit of fluorescence represents the pixel intensity value derived from the 8-bit defined range of 0–255 (the linear range was usually between 42 and 194, slope = 2.5). However, the intensity of transferrin uptake was at the lower end of the linear range of camera output; hence, quantification of total cellular transferrin fluorescence was less reliable. For these reasons, all images were contrast-enhanced by a local contrast enhancement algorithm (18) and linear look-up tables. The images were displayed as 3-dimensional plots to yield a semiquantitative presentation of the data. Due to the lower signal intensity of the transferrin uptake, the coinjection and endocytic marker images were not superimposed, as the liposome images were, but were presented as separate monochrome images that were displayed on a monitor and photographed.

RESULTS AND DISCUSSION

Direct cytoplasmic injection of mAbs provides an approach to study the role of proteins associated with clathrin-coated vesicles (19, 20). To examine the endocytic role of adaptors associated with peripheral clathrin-coated vesicles, adaptor-specific mAbs were produced and characterized. In radioimmunoassays, mAbs AP.6 and AP.7 bound to purified peripheral adaptors designated HA-II (5) or AP-2 (5) by other investigators (data not shown). In immunofluorescence studies, both mAbs stained vesicles in the peripheral region of human fibroblasts (1), HeLa cells, and monkey (CV-1) cells. No prominent staining of the Golgi region was observed (1) and, therefore, we conclude that AP.6 is specific for a component of the peripheral adaptors. Immunoblotting showed that AP.6 reacted with α (a and c) 100-kDa polypeptides (6, 9) in the peripheral adaptors purified from bovine brain coated vesicles and not with the β 100-kDa or 50-kDa components (Fig. 1). The subunit specificity of AP.7 within the peripheral adaptors is unknown since it does not react in immunoblots.

To investigate the effects of these antibodies on endocytosis, we introduced high concentrations of antibody into the cytoplasm of individual cells by needle injection. A previous study suggested that a molar equivalence or excess of mAbs compared to binding sites was required to induce effects on clathrin-mediated processes (19). The number of peripheral adaptors per cell is likely to be similar to the estimated 10^6 clathrin molecules per cell (19). Therefore, a 3- to 30-fold molar excess can be introduced by injecting 20–200 fl of a 20 mg/ml mAb solution. To measure the effects of injected mAbs, the uptake of fluorescent transferrin was studied by VEM. Fluorescent human diferric transferrin was incubated with HeLa cells after injection of mAbs AP.6, X19, and 29B5 (Fig. 2). X19 and 29B5 are mAbs specific for the clathrin heavy chain (14) and dinitrophenol, respectively. Punctate fluorescence (as indicated by spikes in Fig. 2 *B*, *D*, and *F*) in noninjected cells and cells injected with the irrelevant mAb 29B5 (Fig. 2*F*) represents transferrin in coated pits or early endosomes. Inhibition of transferrin uptake, as indicated by a reduced number and intensity of spikes, was observed in most cells injected with AP.6 (Fig. 2*B*). Inhibition was more complete in cells injected with X19 (Fig. 2*D*).

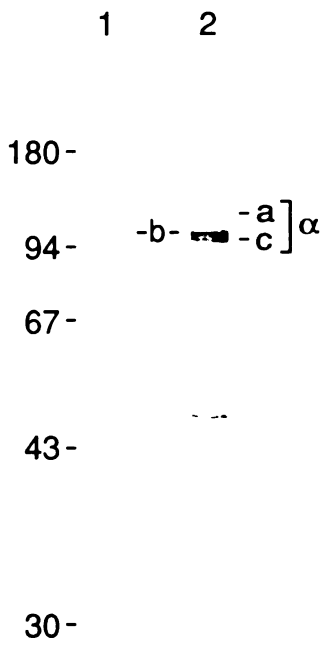
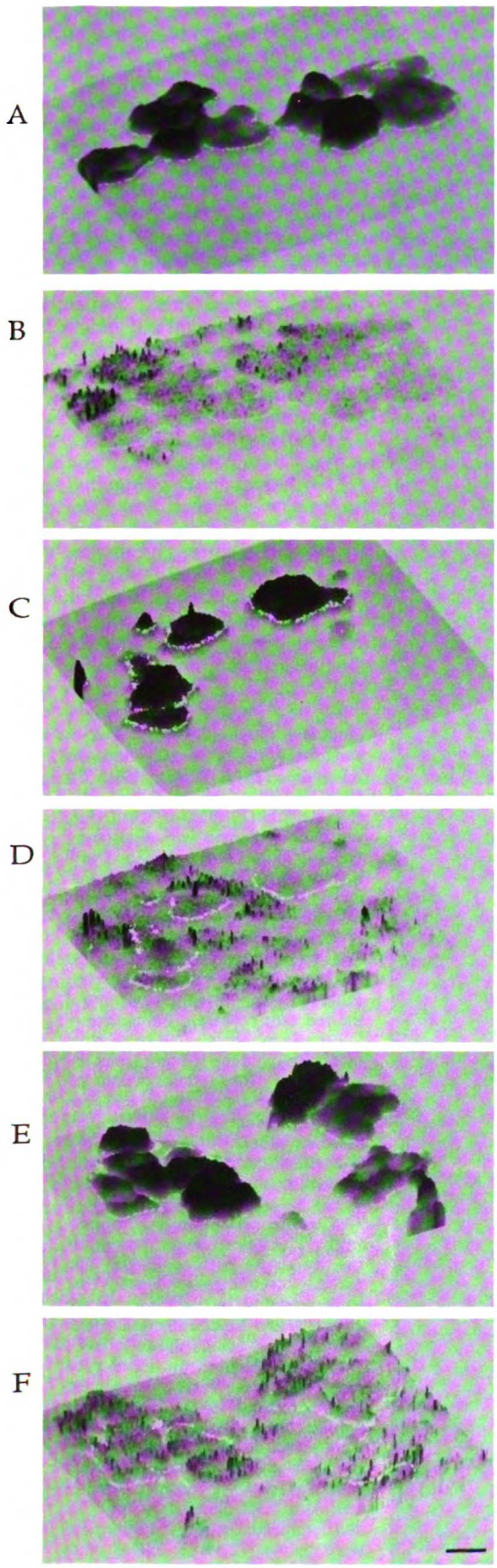


FIG. 1. Specificity of AP.6 and AP.7 for the adaptors associated with peripheral clathrin-coated vesicles. Lane 1, immunoblot of AP.6 binding to purified peripheral adaptors. Lane 2, corresponding adaptor subunits are identified with amido black. The α (a and c) 100-kDa polypeptides are indicated at the right. The b of β 100-kDa polypeptide is indicated in the middle, and the 50-kDa polypeptide is clearly resolved. The migration of molecular mass markers is shown at the left (in kDa). In lane 1, AP.6-staining of the c polypeptide is prominent and the additional weak staining of the a polypeptide has been observed in four independent experiments.

The extent of inhibition of endocytosis was variable in injected cells (Fig. 3). Transferrin uptake was abolished in 49% of AP.6-injected HeLa cells, partially inhibited in 28%, and unaltered in 23%. In contrast, inhibition of endocytosis in X19-injected cells was more homogeneous: 85% of X19-injected cells failed to internalize transferrin. Partially reduced transferrin uptake was found in 18% of noninjected cells or cells injected with the irrelevant mAb 29B5.

To establish the generality of this inhibition by injected mAbs, we examined their effects upon the endocytosis of liposomes. In addition, this allowed us to study the effects of injected antibodies upon accumulation of ligand into late endosomes or lysosomes. We studied the uptake of liposomes loaded with a high concentration of the pH-sensitive dye pyranine (21). Negatively charged liposomes can bind to



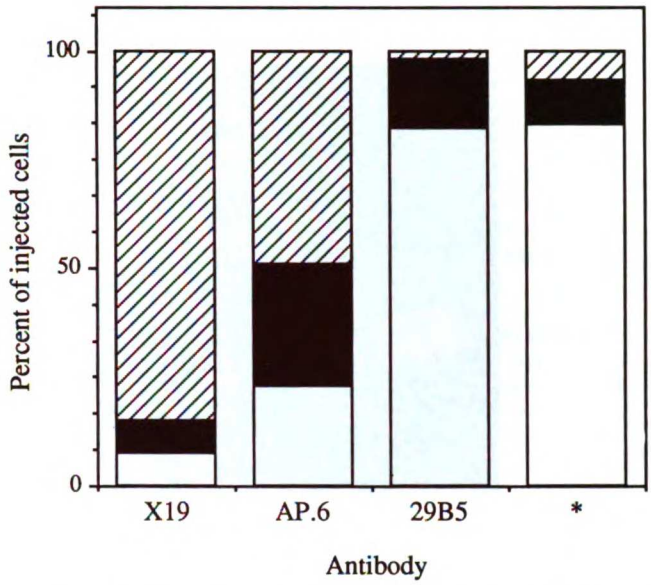


FIG. 3. Inhibition of endocytosis of Texas Red-labeled human transferrin by HeLa cells. Cells were injected with antibodies and incubated with Texas Red-labeled transferrin as in Fig. 2. VEM was used to analyze images. The percent of injected cells showing each type of inhibition is shown in the bar graph. □, Cells in which uptake of transferrin was not affected; ▨, cells that lacked internalized transferrin; ■, cells with reduced transferrin uptake. Fifty-three, 102, and 58 cells were injected with X19, AP.6, and 29B5, respectively. *, Noninjected cells (n = 140) in the same fields. The data shown are representative of three independent experiments.

protease-sensitive site(s) on cell surfaces (R.M.S., unpublished results), are endocytosed by means of coated pits (22), and encounter increasingly acidic compartments. Total cell binding of pyranine-loaded liposomes can be detected by excitation of fluorescence at the pH-insensitive isosbestic point of pyranine, 410 nm. In addition, pyranine enables detection of acidic endocytic vesicle by excitation at 450 nm, a wavelength at which pyranine fluorescence decreases with acidic pH (23).

After CV-1 cells were injected with antibodies, they were incubated for various times prior to a 15-min exposure to pyranine-loaded liposomes (Fig. 4). The live cells were then washed, incubated 90 min at 35°C, and analyzed by VEM. In noninjected cells and in cells injected with 29B5, liposome-encapsulated dye was localized to perinuclear vesicles (Fig. 4*C*). In cells injected with AP.6, the accumulation of pyranine-labeled vesicles often was reduced drastically (Fig. 4*A*). Similar results were found with the AP.7 mAb (data not shown). Whereas noninjected cells showed a clustered perinuclear distribution of vesicles, AP.6-injected cells with a partial internalization block often had vesicles that were more evenly distributed in the peripheral cytoplasm (Fig. 4*D*).

FIG. 2. Effect of microinjected antibodies on endocytosis of Texas Red-labeled transferrin (16). HeLa cells were injected with either AP.6 (*A* and *B*), X19 (*C* and *D*), or 29B5 (*E* and *F*) mixed with fluorescein conjugated to dextran (0.5 mg/ml) as a marker of the volume injected into cells. After injection, cells were incubated for 50 min (AP.6), 30 min (X19), or 10 min (29B5) prior to a 15-min incubation with Texas Red-labeled transferrin. A 3-dimensional plot shows cell-associated transferrin as dark spikes in *B*, *D*, and *F* (the negative of each image was printed). The coinjection marker (*A*, *C*, and *E*) is shown for the same field of cells. To aid image alignment of injected cells with their transferrin uptake, a white outline of the injected cells was applied to paired images. Note that noninjected cells in *B*, *D*, and *F* (revealed by their transferrin uptake) are not visible in *A*, *C*, and *E*, which show only the coinjection marker. (Bar = 20 μm.)

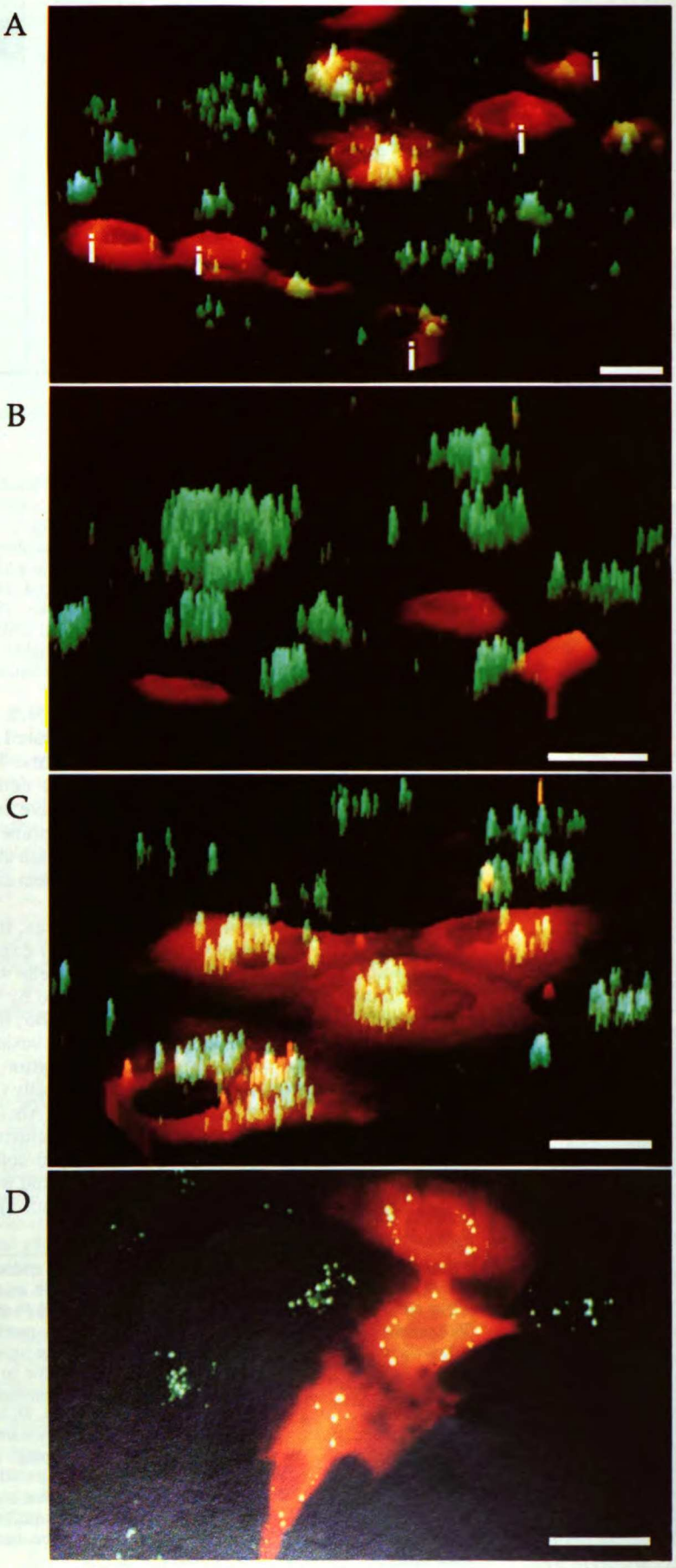


FIG. 4. Effect of microinjected antibodies on endocytosis of liposomes. CV-1 cells were injected with AP.6 (*A* and *D*), X19 (*B*), or 29B5 (*C*). Cells were incubated for 45 min (X19), 65 min (29B5), or 3 hr (AP.6) prior to incubation with liposomes. Ninety minutes after incubation with liposomes, injected cells were analyzed by VEM. The image of injected cells (red) was overlaid with the image of pyranine-loaded liposomes (green). The original images were visualized at 610 nm and 400 nm, respectively. Fluorescent green spikes outside of the red cells represent the uptake of liposomes by noninjected cells. Note that overlaying the red and green images causes the vesicles within injected cells to appear yellow and does not reflect acidity. The relative fluorescence intensity of liposome uptake of the noninjected and injected cells (shown by the white "i") in *A* was 91,988 ± 31,665 (n = 9) and 25,471 ± 33,576 units per cell (n = 5), respectively. The latter number represents the average of both completely and partially blocked cells. (*D*) Cells that were partially blocked for uptake shown in two dimensions rather than the three dimensions shown in *A–C*. This is to illustrate the more even distribution of vesicles in injected cells compared to the surrounding noninjected cells. In *D*, cells that were scored as partially inhibited on the basis of even vesicle distribution had an average fluorescence value of 100,131 ± 5,551 units per cell (n = 3), and noninjected cells in the same field were similar [72,397 ± 25,745 units per cell (n = 5)]. (Bar = 20 μm.)

Analysis of noninjected cells compared to AP.6-injected cells at both 400 nm and 450 nm indicated that clustered perinuclear vesicles were more acidic than those in the peripheral cytoplasm (data not shown). These results suggest an antibody-induced delay of liposome uptake. Fig. 4*B* shows inhibition of liposome uptake in X19-injected cells. Similar results were seen with X19 Fab fragments, but inhibition was less complete, which is expected for lower affinity fragments (19). For cells injected with 29B5 (Fig. 4*C*), 6–10% had reduced liposome internalization, similar to the percentage of noninjected cells with reduced transferrin uptake.

The kinetics of inhibition by AP.6 were examined by incubating injected cells for increasing times prior to liposome incubation. The percentage of cells showing complete or partial inhibition of liposome uptake increased linearly and reached a plateau after a 3-hr postinjection incubation period. At this time, uptake in 40% of the injected cells was completely inhibited. Thirty-five percent of the injected cells showed a partial block that was characterized by a peripheral distribution of internalized liposomes versus the clustered perinuclear pattern in noninjected cells. In contrast, the onset of X19 inhibition was more rapid: 47% of X19-injected cells failed to endocytose liposomes when assayed 30 min after injection. Note that inhibition of transferrin uptake was observed with shorter postinjection incubation periods (50 min for AP.6 and 10 min for X19; Figs. 2 and 3).

The inhibition of endocytosis by AP.6 and X19 was heterogeneous in the total population of injected cells. In cells with a block in endocytosis, inhibition was usually dependent upon the concentration of injected antibody as indicated by injection volume. However, some cells with comparable injection volumes were inhibited while others were not (Fig. 4*A*) or were partially blocked (Fig. 4 *A* and *D*). These results suggest that individual cells vary in their ability to bind and/or endocytose ligands. This explains our observation of 100% inhibition of endocytosis in individual cells, whereas earlier experiments only observed partial inhibition of endocytosis in cell cultures (19, 20). Such stochastic behavior has been observed in many other systems and could be a cell cycle effect (24, 25). In our case, variable susceptibility to AP.6 injection may also be due to differential expression or use of 100-kDa polypeptide isotypes that may be present in subpopulations of peripheral adaptors (9). Collectively, these results emphasize an advantage of single-cell microinjections where individual responses are not averaged as they are in mass biochemistry experiments.

Cells injected with either X19 or AP.7 frequently appeared more spherical. Shape changes were not induced in CV-1 cells 3–10 hr after injection with AP.6 or 29B5. Thus, morphological effects are unlikely to be an injection artifact and may result from the effects of specific injected antibodies upon clathrin sheets on the plasma membrane or upon clathrin and adaptor components associated with clathrin-coated adhesion plaques (26, 27).

The use of two different probes for endocytosis revealed a consistent difference in the rate and extent of inhibition by AP.6 and X19 mAbs. These differences suggest distinct mechanisms of action. X19 can prevent clathrin basket assembly and dissassociates clathrin baskets *in vitro* (28). The inhibitory effects of X19 could be exerted both by uncoating formed lattices and by preventing clathrin assembly. Since the dynamics of adaptor association with membranes are unknown, inhibition by AP.6 could be at the level of adaptor association with receptors or nucleation of clathrin assembly. Our data suggest the α 100-kDa proteins are involved in at least one of these functions. These proteins could also localize clathrin assembly to the plasma membrane and thus be required for endocytosis. These results demonstrate that peripheral adaptors participate in the formation of endocytic coated vesicles *in situ* and are distinct markers for the endocytic pathway.

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Appendix 2: Binding characteristics of assembly polypeptide antibodies

The monoclonals AP.1, AP.6, and AP.7 were first characterized in immunofluorescence. The staining patterns of AP.6 (see Figure 1.4) and AP.7 were similar, and indicated that they bind to the AP2 complex in the cell periphery. AP.1 did not stain in immunofluorescence. Further RIA studies by I. Näthke confirmed that all three antibodies bound to the AP2 complex and not the AP1 complex. Western blotting indicated that AP.6 and AP.7 bind to both the 'a' and 'c' forms of alpha adaptin (see Appendix 1). To determine if these three mAbs have different epitopes, two RIA assays (a sandwich assay and an inhibition assay) were performed. In the sandwich assay AP.1, AP.6 and AP.7 were bound to RIA plates which were then blocked with BSA and incubated with the AP2 complex. Iodinated AP.6 was then used to detect available AP.6 binding sites on the AP2 complex (Figure A2.1). Since much higher levels of AP.1 and AP.7 binding was detected than of AP.6, these studies suggested that AP.1 and AP.7 bind to a different site than AP.6. To reduce the risk of the assembly complexes aggregating and causing misinterpretation of the results, an inhibition assay was performed. In this assay AP2 was bound to RIA plates and AP.1, AP.6, and AP.7 were used to inhibit ^{125}I-AP.6 binding (Figure A2.2). As expected, AP.6 inhibited ^{125}I-AP.6 binding but AP.1, AP.7, and 29B5 (negative control) did not. Thus AP.1 and AP.7

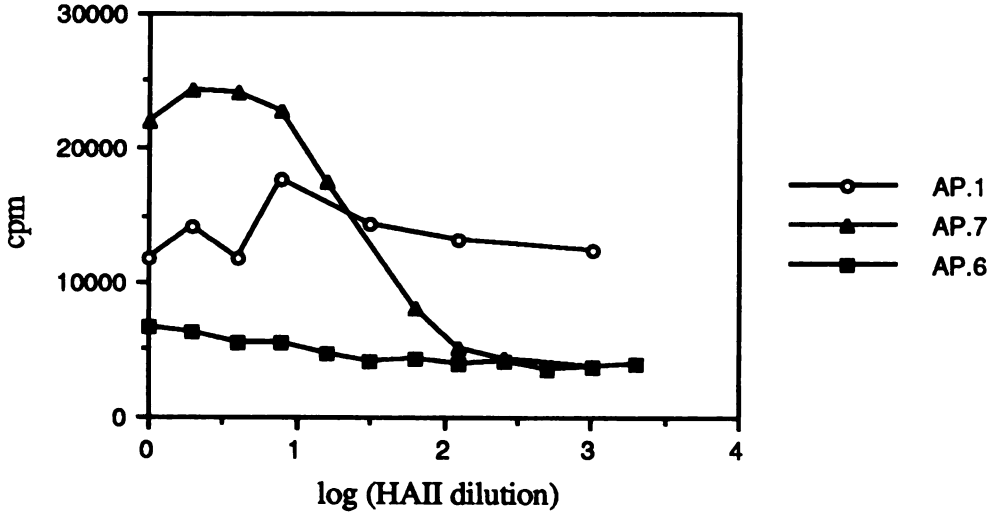

Figure A2.1 Sandwich RIA of anti-adaptin antibodies-AP2 complex- ^{125}I-AP.6

AP.1, AP.6, and AP.7 were plated down separately into RIA plates. After blocking the wells, dilutions of AP2 (HAII) were then incubated in the wells. After washing, ^{125}I-AP.6 was incubated in the wells. The wells were then washed and counted.

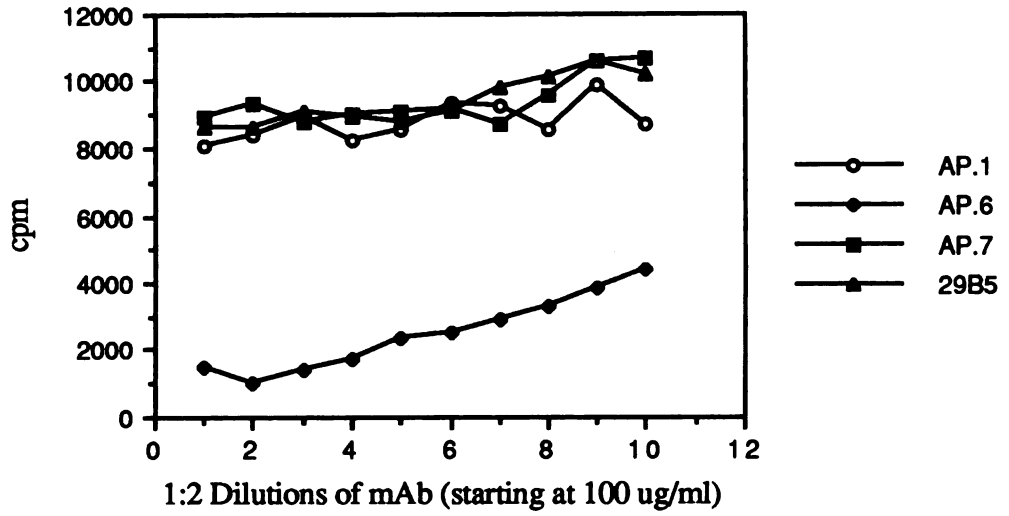

Figure A2.2 Inhibition RIA of ^{125}I-AP.6 binding to AP2 by AP.1, AP.6, AP.7, and 29B5

AP2 was bound to RIA plates, the plates were blocked, and then incubated with AP.1, AP.6, AP.7, and 29B5 in serial dilutions (starting at 100 µg/ml). These antibodies were removed and ^{125}I-AP.6 was added to the wells. The wells were then washed and counted.

bind to a different epitope on the assembly complexes than AP.6. It is also likely that AP.1 and AP.7 do not bind to the same epitope since AP.7 binds in immunofluorescence but AP.1 does not.