Alteration of Clathrin Light Chain Expression by Transfection and Gene Disruption

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The light chain subunits of clathrin, LC_a and LC_b, have been implicated in the regulation of coated vesicle disassembly and other aspects of clathrin cycling within the cell. The potential for functional specialization of each light chain is suggested by tissue-specific variation in the relative amounts of the two light chains and by conservation of differences between LC_a and LC_b sequences during evolution. To investigate whether there might be exclusive roles for LC_a and LC_b in clathrin function, the expression of LC_a was manipulated in C1R lymphoid cells and PC12 pheochromocytoma cells by transfection with light chain cDNA. These two cell lines differ in their ratios of LC_a to LC_b, expressing 86 and 25% LC_a, respectively. After transfection with exogenous human LC_a cDNA, a PC12 cell derivative was produced that completely lost the ability to manufacture LC_a. Loss of LC_a expression was found to be because of gene disruption and consequent lack of mRNA transcription. In C1R cells, the normally high level of LC_a expression was reduced to 25% by overexpression of transfected LC_b cDNA under the control of an inducible promoter. The C1R transfectants with reduced levels of LC_a and the LC_a-negative PC12 transfectant grow normally and show no change in clathrin distribution, clathrin assembly level, or impairment of endocytosis or secretion compared with wild-type cells and cells transfected with vectors lacking light chain cDNA. However, subtle alterations in the hsc70-mediated clathrin uncoating process were observed for vesicles derived from the LC_a-negative cells, reflecting the preferential activity of LC_a in stimulating the *in vitro* uncoating reaction.

INTRODUCTION

Clathrin-coated pits and vesicles internalize selected macromolecules by receptor-mediated endocytosis and transport receptor-bound lysosomal enzymes from the *trans*-Golgi network to a prelysosomal compartment. In cells with a regulated secretory pathway, clathrin-coated structures play a role in the formation of secretory granules and rapid reuptake of membrane after regulated secretion (reviewed in Brodsky, 1988). The clathrin coat, a lattice of hexagons and pentagons, is formed by assembly of triskelion-shaped clathrin molecules composed of three clathrin heavy chains and three light chains. The clathrin heavy chain provides the structural

backbone for the triskelion shape and the interactions necessary for self-polymerization. The clathrin light chains have a regulatory effect on clathrin assembly (Ungewickell and Ungewickell, 1991) and provide the recognition site for coated vesicle disassembly by hsc70 (Schmid *et al.*, 1984). Many structural features of the light chains suggest that they play additional roles in influencing clathrin dynamics within the cell (reviewed in Brodsky *et al.*, 1991).

There are two forms of clathrin light chain in mammalian and avian cells, LC_a and LC_b, whereas there is only one in yeast (Silveira *et al.*, 1990). The light chain sequences suggest evolutionary duplication of an ancestral light chain gene and possible diversification of function for the resulting LC_a and LC_b gene products. The protein sequences of LC_a and LC_b are 60% identical

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(Jackson *et al.*, 1987), and the differences are well conserved between mammalian species with 97% identity between LC_a sequences from rat, human, and cattle and a similar degree of identity between LC_b sequences (Jackson and Parham, 1988). Both light chains bind calcium (Mooibroek *et al.*, 1987; Näthke *et al.*, 1990) and calmodulin (Lisanti *et al.*, 1982), but other biochemical characteristics differ. LC_b can be phosphorylated at a casein kinase II acceptor site near the amino terminus (residues 11 and 13), which is absent from the LC_a sequence (Hill *et al.*, 1988). In vitro, LC_a has been shown to be a better substrate for hsc70 than LC_b, correlating with the presence of an hsc70 ATPase stimulatory sequence (residues 47 to 71) absent from LC_b (DeLuca-Flaherty *et al.*, 1990).

Previous studies have shown that LC_a and LC_b compete for binding to clathrin heavy chains (Winkler and Stanley, 1983; Brodsky *et al.*, 1987). Heavy chain-bound LC_a and LC_b turn over independently, with half-lives of 24 and 45 h, respectively (Acton and Brodsky, 1990), whereas free light chain is degraded with a half-life of less than 30 min (Brodsky, 1985b). This competition between light chains allowed us to manipulate the levels of the heavy chain-associated pool of each type of light chain to investigate the functional role of light chain polymorphism in mammalian cells. In these studies, cells were transfected with exogenous LC_a or LC_b cDNA linked to an inducible promoter so that expression of the transfected light chain could be increased by induction with dexamethasone. This resulted in competition with and degradation of other light chains made by the cell. Because different cell types differ in the ratios of LC_a to LC_b expressed (Acton and Brodsky, 1990), we selected as targets for these transfection experiments a human lymphoblastoid cell line C1R with a high LC_a to LC_b ratio and the rat pheochromocytoma cell line PC12 that has a low LC_a to LC_b ratio. We demonstrate that reduction of LC_a expression in C1R cells and complete loss of LC_a expression by PC12 cells had no measurable effect on clathrin distribution and assembly or endocytosis and secretion compared to wild-type cells. However, a subtle change in the hsc70-mediated uncoating of clathrin-coated vesicles from the LC_a-negative cells was observed.

MATERIALS AND METHODS

Materials

C1R cells, obtained from P. Cresswell (Yale University) (Salter *et al.*, 1987), were grown in RPMI 1640 medium with 10% fetal calf serum (FCS). PC12 cells obtained from R. Kelly (University of California, San Francisco) were grown in Dulbecco's modified Eagle's medium with 4.5 g/l glucose (DME-H21) plus 10% horse serum (HS) and 5% FCS. Tissue culture media were obtained from the University of California, San Francisco Cell Culture Facility. Production and characterization of anti-clathrin heavy chain monoclonal antibody (mAb) X32 (Blank and Brodsky, 1986) and mAb TD.1 against the heavy chain terminal domain (Näthke *et al.*, 1992), anti-LC_a mAb X16 (Brodsky, 1985a) and rabbit anti-light chain antisera to peptides 2

and 1a (DeLuca-Flaherty *et al.*, 1990) were previously described. mAb 29B5 (anti-dinitrophenol) was obtained from L. Herzenberg (Stanford University). Goat anti-rabbit IgG was iodinated using chloramine-T. Human transferrin and streptavidin were iodinated using Iodobeads (Pierce Chemical, Rockford, IL). Rat apotransferrin (Sigma Chemical, St. Louis, MO) was iron-loaded (Karin and Mintz, 1981) and iodinated using Iodobeads.

Transfections and Induction

Full-length cDNA encoding human clathrin light chains lacking neuron-specific sequences (Jackson and Parham, 1988), including the 5' and 3' untranslated regions, were subcloned into the mammalian expression vector pMAMneo (Clontech Laboratories, Palo Alto, CA) with the dexamethasone-inducible mouse mammary tumor virus promoter and the G418 selectable neomycin resistance gene. C1R cells were transfected by electroporation (Chu *et al.*, 1987), and stable transfectants were isolated by selection in 1 mg/ml G418 for 2 wk then cloned by limiting dilution. Positive clones were tested for integrated constructs by Southern blotting using light chain cDNA as hybridization probe (Sambrook *et al.*, 1989). PC12 cells were transfected by Lipofectin (BRL, Bethesda, MD) according to kit instructions, and stable transfectants were isolated by selection in 0.5 mg/ml G418 for 3 wk.

For induction of light chain expression, 100 ml of exponentially growing C1R cells (3×10^5 /ml) were induced by adding 0.1 ml of a 1 mM dexamethasone stock solution in ethanol and then were cultured 48 hr. Identical control cultures received 0.1 ml of ethanol. Induction of light chain expression in PC12 cells used similar conditions.

Northern and Southern Blots

All protocols for RNA purification, electrophoresis, synthesis of strand-specific probes, DNA extraction from cells, restriction digests, transfer, and hybridization were exactly as described in Ausubel *et al.* (1989).

Total RNA for Northern blots was isolated from about 5×10^7 cells. Twenty micrograms of total RNA was separated by denaturing formaldehyde/agarose gel electrophoresis (0.8% agarose gel) and was blotted onto Hybond C (Amersham, Arlington Heights, IL). The filters were baked at 80°C for 2 h, prehybridized 5 h in 6× standard saline citrate (SSC), 5× Denhardt's solution, 100 µg/ml salmon-sperm DNA, 0.1% sodium dodecyl sulfate (SDS), and 50% formamide at 42°C. Bags were emptied, fresh prehybridization solution was added containing 2×10^6 cpm strand-specific LC_a probe ($\sim 10^8$ cpm/µg), and incubation at 42°C continued overnight. Blots were washed 30 min in 6× SSC, 0.1% SDS at room temperature then twice at 62°C in 0.1× SSC for 30 min each. The γ-actin probe was labeled by the random primed method (Feinberg and Vogelstein, 1983) with the use of the Boehringer Mannheim random primer kit (Indianapolis, IN).

High molecular weight DNA for Southern blots was isolated from about 10^7 cells. Twenty micrograms of DNA was digested overnight at 37°C with restriction enzymes EcoRI and Sma I. The DNA was separated on an 0.8% agarose gel and blotted onto Hybond C. The filters were baked at 80°C for 2 h and prehybridized 5 h in 6× SSC, 5× Denhardt's solution, 100 µg/ml salmon sperm DNA, 0.1% SDS, and 50% formamide at 42°C. Bags were emptied, fresh prehybridization solution was added containing 5×10^6 cpm LC_a probe ($\sim 10^9$ cpm/µg), and incubation at 42°C continued overnight. Blots were washed 30 min in 6× SSC, 0.1% SDS, at room temperature then twice at 62°C in 0.1× SSC for 30 min each. The full-length LC_a probe was labelled by the random primed method using the Boehringer Mannheim random primer kit.

Measurement of LC_a/LC_b Ratios in Cells

Cell lysates of induced and uninduced C1R cells were prepared in 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer pH 7.0, 150 mM NaCl, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N-N'-N',N'-tetraacetic acid (EGTA), 0.5 mM MgCl₂, 0.02%

NaN₃, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% Triton X-100 (Fisher, Pittsburgh, PA) and centrifuged at $1\,000 \times g$. The supernatant was boiled for 10 min and centrifuged at $10\,000 \times g$. The heat-stable proteins, which include clathrin light chains, were acetone-precipitated and separated by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). Light-chain ratios in C1R cells were determined by quantitative immunoblotting (Acton and Brodsky, 1990) using an affinity-purified rabbit antiserum against peptide 2, a 22 amino acid sequence identical in LC_a and LC_b (DeLuca-Flaherty *et al.*, 1990), detected with ¹²⁵I-labeled goat anti-rabbit IgG. Autoradiographs of the immunoblots (on preflashed X-ray film) were scanned with a LKB Ultrosan XL (Pharmacia, Piscataway, NJ). Measurement of LC_a/LC_b ratios in PC12 cells was the same as for C1R cells except 0.5% Triton X-100 was used in the cell lysis buffer and proteins were not acetone-precipitated before electrophoresis. Biotinylated goat anti-rabbit IgG (Organon Technika, Durham, NC) and ¹²⁵I-streptavidin were used to detect primary antibody binding to immunoblots.

To determine the ratio of LC_a/LC_b associated with clathrin heavy chain, clathrin was quantitatively immunoprecipitated from cell lysates with preformed immune complexes (Brodsky, 1985b) containing the anti-clathrin heavy chain monoclonal antibody X32 (Blank and Brodsky, 1986) and sheep anti-mouse IgG and then processed for immunoblotting with anti-peptide 2 antiserum.

Measurement of Assembled and Unassembled Clathrin

Cells were homogenized in Buffer D (10 mM HEPES pH 7.2, 150 mM NaCl, 1 mM EGTA, 0.5 mM MgCl₂, 0.02% NaN₃, 0.005% PMSF, 10 μg/ml leupeptin, 0.7 μg/ml pepstatin) and spun at $1\,000 \times g$. Supernatants were then spun at $100\,000 \times g$ for 30 min at 4°C in a Beckman TL100 tabletop ultracentrifuge (Fullerton, CA). Resulting supernatants (unassembled clathrin) were recovered, and pellets (assembled clathrin) were resuspended in Buffer D at the same volume as supernatants. These fractions were then electrophoresed and transferred to nitrocellulose; blots were probed with anti-heavy chain mAb TD.1, followed by biotinylated horse anti-mouse-IgG and ¹²⁵I-streptavidin. Density of bands on exposed film was measured with a Beckman DU64 Spectrophotometer. Linearity of film exposure was tested by titrating purified clathrin heavy chain on the same blots.

Vesicle Uncoating by hsc70

Three 15-cm diameter tissue culture plates each of LC_a-negative PA35 cells and PV6 (PC12 cells transfected with vector without light chain cDNA) were rinsed with phosphate-buffered saline (PBS) pH 7.4. Cells were removed by scraping, resuspended in 900 μl uncoating buffer (40 mM HEPES pH 7.4, 75 mM KCl, 4.5 mM MgCl₂, 1.5 mM CaCl₂), supplemented with protease inhibitors (2 μg/ml aprotinin, 1 μg/ml leupeptin, 0.005% PMSF), and homogenized with five strokes in a Potter-Elvehjem homogenizer. The postnuclear supernatants (generated by spinning homogenate 15 min at $1000 \times g$) were spun for 10 min at $100\,000 \times g$ in a Beckman TL100 tabletop ultracentrifuge to pellet a crude membrane fraction. The pellets were resuspended in 900 μl of uncoating buffer + 0.5% NP-40, incubated on ice for 30 min, and spun 10 min at $100\,000 \times g$ to dissolve uncoated and partially coated membranes and generate a crude clathrin-coated vesicle fraction. Detergent treatment also removes a contaminating ATPase activity found associated with clathrin-coated vesicles (Braell *et al.*, 1984). Pellets were rinsed to remove excess detergent, resuspended in 900 μl uncoating buffer, and left at room temperature for 15 min to remove any loosely associated clathrin. During this incubation, the protein concentration of the crude clathrin-coated vesicle preparation was determined using the Bradford assay with bovine serum albumin (BSA) as the protein standard (United States Biochemical, Cleveland, OH) to normalize for amount of material added to uncoating reactions. Clathrin-coated vesicle fractions were spun 10 min at $100\,000 \times g$, and pellets were resuspended in 900 μl uncoating buffer. Fifty microliters of this material (~100 μg/ml) was used for each uncoating

reaction time point that was initiated by adding 0.25 mM ATP and 25 μg/ml purified bovine brain hsc70 (gift from D. McKay, Stanford University) and allowed to proceed for the indicated time (DeLuca-Flaherty *et al.*, 1990). For the background uncoating controls, ATP was pretreated with 10 U/ml apyrase (Sigma). Each reaction time point was then collected and placed into a prechilled microfuge tube and spun at $100\,000 \times g$ for 10–60 min. Supernatants (clathrin removed by hsc70) were collected and electrophoresed on an 8% SDS-polyacrylamide gel and transferred to nitrocellulose. Blots were probed with mAb TD.1 followed by horse radish peroxidase-conjugated goat-anti mouse IgG and developed with electrochemiluminescence (ECL) substrate (Amersham).

Transferrin Recycling

To measure transferrin uptake and recycling for C1R cells (Hopkins and Trowbridge, 1983), $\sim 2 \times 10^7$ cells were suspended in 10 ml binding buffer (PBS with 10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) [PIPES], 5 mM glucose, 1 mg/ml BSA, pH 7.4). After 1 h at 37°C, cells were centrifuged ($500 \times g$, 10 min) and resuspended in 1 ml of cold binding buffer containing 3×10^7 cpm ¹²⁵I-labeled iron-loaded human transferrin for 90 min at 4°C. After washing cells were resuspended in 1 ml cold binding buffer, and uptake was initiated by adding the cells to 9 ml of binding buffer at 37°C. Every minute 0.5 ml aliquots were removed and added to precooled tubes containing 0.5 ml ice-cold binding buffer. After centrifugation ($500 \times g$, 3 min) supernatants were removed for counting to measure recycled transferrin. Pellets were resuspended in 0.25 ml of acetic acid wash buffer (0.2 M acetic acid, 0.5 M NaCl), left for 5 min, and centrifuged again to measure released surface-bound transferrin. Then cell pellets were dissolved in 0.25 ml of 1 M NaOH and counted to measure internalized transferrin.

For PC12 transferrin internalization experiments in which recycling was inhibited, cells growing on six-well tissue culture plates coated with poly-D-lysine for 1–2 d were preincubated in binding buffer for 4 h at 37°C. Iodinated iron-loaded rat transferrin at 500 000 cpm/ml in binding buffer was prebound for 1.5 h at 4°C. The cells were shifted to 16°C for the indicated time periods and then rapidly placed on ice to stop internalization. After washing in PBS, pH 7.4 at 4°C, the cells were dissolved in 1 ml 1 M NaOH and counted.

For steady state transferrin internalization experiments, PC12 cells growing on six-well tissue culture plates coated with poly-D-lysine for 1–2 d were preincubated in binding buffer for 2 h at 37°C. Iodinated iron-loaded rat transferrin at 500 000 cpm/ml in binding buffer was prebound for 1 h at 4°C. The cells were shifted to 37°C for the indicated time periods and then rapidly placed on ice to stop internalization. After washing in PBS, pH 7.4 at 4°C, the cells were dissolved in 1 ml 1 M NaOH and counted.

Immunofluorescence

Immunofluorescence was performed as previously described (Wong *et al.*, 1990). Cells were grown on Lab-tek chamber slides coated with poly-D-lysine (Nunc, Naperville, IL) in the presence of 100 ng/ml of the β-subunit of nerve growth factor (β-NGF, Boehringer Mannheim). Images were obtained by confocal microscopy on a Bio-Rad MRC 600 confocal microscope system (Richmond, CA), equipped with a 25 mW argon laser and mounted on a Nikon Optiphot with a Nikon CFN Plan Apo 60/1.40 oil lens, WD 0.17 mm (Garden City, NY). Each image is the arithmetic average of 10 frames, and the section thickness was ~1.0 μm. Images were enhanced by maximizing the dynamic range and photographed using TMAX-100 film (Eastman Kodak, Rochester, NY).

Norepinephrine Secretion

PC12 cells were grown 4–5 d on 100-mm tissue culture plates coated with poly-D-lysine, incubated for 2 h with 10 μCi/plate ³H-norepinephrine (Amersham) in DME-H21 medium containing 0.1 mg/ml

Figure 1. PC12 clones transfected with human LC α . PC12 cells were transfected with cDNA for human LC α in the pMAMneo vector and individual clones isolated. Postnuclear supernatants of transfected cell lysates were electrophoresed by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-clathrin light chain antibodies. (A) Immunoblot with anti-peptide 2 antiserum (anti-LC α /LC β). Lanes 1–7 are PC12 transfectant clones PA5, PA6, PA11, PA17, PA25, PA28, PA31, respectively, after culture in 1 μ M dexamethasone for 48 h. (B) Immunoblot of LC α -negative PC12 clone (PA35) and controls. Lanes 1–3: anti-peptide 2 antiserum. Lanes 4–6: X16 mAb (LC α -specific). Lanes 1 and 4: clone PA35 lysate; lanes 2 and 5: clone PV6 lysate; lanes 3 and 6: clone PA6 lysate. Clone PV6 was transfected with pMAMneo, and clone PA6 was transfected with pMAMneo-LC α . For the experiment in B, all clones were cultured in the absence of dexamethasone. r indicates rat LC α and h indicates human LC α .

pargyline, and washed four times with 5 ml serum-free DME-H21 (Lowe *et al.*, 1988). For the zero time point, cells were left 2 min in 5 ml serum-free DME-H21 (37°C) and medium was saved. Cells were then incubated in 37°C serum-free medium with 55 mM KCl and at intervals of 2 min medium was removed, saved, and replaced with new medium. Control cells were incubated in serum-free medium without 55 mM KCl. After removal of medium for the fifth time point (8'), cells were lysed with 5 ml 1 M NaOH. One milliliter of each time point was counted in liquid scintillation counter. Percent secreted is calculated as cpm secreted in medium/total cpm taken up by cells.

Sulfate Labeling and Secretion

PC12 cells grown 4–5 d on poly-D-lysine coated six-well plates were rinsed in PBS, incubated for 6 h at 37°C in SO $_4$ -free DME-H21 medium containing 0.5 mCi/ml 35 S-SO $_4$ (1 ml/well), and then rinsed twice in 2 ml of warm PBS (Tooze and Huttner, 1990). Chase medium (DME-H21 medium, 5% FCS, 10% HS) was added (1 ml/well) and cells were incubated 15 h at 37°C, then rinsed four times with 5 ml warm serum-free medium. Stimulation medium (serum-free DME-H21, 55 mM KCl) or control medium (serum-free DME-H21) was added (1 ml/well), and cells were incubated 5 min at 37°C, and the medium was removed for precipitation with 10% trichloroacetic acid (TCA). The cells were then lysed in 500 μ l of lysis buffer and spun at 1 000 $\times$ g. TCA-precipitated medium samples were spun at 10 000 $\times$ g, pellets were washed in –20°C acetone, and 50 μ l 6 M urea was added. After incubation at 40°C for 5 min, 50 μ l electrophoresis sample buffer (Laemmli, 1970) was added, and the samples were incubated 5 min at 40°C. Cell lysates were not TCA-precipitated but instead electrophoresis sample buffer was added directly and samples were boiled 10 min. Samples were analyzed on 8% acrylamide/0.4% bis-acrylamide gels, which were soaked in Enlightening (New England Nuclear, Boston, MA) 20 min before 48 h exposure to film.

RESULTS

Production of Light Chain Transfectants

To investigate whether clathrin light chains LC α and LC β perform exclusive functions in mammalian cells,

experiments aimed at the elimination of expression of one or the other light chain were carried out. The human B lymphoblastoid cell line C1R and the rat pheochromocytoma cell line PC12 were transfected with human LC α or LC β cDNA under the control of the mouse mammary tumor virus promoter (pMAMneo vector) inducible with dexamethasone. Six stable C1R clones transfected with the LC β cDNA (clones designated CB1, CB2, etc.) and four stable C1R clones transfected with the LC α cDNA were isolated from a total of 8 G418-resistant LC β -transfectants and 10 G418-resistant LC α -transfectants, respectively. Two stable C1R clones, transfected with the pMAMneo vector without light chain cDNA inserts (e.g., clone CV10), were isolated to use as controls. After transfection of PC12 cells with the human LC α cDNA, 11 of 59 G418-resistant clones (clones designated PA1, PA2, etc.) expressed human LC α after induction with dexamethasone, and one clone, PA35, was completely devoid of expression of both the transfected LC α gene and the endogenous LC α gene in the presence or absence of dexamethasone (Figure 1). In addition, 22 G418-resistant clones of PC12 cells transfected with vector without light chain cDNA were isolated to use as control cells (clones designated PV1, PV2, etc.).

The fact that no LC α expression was detected in LC α -transfected PC12 clone PA35 using either mAb X16 (recognizing LC α residues 125–144) (Brodsky *et al.*, 1987) or anti-peptide 2 serum (recognizing LC α residues 23–44) (DeLuca-Flaherty *et al.*, 1990), suggested that the protein products of both transfected human and endogenous rat LC α DNA were absent (Figure 1). A Northern blot of RNA extracted from PA35 cells showed that no mRNA encoding LC α could be detected in these cells (Figure 2).

To investigate the LC α -expression defect at the genomic level, DNA was prepared from the LC α -negative

Figure 2. Northern blot of PC12 clones transfected with human LC α . RNA from LC α -expressing PC12 transfectant PA6 (lane 1) and LC α -negative PC12 transfectant PA35 (lane 2) was electrophoresed, transferred to Hybond filters, and blotted with probes for LC α (sense strand), γ -actin or LC α (anti-sense strand). The same nylon filter was used for each blot shown after stripping bound probes. Positions of ribosomal RNA markers are shown at the left. The LC α probes were prepared from human LC α cDNA but crossreact under these hybridization conditions with rat LC α .

transfectant PA35, the LC_a-expressing transfectant PA6, and the parent PC12 cell line and digested with *EcoRI* or *Sma I* (Figure 3). Southern blot analysis with a probe for LC_a showed that in PC12 cells, the endogenous LC_a gene yields a single band at 7 kilobase (kb) after *Sma I* digestion and two bands at 6.5 and 5 kb with *EcoRI*. These bands are also present in the digest of PA6 DNA, indicating that in this LC_a-expressing transfectant the endogenous LC_a gene remains intact and the LC_a-pMAMneo cDNA construct is integrated nonhomologously into the genome. Extra bands in the PA6 digests at 3.2 kb (*Sma I*) and 5.4 kb (*EcoRI*) are of the size expected from a functionally integrated LC_a-pMAMneo construct. Interestingly, their intensity is noticeably greater than that for most of the other bands. It is likely that this reflects the presence of multiple integrated copies of human LC_a cDNA. Other bands in the *Sma I* digest of PA6 at 9 and 6.5 kb cannot be readily explained but may represent nonfunctional integration of some of the LC_a cDNA copies. On the other hand, none of the bands corresponding to the functionally integrated LC_a-pMAMneo construct are present in the DNA digests from the LC_a-negative PA35 cells. Moreover, the band corresponding to the endogenous LC_a gene is absent from the *Sma I* digest altogether and instead there is a band at 13 kb. Because this is the only band present in the lane, it indicates that the integration of the LC_a cDNA construct must have occurred within or very close to the endogenous LC_a gene. The pattern for the *EcoRI* digest of PA35 DNA is also consistent with this interpretation; one of the two endogenous LC_a bands (at 6.5 kb) remains intact, whereas there is a new band at 5.2 kb.

The most likely explanation for these results is that a homologous recombination event has occurred in PA35 cells between the transfected LC_a pMAMneo construct and the endogenous LC_a gene that has disrupted the gene and abolished transcription of mRNA and expression of the LC_a protein. This event would have been favored if the parent PC12 cells used for transfection were hemizygous for the LC_a gene, a likely possibility considering their long history as a cultured cell line (Burstein and Greene, 1982). Without further and much more extensive characterization we cannot determine the exact position of the gene disruption in the PA35 transfectant. However, Southern blotting data, using additional enzymes to digest PA35 DNA, suggested that most of the endogenous LC_a gene in PA35 cells has remained intact, and therefore the integration event probably occurred near either the 5' or 3' end.

Changes in Light Chain Ratios of C1R and PC12 Transfectants

Isolation of the PC12 and C1R transfectants provided the opportunity to study the effect of LC_a reduction and depletion on the functions of clathrin in C1R cells that

Figure 3. Southern blot of PC12 cells and clones transfected with human LC_a. DNA from LC_a-expressing PC12 transfectant PA6, LC_a-negative PC12 transfectant PA35, and the parent PC12 cell line was digested with either *EcoRI* (E) or *Sma I* (S). After electrophoresis and transfer to nitrocellulose, samples were probed with a full length LC_a probe prepared from human LC_a cDNA and labeled by random priming. Migration of molecular weight markers is indicated at the left in kilobases.

normally express a sixfold excess of LC_a relative to LC_b or in PC12 cells that normally express three times as much LC_b as LC_a (Figures 1 and 4). To carry out these studies, it was necessary first to characterize the induction conditions required to maximize the expression of the exogenous LC_b cDNA in C1R transfectants and to determine whether overexpression resulted in depletion of clathrin heavy chain-associated LC_a.

Increased expression of LC_b was demonstrated in four C1R clones stably transfected with LC_b cDNA and cultured in the presence of 1 μ M dexamethasone for 48 h (Figure 4). The LC_a:LC_b ratio in untransfected C1R cells and in C1R clones transfected with vector without light chain cDNA was found to be about 6:1 by quantitative immunoblotting with anti-peptide 2 serum similar to the high LC_a:LC_b ratio in another B lymphoblastoid cell line, LB (Acton and Brodsky, 1990). The C1R clones transfected with LC_b, when uninduced, had somewhat lower LC_a:LC_b ratios than control cells. When fully induced with the culture conditions described above, the LC_a:LC_b ratios were changed to about 1:4. The changes in LC_a:LC_b ratio after induction of LC_b expression were also reflected in the proportion of LC_a and LC_b in triskelions and coated vesicles (Figure 5), showing that the expressed exogenous LC_b light chain was incorporated into functional clathrin triskelions and that the relative amount of heavy chain-associated LC_a was reduced. Because LC_a and LC_b compete with each other for heavy chain binding (Brodsky *et al.*, 1987), it is expected that the presence of excess LC_b would increase the level of unbound LC_a if binding sites on the heavy chain were limiting. Unbound LC_a is rapidly degraded when dissociated from the heavy chain (Brodsky, 1985b), possibly accounting for the fact that the over-

Figure 4. Induction of LC_b expression and relative reduction of LC_a in transfected C1R clones. (A) Transfected clones CV10 (control containing only pMAMneo vector) and CB1-4 (clones containing pMAMneo- LC_b) were cultured with (+) and without (-) $1 \mu M$ dexamethasone for 48 h, lysed, and post-nuclear supernatants of the lysates were electrophoresed by SDS-PAGE. After transfer to nitrocellulose, blots were probed with anti-peptide 2 antiserum (anti- LC_a/LC_b). (B) Densitometry of the autoradiogram shown in A demonstrating relative reduction of LC_a in dexamethasone-treated (LC_b -transfected and induced) cells (CB4) compared to control transfected cells (CV10). Tracings 1 and 2 are CV10 minus and plus dexamethasone, respectively. Tracings 3 and 4 are CB4 minus and plus dexamethasone, respectively. Absorbance at 500 nm is shown relative to distance from the bottom of the gel.

expression of LC_b appears to reduce the total levels of LC_a observed. Preliminary analysis of the 11 PC12 and 4 C1R transfectants that expressed LC_a after dexamethasone induction indicated that overexpression of LC_a and concomitant reduction of LC_b was not as readily achieved as reduction of LC_a in the LC_b -transfectants. This might be expected because the half-life of heavy chain-associated LC_b (45 h) is almost twice as long as the half-life of bound LC_a (24 h), increasing the time required for efficient competition by the overexpressed light chain.

To determine whether the LC_a -negative PC12 transfectant PA35 compensated for the loss of LC_a with an increase in heavy chain-associated LC_b , PA35 cells were compared with control PC12 cells for the relative levels of LC_b and heavy chain (Table 1) by quantitative immunoblotting with anti-peptide 2 and TD.1 antibodies, respectively. Relative to the level of clathrin heavy chain present in the cells, the level of LC_b in the LC_a -negative PC12 transfectant was increased compared with the

Figure 5. Light chains associated with clathrin heavy chain in C1R clones induced to overexpress LC_b . LC_b -transfected C1R clones CB3 and CB4 and control transfectant CV10 were cultured with (+) and without (-) $1 \mu M$ dexamethasone for 48 h, lysed, and clathrin isolated from postnuclear supernatants of the lysates by immunoprecipitation with X32 (anti-clathrin heavy chain mAb). Samples were separated by SDS-PAGE, transferred to nitrocellulose, and blots were probed with anti-peptide 2 antiserum (anti- LC_a/LC_b).

level in parent PC12 cells. These studies suggest that the heavy chain sites normally occupied by LC_a in PC12 cells are not empty in the LC_a -negative transfectants but are occupied by LC_b .

The growth rate and morphology of the LC_a -negative PA35 cells was within the normal range of variation of parent PC12 cells and PC12 clones transfected with the pMAMneo vector with and without light chain cDNA. Similarly, C1R clones that were induced to overexpress the transfected LC_b cDNA did not appear to differ in growth rate and morphology from control C1R clones cultured under identical conditions (48 h in the presence of $1 \mu M$ dexamethasone). The growth rate of all C1R clones (control and LC_b -transfected) was slower in dexamethasone. However, all recovered to rapid growth rates, with indistinguishable kinetics, 48 h after removal of dexamethasone.

Distribution and Sorting Functions of Clathrin in LC_a -depleted Transfectants

Clathrin is localized in the Golgi region and at the plasma membrane of all cells, reflecting the participation of clathrin-coated vesicles in receptor-mediated sorting in the *trans*-Golgi network (e.g., of lysosomal enzymes) and in receptor-mediated endocytosis. Previous studies of cells expressing both LC_a and LC_b have revealed that LC_a and LC_b are both found in the Golgi region and the plasma membrane with no apparent specialized lo-

Table 1. Relative levels of light chains and heavy chains in PC12 and PC12 transfectants^a

Cell	LC_a/HC	LC_b/HC	$(LC_a + LC_b)/HC$
PC12	0.21	0.79	1.00
PV5	0.22	0.79	1.02
PA35 (LC_a^-)	0.0	1.04	1.04

^a Light chain and heavy chain levels were determined by quantitative immunoblotting. 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 $(LC_a + LC_b)/HC$ equaled 1.00 for PC12 cells.

calization for either light chain (Puszkin *et al.*, 1989). However, cells expressing both light chains have triskelions with both light chains (Kirchhausen *et al.*, 1983), which makes it impossible to determine whether individual light chains might have a preferred subcellular localization. Analysis of clathrin distribution in the LC_a-negative PC12 transfectant PA35 by immunofluorescence with anti-clathrin antibodies provided an opportunity to examine the subcellular localization of triskelions with only LC_b light chain (Figure 6, A and B). Staining of PA35 cells with anti-heavy chain antibody indicated that clathrin was localized both at the plasma membrane and in the Golgi region, demonstrating no requirement of LC_a for intracellular localization and no preference influenced by LC_b. Anti-peptide 1a serum (anti-LC_a) stained both the Golgi and plasma membrane in PV5 cells (PC12 cells transfected with vector without light chain cDNA), but, as expected in LC_a-negative, cells gave low background staining (Figure 6, B and E) similar to a negative control antibody, confirming that the PA35 cells do not produce LC_a. C1R transfectants, induced to overexpress LC_b under conditions that max-

imally reduce the intracellular level of LC_a, showed no apparent difference in their intracellular distribution of clathrin from similarly treated control cells. However, it should be pointed out that because of the limited amount of cytoplasm in B cells, subtle changes in clathrin distribution in C1R cells would not be evident.

To determine if the absence of LC_a might affect the sorting of intracellular proteins by clathrin, the immunofluorescence staining pattern for secretogranin I (Figure 6, C and F) and for the 215KD mannose-6-phosphate receptor was analyzed. Secretogranin I is packaged into secretory granules in the parent PC12 cells (Rosa *et al.*, 1985) and appears to be similarly localized in the LC_a-negative PC12 transfectant PA35 and PV5 (PC12 clone transfected with vector lacking light chain cDNA). The mannose-6-phosphate receptor is sorted to a prelysosomal compartment by clathrin-coated vesicles in the *trans*-Golgi network and was observed in intracellular vesicles with a similar distribution pattern in PA35 LC_a-negative transfectants and PV5 control transfectants. Immunofluorescence would only detect gross differences in intracellular distribution of

Figure 6. Localization of clathrin and secretogranin I in LC_a-negative and control cells. Control and LC_a-negative PC12 cells, grown in the presence of 100 ng/ml β -NGF, were fixed, permeabilized, and incubated with primary antibody. Rhodamine conjugated goat-anti-mouse Ig or goat-anti-rabbit Ig was used to detect primary antibodies. (A–C) Control PC12 clone PV5 (transfected with pMAMneo without light chain cDNA). (D–F) LC_a-negative PC12 clone PA35, produced after transfection with pMAMneo-LC_a. (A and D) Clathrin heavy chain localized with mAb X22. (B and E) Clathrin LC_a localized with anti-peptide 1a serum, specific for LC_a. (C and F) Secretogranin I localized with anti-secretogranin I mAb. Bar, 10 μ m.

molecules but at this level, the intracellular sorting functions of clathrin in the *trans*-Golgi network of PC12 cells seem to be unaffected by the absence of LC_a.

Assembly of Clathrin in LC_a-depleted Transfectants

Both LC_a and LC_b have a binding site for hsc70 (Schmid *et al.*, 1984; DeLuca-Flaherty *et al.*, 1990). This heat shock cognate protein is believed to control disassembly of clathrin-coated vesicles in the cell, because it causes disassembly of clathrin-coated vesicles *in vitro*. Of the two clathrin light chains, LC_a has been shown to be a preferred substrate for *in vitro* stimulation of ATP hydrolysis by hsc70 (DeLuca-Flaherty *et al.*, 1990), suggesting that disassembly of clathrin-coated vesicles with only LC_b might be less efficient than disassembly of clathrin-coated vesicles with LC_a and LC_b. To investigate this possibility, the assembly state of clathrin was examined in LC_a-depleted transfectants. Cell fractions containing assembled and unassembled clathrin were prepared by differential centrifugation of cell homogenates, and the clathrin in each fraction was quantitated by immunoblotting with anti-clathrin heavy chain mAb TD.1 (Figure 7). Densitometry revealed no difference between control PC12 transfectants and the LC_a-negative transfectant PA35 in the percentage of clathrin heavy chain assembled (Table 2). In addition, no difference was found in the assembly states of clathrin in a C1R clone induced to express different light chain ratios compared with the assembly state of clathrin in a control C1R clone exposed to induction conditions (Table 2). Thus, a change in the light chain ratio does not appear to affect the level of clathrin assembly, regardless of whether most of the intracellular clathrin is normally assembled, as it is in C1R cells (87%), or disassembled, as in PC12 cells with 36% of the total clathrin assembled at steady state.

Uncoating of Membranes Isolated from the LC_a-negative Transfectant

Although steady state levels of assembled and disassembled clathrin were not changed in cells with altered

Figure 7. Immunoblot of clathrin heavy chain in subcellular fractions separating assembled and unassembled clathrin. Lysates of PC12 clones were spun at $100\,000 \times g$ to separate assembled (pellet) and unassembled (supernatant) clathrin. Samples were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with mAb TD.1 (anti-clathrin heavy chain), detected with biotinylated horse-anti-mouse Ig and ^{125}I -streptavidin. The autoradiograph of clathrin heavy chain (hc) is shown. Even lanes are pellets and odd lanes are supernatants. LC_a-negative transfectant PA35 (lanes 1–2), PC12 clone PA1 transfected with pMAMneo-LC_a (lanes 3–4), PC12 clone PV6 transfected with pMAMneo without light chain cDNA (lanes 5–6), and parent PC12 cells (lanes 7–8). All clones were cultured in the absence of dexamethasone (uninduced).

Table 2. Heavy chain assembly state in LC_a-negative and control PC12 cells

Cell	% Heavy chain assembled ^a
PC12 cells and transfectants:	
PC12 parent	33 ± 2 (3)
PV6 (transfected with pMAMneo)	38 ± 5 (3)
PA1 (transfected with pMAMneo-LC _a , nonproducer)	39 ± 2 (2)
all controls	36 ± 4 (8)
PA35 (LC _a -negative)	35 ± 8 (3)
C1R cell transfectants (dexamethasone-treated) ^b :	
CV10 (transfected with pMAMneo)	87 ± 7 (3)
CB3 (transfected with pMAMneo-LC _b)	90 ± 4 (3)

^a Clathrin heavy chain levels in supernatants (containing unassembled clathrin) and pellets (containing assembled clathrin) produced by centrifugation of cell lysate at $100\,000 \times g$ were determined by quantitative immunoblotting. Percentage of heavy chain assembled was calculated as assembled/total + unassembled. Numbers in parentheses indicate the number of experiments in which the clathrin assembly state was measured for each cell line.

^b C1R cells were grown in $1 \mu\text{M}$ dexamethasone for 48 h (after dexamethasone treatment, LC_a:LC_b ratios in CV10 and CB3 were 7:1 and 1:3, respectively).

clathrin light chain expression, it is still possible that loss of LC_a expression affects the uncoating process. To investigate uncoating directly, membrane fractions containing clathrin-coated vesicles were prepared from the PC12 transfectants and tested as substrates for ATP-dependent clathrin release by purified hsc70 *in vitro*. When the uncoating of vesicle membranes from LC_a-negative PA35 cells was compared with uncoating of membranes from a control PC12 clone transfected with vector-lacking light chain cDNA (PV6), some differences were observed (Figure 8). In 10 out of 13 experiments (Table 3), where care was taken to equalize amounts of PA35 and PV6 membranes used as substrate, the uncoating reaction was less efficient for PA35 membranes. In one experiment, no uncoating of PA35 membranes was observed under conditions where PV6 control membranes were uncoated. In three experiments the rate of PA35 membrane uncoating was slower than the rate of PV6 membrane uncoating, and in 6 experiments, less clathrin was released from PA35 membranes than was released from equivalently coated PV6 membranes. The variability in PA35 uncoating compared to PV6 uncoating suggests that loss of LC_a has only a subtle effect on uncoating by hsc70. This is predicted from the biochemical studies demonstrating that whereas LC_a is more efficient at stimulating hsc70 activity, LC_b can still serve as a substrate (DeLuca-Flaherty *et al.*, 1990).

Endocytic Function in LC_a-depleted Transfectants

To determine if endocytic function might be altered in cells with reduced LC_a, transferrin uptake and recycling

Figure 8. Altered hsc70-mediated uncoating of membrane vesicles from LC_{α} -negative transfectants. Purified hsc70 was combined with membranes isolated from LC_{α} -negative PA35 cells and from control PC12 transfectant PV6. Clathrin released from these membranes after the indicated reaction times (minutes) was detected by immunoblotting with anti-clathrin heavy chain mAb TD.1, after electrophoresis and transfer to nitrocellulose. All reactions were carried out in the presence of ATP, except the background reaction (bkg), for which the ATP was pretreated with apyrase. This background sample represents the spontaneous dissociation of clathrin from the membrane vesicles under the reaction conditions for the longest reaction time tested. Experiments I-V show examples of the different types of results observed when comparing uncoating of PA35 membranes to uncoating of equivalent quantities of PV6 membranes. See text for details and Table 3 for the frequency with which these results were observed.

was measured in C1R transfectants cultured under conditions for induction of LC_{β} expression (1 μ M dexamethasone, 48 h) (Figure 9). Iodinated iron-loaded transferrin was bound to these cells at 4°C, and after removal of the unbound transferrin by washing, bound transferrin was induced to internalize by incubation of the cells at 37°C. At defined time intervals during the incubation, the medium was sampled for recycled transferrin, and the level of internalized transferrin was measured after removal of surface-bound transferrin by exposure of the cells to a low pH buffer (Hopkins and Trowbridge, 1983). No significant difference in endocytic or recycling rates was found between LC_{β} -transfected C1R clones with a low LC_{α} : LC_{β} ratio and control C1R clones (transfected with vector without light chain cDNA) with a high LC_{α} : LC_{β} ratio. Some variation in rates was observed depending on the growth state of individual cell cultures with exponentially growing cells showing the fastest rates of both uptake and recycling. However, when comparisons were done with C1R clones in the same growth state, the control and LC_{α} -reduced clones showed identical endocytic behavior (Figure 9).

To compare endocytic function in the LC_{α} -negative and control PC12 cell transfectants, an alternative assay of transferrin endocytosis from that used for C1R cells

Table 3. Differences in uncoating of vesicles from LC_{α} -negative PA35 transfectants relative to uncoating of control PV6 transfectants

PA35 uncoating relative to PV6 uncoating ^a	No. of experiments ^b
I no uncoating	1
II less uncoating	6
III slower rate	3
IV no difference	1
V faster uncoating	2

^a See figure 8 for examples.

^b Number of experiments in which these relative phenotypes were observed.

was necessary because of the different properties of the PC12 cells. Their slow growth rate does not require the rapid rates of transferrin uptake observed for the faster

Figure 9. Endocytosis and recycling of transferrin in control and LC_{α} -reduced C1R transfectants. Iodinated transferrin was prebound at 4°C for 1 h and excess rinsed away. The cells were shifted to 37°C and at intervals of 1 min were put on ice. After spinning at 1000 $\times$ g, the supernatant (■) was counted (recycled transferrin). Cell-surface transferrin (●) was measured as counts stripped from the cells with a 5 min wash in acetic acid buffer pH 2.5. Remaining counts represent internalized transferrin (▲). The percent of counts in each pool is reported for each time point to normalize for slight variation in sample size. (A) Clone CV10 (C1R control transfected with pMAMneo) treated with 1 μ M dexamethasone for 48 h (LC_{α} : LC_{β} ratio at 6:1); (B) Clone CB3 (C1R transfected with pMAMneo- LC_{β}) treated with 1 μ M dexamethasone for 48 h (LC_{α} : LC_{β} ratio at 1:4).

growing C1R cells, so initial uptake rates were difficult to measure accurately due to simultaneous recycling. Furthermore, because of the highly invaginated surface of PC12 cells, exposure of cells to low pH buffer was only ~40% effective in removing surface-bound transferrin, even after incubation times of up to 15 min, so it was not possible to distinguish between surface-bound and internalized transferrin. To circumvent these problems, iodinated iron-loaded transferrin was prebound to PC12 transfectants at 4°C and then (without prior washing) cells were incubated in the presence of labeled transferrin at 16°C, a temperature at which endocytosis proceeds but recycling and degradation are inhibited (Hopkins and Trowbridge, 1983) (Figure 10A). Continuous transferrin uptake was measured as a function of cell-associated (surface and internalized) transferrin when aliquots of cells were washed at the indicated time. The prebinding step ensured that the rate of binding would not contribute to the rate of accumulation of transferrin. Measurement of transferrin accumulation over time at 16°C showed no significant difference in endocytic functions of the LC_a-negative and a control PC12 transfectant (PV5, transfected with vector without light chain cDNA).

Steady state accumulation of transferrin at 37°C, during which recycling can take place, was then examined in both PA35 and control transfectant PV6. Iodinated iron-loaded transferrin was pre-bound to PC12 transfectants at 4°C and then without prior washing, accumulation at 37°C was measured as a function of cell-associated (surface and internalized) transferrin at indicated time points. There appeared to be no difference in transferrin accumulation between the two cell lines suggesting that recycling was not substantially altered in LC_a-negative clones (Figure 10B).

Secretory Function in LC_a-negative PC12 Cells

Because clathrin is involved in the formation of secretory granules (Orci *et al.*, 1984; Tooze and Tooze, 1986), it was of interest to determine whether cells lacking LC_a could be induced to secrete normally. It has been shown previously that PC12 cells accumulate exogenously supplied ³H-norepinephrine and will release accumulated radioactivity in response to 55 mM KCl, a stimulus that induces regulated secretion (Lowe *et al.*, 1988). This process was tested for the LC_a-negative PA35 transfectant and control PC12 transfectant (Figure 11). Cells were grown on poly-D-lysine for 4–5 d and then incubated for 2 h with ³H-norepinephrine. After extensive washing the cells were exposed to 55 mM KCl. Both control PC12 transfectants and the LC_a-negative PA35 transfectant were induced to secrete their accumulated ³H-norepinephrine and responded to the 55 mM KCl stimulus with identical kinetics and efficiency of secretion.

It is not clear whether ³H-norepinephrine accumulated by PC12 cells is restricted to dense core secretory

Figure 10. Comparison of ¹²⁵I-labeled transferrin internalization in LC_a-negative transfectant PA35 and control PC12 transfectants. (A) Rate of ¹²⁵I-labeled transferrin uptake at 16°C. LC_a-negative PA35 cells and PC12 clone PV5 (transfected with vector without light chain cDNA) grown 2 d on poly-D-lysine-coated tissue culture dishes were preincubated 4 h, 37°C in binding buffer (PBS, 10 mM PIPES, 5 mM glucose, 1 mg/ml BSA, pH 7.4). After washing, 10⁶ cpm/ml ¹²⁵I-transferrin was prebound at 4°C for 90 min, and cells were shifted to 16°C for indicated times. The cells were washed three times in PBS and dissolved in 1 M NaOH for counting. Each time point is the mean cpm of triplicate samples. Differences in total cell associated cpm between the two transfectants are due to minor variations in cell number. (B) Steady state internalization and recycling of ¹²⁵I-labeled transferrin at 37°C. LC_a-negative PA35 cells and PC12 clone PV6 (transfected with vector without light chain cDNA) grown 2 d on poly-D-lysine-coated tissue culture dishes were preincubated 2 h 37°C in binding buffer (PBS, 10 mM PIPES, 5 mM glucose, 1 mg/ml BSA, pH 7.4). After washing, 10⁶ cpm/ml ¹²⁵I-transferrin was prebound at 4°C for 60 min, and cells were shifted to 37°C for indicated times. The cells were washed three times in PBS and dissolved in 1 M NaOH for counting. Each time point is the mean cpm of triplicate samples.

granules or also sequestered within and released from synaptic vesicles (Südhof, 1989). To examine secretion specifically the result of release from dense core granules, the secretion of two dense core proteins, secretogranins I and II, was measured for the LC_a-negative clone PA35 and parent PC12 cells (Figure 12). On the basis of immunofluorescent staining for secretogranin I in the LC_a-negative PC12 clone PA35, it appeared this

Figure 11. Norepinephrine secretion in LC_a -negative and control PC12 cells. Cells grown 4 d in 100 mm poly-D-lysine-treated tissue culture plates were incubated for 2 h with 10 μ Ci 3H -norepinephrine in serum-free medium with 0.1 mg/ml pargyline, rinsed four times and placed in medium with (closed points and x) or without (open points and line) 55 mM KCl for 2-min intervals. Medium was recovered and saved at each interval for counting. Medium without 55 mM KCl was used for zero time point. PC12 parent cells (●, ○), PA35 (LC_a -negative) (x, —), PV2 (■, □), PV5 (▲, △), and PC12 clones are shown. PV2 and PV5 were PC12 clones transfected with pMAMneo without light chain cDNA.

protein was appropriately localized in secretory granules (Figure 6F). To determine whether it could be released, cells were labeled for 6 h with ^{35}S -SO₄, which labels primarily the secretogranins and some proteoglycans. The cells were then washed and incubated in medium with serum for 15 h to allow secretion of all sulfated proteins targeted to the constitutive secretory pathway (Tooze and Huttner, 1990). The cells were then induced to secrete granules by exposure to 55 mM KCl. As in the secretion of 3H -norepinephrine, the LC_a -negative PC12 transfectant PA35 and parent PC12 cells showed identical efficiency of secretion. Thus LC_a is not necessary for the formation of or secretion from dense-core granules. These studies also indicate that sulfation, a modification which occurs in the *trans*-Golgi network (Baeuerle and Huttner, 1987), can occur efficiently in cells with only LC_b light chain. In preliminary studies (P. Bresnahan and G. Thomas, unpublished data) where PA35 cells were transiently transfected with the cDNA for pro- β -NGF (Bresnahan *et al.*, 1990), the expressed pro- β -NGF was processed as in parent PC12 cells transfected with the same construct. These results suggest the processing enzymes still have access to the secretory pathway in the PA35 cells.

DISCUSSION

Cells derived from different tissues characteristically express different ratios of clathrin light chains, LC_a and LC_b (Acton and Brodsky, 1990). Consequently, tissues have variable levels of four types of clathrin triskelion,

each type with a different threefold combination of the two clathrin light chains (aaa, aab, abb, bbb). To study the functional role of clathrin light chain polymorphism, the relative levels of expression of the LC_a and LC_b light chains were altered by genetic manipulation of two cell lines that normally express very different ratios of LC_a to LC_b . C1R cells that characteristically have 86% LC_a and 14% LC_b were induced to overexpress transfected LC_b cDNA, such that LC_a was reduced to 25% of the light chain pool. The changed light chain levels were reflected in the light chain composition of the clathrin heavy chain in the induced, transfected cells. A clone of PC12 cells, in which LC_a is normally 25% of the light chain pool, expressed 100% LC_b when LC_a expression was lost after transfection with a vector containing cDNA for human LC_a . This appeared to be a result of gene disruption. LC_b replaced the lost LC_a in the clathrin triskelions in the LC_a -negative PC12 cells, making it possible to analyze the function of a cell with a single type of clathrin light chain and therefore a single species of clathrin triskelion.

When the C1R clones with reduced LC_a and the LC_a -negative PC12 cells were tested for a variety of clathrin functions, they were found to mediate these functions with the same kinetics and efficiency as control cells. The intracellular distribution of clathrin was not affected in these cells. In conjunction with this finding, neither the sorting by clathrin in the *trans*-Golgi network nor the sorting at the plasma membrane appeared to be selectively affected by LC_a depletion. Localization of mannose-6-phosphate receptor and secretogranin I, as well as packaging and release of secretory granules, was the same in LC_a -negative PC12 cells compared to control

Figure 12. Secretion of secretogranins in LC_a -negative and control PC12 cells. Cells were labeled with ^{35}S -SO₄ for 6 h, chased in normal medium for 15 h, and then incubated with or without 55 mM KCl. Medium was collected and TCA-precipitated, solubilized in 6 M urea and subjected to SDS-PAGE (8% acrylamide, 4% bisacrylamide). Unsecreted secretogranins were visualized concurrently by electrophoresis of cell lysates. Lanes 1–4: Parent PC12 cells; lanes 5–8: Clone PA35 (LC_a -negative); lanes 1, 2, 5, and 6: no KCl stimulation; lanes 3, 4, 7, and 8: stimulation with KCl; odd numbered lanes: cell lysates; even numbered lanes: medium. SgI and SgII are secretogranins I and II, respectively. Migration positions of molecular weight marker proteins are indicated at the right in kilodaltons.

transfected PC12 cells. Also, endocytosis of transferrin was not measurably altered in LC_a-negative PC12 cells or C1R cells with reduced levels of LC_a. The only detectable effect of LC_a depletion was a reduction in efficiency of hsc70-mediated uncoating of vesicles isolated from LC_a-negative PC12 cells. The difference in uncoating was observed in *in vitro* reactions but did not appear to influence steady state levels of clathrin assembly in the LC_a-negative PC12 cells. Assembly levels of clathrin were also unchanged in C1R cells with reduced expression of LC_a.

The absence of a dramatic phenotype after loss of LC_a light chain expression is reminiscent of many gene knockout experiments showing that mice lacking a particular gene can survive under laboratory conditions (Soriano *et al.*, 1991; Rajewsky, 1992). In some of these cases, the disrupted genes participate in the immune system or in kinase cascades, where alternate pathways may compensate for the defect. Given that cell lines have no developmental constraints and that the two mammalian clathrin light chains have 60% identity of sequence (Jackson and Parham, 1988), it is not surprising that the cells with disruption of only one of the mammalian clathrin light chains survive. This property has made it possible to derive information about clathrin light chain function and expression from these cell lines.

First, the intracellular distribution of clathrin in the LC_a-negative cells demonstrates that light chain composition does not preferentially influence localization to the plasma membrane or the *trans*-Golgi network, where clathrin carries out distinct functions. Second, the reduction in efficiency of hsc70 uncoating of LC_a-negative vesicles provides a biological correlate to earlier biochemical studies in which it was shown that LC_a is the favored substrate for stimulation of the hsc70 ATPase reaction and that purified LC_a preferentially inhibits uncoating of clathrin-coated vesicles (DeLuca-Flaherty *et al.*, 1990). The LC_a-negative cells therefore provide a system in which the *in vivo* role of hsc70 in the uncoating reaction can be examined. So far, no gross effects of the reduced efficiency of uncoating have been observed with regard to steady state levels of clathrin assembly or on endocytosis and recycling. Because these are relatively slow processes in PC12 cells, the uncoating step may not be rate limiting. However, the possibility should be considered that hsc70 is not involved in uncoating in the cell, but can be induced to uncoat vesicles *in vitro* because it has disaggregation functions as a chaperone. Further study of the LC_a-negative cells may help to resolve the biological role of hsc70 in the clathrin cycle.

Finally, study of cells in which clathrin light chain expression was altered reveals *in vivo* dynamics of regulation of triskelion light chain composition that were predicted from previous studies of light chain turnover and the biochemistry of light chain-heavy chain interactions (Ungewickell, 1983; Winkler and Stanley, 1983;

Brodsky *et al.*, 1987; Acton and Brodsky, 1990). In the C1R transfectants, overexpressed LC_b competed efficiently for LC_a binding sites on the clathrin heavy chain. However, preliminary attempts to overexpress LC_a and eliminate LC_b demonstrated that this competition was less efficient, suggesting that if LC_b is present, it has preferential association with the heavy chain. The longer half-life of heavy chain-associated LC_b (45 h) compared to that of heavy chain-associated LC_a (24 h) is further evidence for preferential binding of LC_b (Acton and Brodsky, 1990). Thus, although light chain distribution on triskelions is random (Kirchhausen *et al.*, 1983), the binding of LC_b seems to be favored. Previous studies demonstrated a predominance of LC_b in cells with a regulated secretory pathway (Acton and Brodsky, 1990), suggesting a specialized role for LC_b in the secretory process. In the LC_a-negative PC12 cells, LC_b could substitute for LC_a in normal clathrin functions and still play a specialized role in secretion. Although secretory activities were not exhaustively analyzed in the LC_a-negative cells, it might be expected that they would only be affected by elimination of the LC_b light chain, an experimental challenge for the future.

It is possible, of course, that the clathrin functions we tested are indifferent to changes in light chain ratios because neither light chain is required for these functions or that LC_a and LC_b functions are not as differentiated as their primary structures seem to indicate. 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. Other explanations for the dispensability of LC_a include the possibilities that the mammalian LC_a is highly specialized or even the vestigial product of a duplicated light chain gene. If vestigial, then the high degree to which the LC_a sequences have been conserved between species is unusual (Jackson and Parham, 1988). If LC_a only functions in a limited specialized capacity, then the tests of clathrin functions carried out in these studies could have overlooked its specialized role. However, there are several blood cell types in which LC_a is the dominant light chain (80–90% of the light chain pool) (Bar-Zvi *et al.*, 1988; Acton and Brodsky, 1990), so it is likely that in these cells LC_a mediates some housekeeping functions of clathrin in addition to any potential highly specialized role. It is also possible that only the regions of LC_a and LC_b that are highly similar in sequence encode the functional portion of the protein and that the highly differentiated regions are not relevant to function directly but might be involved in regulation of function. For example, LC_b, but not LC_a, is found phosphorylated *in vivo* (Bar-Zvi *et al.*,

1988). If LC_b function is regulated by phosphorylation whereas LC_a is not, then perhaps using LC_b costs more energy than using LC_a . Thus LC_b expression might be increased only in cells in which more regulation of clathrin function is required. Those cells that 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 LC_b , whereas cells that are undergoing rapid and continuous nonregulated endocytosis, such as reticulocytes or activated B lymphocytes, have much more LC_a than LC_b (Bar-Zvi *et al.*, 1988; Acton and Brodsky, 1990). Finally, it is possible that the LC_a -negative PA35 clone may have undergone mutation in an additional gene that compensates for loss of LC_a function.

The results presented here suggest either that further functional assays for clathrin must be defined to find functional differences between LC_a and LC_b , or that there is sufficient redundancy in light chain functions such that LC_b can effectively compensate for lack of LC_a .

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