# Predominance of Clathrin Light Chain $LC_b$ Correlates with The Presence of a Regulated Secretory Pathway

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*Abstract.* Two forms of clathrin light chains, $LC_a$ and $LC_b$, are expressed in all mammalian and avian tissues that have been examined, whereas only one type is found in yeast. Regions of structural dissimilarity between $LC_a$ and $LC_b$ indicate possible functional diversity. To determine how $LC_a$ and $LC_b$ might differentially influence clathrin function, light chain expression patterns and turnover were investigated. Relative expression levels of the two light chains were determined in cells and tissues with and without a regulated secretory pathway. $LC_a/LC_b$ ratios ranged from 5:1 to 0.33:1. A higher proportion of $LC_b$ was observed in cells and tissues that maintain a regulated pathway of secretion, suggesting a specialized role for the $LC_b$ light chain in this process. The ratio of light chains in assembled clathrin was found to reflect the levels of total light chains expressed in the cell, indicating no preferential incorporation into triskelions or coated vesicles. The half-lives of $LC_a$, $LC_b$, and clathrin heavy chain were determined to be 24, 45, and 50 h, respectively. Thus, $LC_a$ is turned over independently of the other subunits. However, the half-lives of all three subunits are sufficiently long to allow triskelions to undergo many rounds of endocytosis, minimizing the possibility that turnover contributes to regulation of clathrin function. Rather, differential levels of $LC_a$ and $LC_b$ expression may influence tissue specific clathrin regulation, as suggested by the predominance of $LC_b$ in cells maintaining a regulated secretory pathway.

CLATHRIN is the major coat protein of coated pits and vesicles that mediate the selective internalization and transport of receptors (for review, see Brodsky, 1988). Cells use clathrin to endocytose nutrients and hormones as well as to transport lysosomal enzymes from the Golgi apparatus to a prelysosomal compartment (Griffiths et al., 1988). In cells that 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). In all these processes, clathrin polymerizes into a polyhedral protein lattice followed by membrane invagination, coated vesicle formation, and finally clathrin depolymerization.

The clathrin molecule has a triskelion (three-legged pinwheel) shape (Ungewickell and Branton, 1981). It is composed of three identical heavy chains and three light chains (for review see Pearse and Crowther, 1987). The backbone of the coat lattice is formed by the clathrin heavy chain. The contribution(s) of the light chains are not yet defined, although many of their properties suggest they play a regulatory as well as structural role in clathrin function (Schmid et al., 1984; Bar-Zvi et al., 1988). In mammalian and avian cells there are two types of light chains, $LC_a$ and $LC_b$, while yeast has only one type (Payne and Schekman, 1985). $LC_a$ and $LC_b$, which are encoded by different genes, are 60% identical in protein sequence and have several domains that are very different in primary structure implying that they serve functionally different roles (Jackson and Parham, 1988). Major sequence dissimilarities between $LC_a$ and $LC_b$ include the distribution of cysteines at the COOH terminus (Parham et al., 1989), a region of low homology on the $NH_2$-terminal side of the heavy chain binding region (Jackson et al., 1987) and a phosphorylation site that is present in $LC_b$ but not $LC_a$ (Hill et al., 1988; Bar-Zvi and Branton, 1986). These differences are maintained between species (95–98% identity in nucleotide sequence) (Jackson and Parham, 1988) indicating that $LC_a$ and $LC_b$ perform distinct functions in mammalian and avian cells that are not required of clathrin light chain in yeast.

To gain insight into differential roles for the two light chains, two major differences between $LC_a$ and $LC_b$ were characterized. 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. $LC_b$ was found to predominate in cells and tissues maintaining a regulated secretory pathway, suggesting that $LC_b$ plays a specialized role in either secretory granule formation and/or rapid membrane retrieval after secretion. This is the first evidence for a differentiated function for $LC_b$. A second difference between $LC_a$ and $LC_b$ was investigated to follow up earlier studies indicating the possibility of differential turnover of the two light chains (Brodsky, 1985*a*). A very short half-life for one of the clathrin light chains would render clathrin susceptible to regulation by the availability of that light chain. Although half-lives of $LC_a$ and $LC_b$ 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. The results of these studies suggest that it is the differential levels of $LC_a$ and $LC_b$ expression that may influence clathrin function and regulation in different cell types.

## Materials and Methods

### Materials

EBTr and MDBK cells were obtained from the American Type Culture Collection (Rockville, MD). LB, an Epstein Barr virus–transformed human B cell line, was from V. Engelhard (University of Virginia, Charlottesville), Supe T (a human T cell line) was from G. Davis (University of California, San Francisco), PC12 cells were from L. Reichardt (University of California, San Francisco), and AtT20 cells were from H.-P. Moore (University of California, Berkeley). All cells were grown at 37°C in 5% $CO_2$. EBTr, MDBK, and Supe T cells were grown in RPMI 1640 with 10% horse serum. PC12 and AtT20 cells were grown in DME (high glucose) with 7% horse serum and 7% FCS. LB cells were grown in RPMI 1640 with 10% FCS. The anticlathrin monoclonal antibodies LCB.1, X16, X22, X32, and X43 have been previously described (Brodsky et al., 1987; Blank and Brodsky, 1986; Brodsky, 1985*a*). 29B5 (control, antidinitrophenol) was from L. Herzenberg (Stanford University). Rabbit anti-mouse IgG was iodinated using Iodobeads (Brodsky, 1985*b*). All bovine tissues were obtained from the Ferrara Meat Co. Inc. (San Jose, CA) and were processed within 2 h of slaughter. Bovine adrenal and brain coated vesicles were prepared as described previously (Blank and Brodsky, 1986) with the modification that buffer D (10 mM Hepes, 150 mM NaCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% $NaN_3$, pH 7.2) was used in all steps and purification beyond the sucrose gradient step was omitted. Adrenal light chains were prepared from adrenal coated vesicles as described (Parham et al., 1989) using DE52 chromatography (Ungewickell, 1983). Since the $LC_b$ obtained was slightly more pure than the $LC_a$, comparative quantities were determined by densitometry of Coomassie blue–stained bands from SDS-PAGE. Primary lymphocytes were isolated from bovine blood by density centrifugation over Ficoll-Hypaque (Brodsky et al., 1981).

### Pulse Chase Labeling

LB cells were pulse labeled for 10 min with 1 mCi [$^{35}$S]methionine (NEG-009T, New England Nuclear, Boston, MA) per $2 \times 10^7$ cells at $4 \times 10^6$ cells/ml, diluted in medium with excess unlabeled methionine, washed, and then cultured at $8 \times 10^6$ cells/ml in medium with 10× unlabeled methionine (chase medium) (Brodsky, 1985*b*). 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 to maintain the viability of the cells for up to 60 h. Aliquots of $2 \times 10^6$ cells were taken immediately after addition of chase medium and at various time points during the chase incubation and washed twice in cold serum-free medium. Cell aliquots were then solubilized in 150 $\mu$l of cell lysis buffer (10 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 0.005% PMSF, 0.0007% pepstatin), left 30 min, 4°C, and then centrifuged 1,000 *g*, 10 min, 4°C. The supernatant (cell lysate) was recovered and frozen at −80°C.

### Immunoprecipitation

Preformed immune complexes of mAb and sheep anti-mouse IgG were used for immunoprecipitation in all experiments (Brodsky, 1985*b*). Thawed cell lysates were preabsorbed for two 30-min periods with 25 $\mu$l of a control antibody (29B5) immune complex. The lysate was then incubated with 100 $\mu$l of specific immune complex for 1 h at 4°C. Immunoprecipitates were washed twice in buffer E (50 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.02% $NaN_3$), and once in buffer E + 0.5 M NaCl, then resuspended in 100 $\mu$l of reducing sample buffer (Laemmli, 1970), and boiled 10 min. The samples were analyzed by PAGE (Laemmli, 1970). Gels were incubated with Enlightening (New England Nuclear) before drying and exposure to film. Clathrin heavy chain and associated light chains were immunoprecipitated with X32 or X22 (both anticlathrin heavy chain) immune complexes. X32 was used for quantitative immunoprecipitation in pulse chase studies. For immunoprecipitation of total cellular light chains, cell lysates were first boiled for 10 min and spun at 1,000 *g*. Light chains were quantitatively immunoprecipitated from the supernatant with a combination of X43 (anti-$LC_b$) and X16 (anti-$LC_a$) immune complexes. Before gel analysis of light chains, X16/X43 and X22 immunoprecipitates were boiled for 10 min in 50 $\mu$l of buffer E, spun at 1,000 *g*, and the supernatant (containing the light chains) recovered. All pulse chase experiments included a second round of specific immunoprecipitation to ensure that the immunoprecipitation was quantitative.

### Autoradiography and Densitometry

Dried gels and nitrocellulose blots were exposed to film at −80°C for varying periods. Film exposure was tested for linearity with radioactivity by running a standard curve or was made linear by preflashing the film (Hames and Rickwood, 1981). Autoradiographs were scanned using a DU-64 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA) with gel scanning accessories and software to integrate the peak areas. Data from pulse chase experiments were plotted as natural log of the peak area versus time. This yielded linear plots with slope equal to the degradation rate constant given by the first order equation: $\ln[P_t/P_0] = -k_d t$ (Goldberg and Dice, 1974). The half-life is calculated as: $t_{1/2} = \ln 2/k_d$. Half-life studies were repeated to ensure reproducibility of results.

### Quantitation of Light Chains

Light chain levels were determined by quantitative immunoblotting. Tissues and cells were homogenized in buffer D (10 mM Hepes, 150 mM NaCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% $NaN_3$, 0.05% PMSF, pH 7.2), and then centrifuged at 1,000 *g*, 30 min. The resulting supernatants were boiled for 10 min, and then centrifuged at 10,000 *g* for 10 min, leaving the clathrin light chains in solution while the heavy chains precipitate along with most of the other cellular proteins (Brodsky et al., 1983). Preliminary studies were conducted to ensure that both light chains were equally boiling resistant. Boiled tissue homogenates were reduced and run on a pair of 10% polyacrylamide gels. Dilutions of either purified adrenal $LC_a$ or $LC_b$ were also run on each gel (starting at 100 ng) to generate a standard curve. Proteins were transferred to nitrocellulose (14 V, 90 min) and blots incubated overnight with 1% nonfat milk in TBS (10 mM Tris; 150 mM NaCl), pH 7.4, followed by primary antibody (10 $\mu$g/ml in 1% milk in TBS) for 4 h at room temperature. mAbs X16 (anti-$LC_a$) and LCB.1 (anti-$LC_b$) were used for $LC_a$ and $LC_b$ quantitation, respectively. Antibody binding was detected following a 2-h incubation with $^{125}$I-rabbit anti-mouse IgG ($0.5 \times 10^6$ cpm/ml in TBS) and autoradiography (Brodsky, 1985*a*). Standard curves were plotted as band density versus nanograms of light chain standard yielding linear plots. Only those standard curves of four points or more with an $r^2$ value >0.95 were used to calculate quantities in tissue and cell samples. In addition, only numbers that fell within the standard curve were acceptable since there was no guarantee of linearity upon extrapolation. The relative quantities of light chains obtained for unknown samples are reported in Table I as a ratio of $LC_a$ to $LC_b$ (ng $LC_a$/ng $LC_b$).

Light chain ratios in PC12 and AtT20 cells were estimated after immunoprecipitation of triskelions with mAb X22 (anti-heavy chain) since mAb LCB.1 (used above) does not bind mouse or rat $LC_b$ (see text). $1 \times 10^7$ LB, PC12, and AtT20 cells were each labeled with 0.5 mCi [$^{35}$S]cysteine (New England Nuclear) for 10 h. Cells were then solubilized in cell lysis buffer, 30 min, 4°C, and the lysate was used for immunoprecipitation, as above.

### Assembled and Unassembled Clathrin

Tissues or cells were homogenized in buffer D with 0.05% PMSF. Control experiments similar to those of Goud et al. (1985) were performed to ensure that clathrin did not assemble or disassemble in this buffer during this procedure. The homogenate was spun at 1,000 *g* and a known volume of the resulting supernatant was then spun at 100,000 *g* in an ultracentrifuge (TL100; Beckman Instruments, Inc., Palo Alto, CA). The 100,000 *g* supernatant was measured and adjusted to the original volume. The pellet was

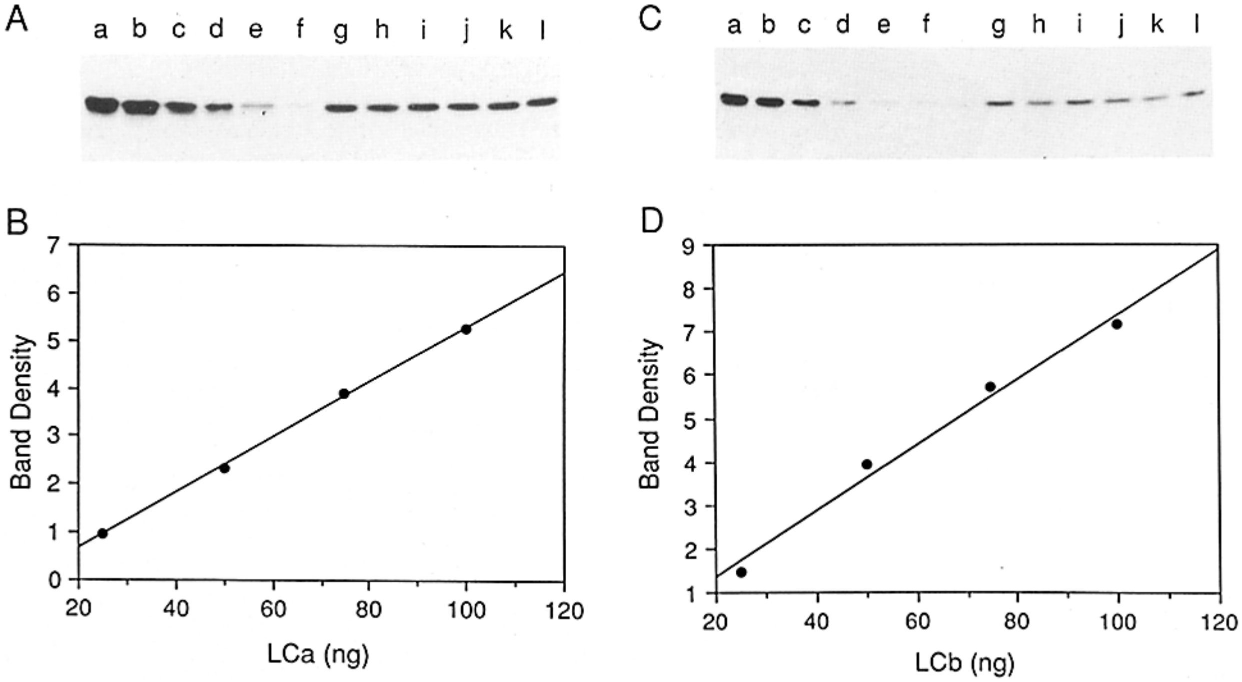

*Figure 1.* Method for quantitation of clathrin light chain ratios. Purified adrenal $LC_a$ or $LC_b$ was run on SDS polyacrylamide gels (10%) alongside homogenates of kidney cortex and medulla. Proteins were transferred to nitrocellulose and reacted with X16 (anti-$LC_a$) or LCB.1 (anti-$LC_b$). Bound 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 $LC_a$. Lanes *a–f* are purified adrenal $LC_a$. (*a*) 100 ng; (*b*) 75 ng; (*c*) 50 ng; (*d*) 25 ng; (*e*) 12.5 ng; and (*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*) Standard curve from densitometry of purified $LC_a$. $y = -0.4665 + 57.388x$ with a correlation coefficient of 0.999. (*C*) LCB.1 binding to $LC_b$. Lanes *a–f* contain purified adrenal $LC_b$. (*a*) 100 ng; (*b*) 75 ng; (*c*) 50 ng; (*d*) 25 ng; (*e*) 12.5 ng; and (*f*) 6.3 ng. Lanes *g–l* as in *A*. (*D*) Standard curve from densitometry of purified $LC_b$. $y = -0.1365 + 75.332x$ 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 that could be interpolated on the curve were used. The light chain ratios are determined as $LC_a/LC_b$ (ng/ng).

resuspended in buffer D by gentle hand homogenization, and this volume also adjusted back to the original volume. This procedure yielded the assembled and unassembled clathrin at the original concentration before the 100,000 *g* spin. These fractions were then analyzed for light chain composition as above. Percent of light chain assembled was calculated as $LC_x$ (assembled)/[$LC_x$(assembled) + $LC_x$(unassembled)], where $x = a$ or $b$ (all values are in nanograms).

## Results

### Differential Light Chain Expression

To investigate whether $LC_a$ and $LC_b$ contribute differentially to the role of clathrin in the regulated secretory pathway, levels of the light chains in two tissues specializing in regulated secretion, brain cortex, and adrenal medulla were compared to levels in five other tissues (Fig. 1). Bovine tissue homogenates were run on SDS polyacrylamide gels alongside dilutions of known quantities of purified bovine adrenal $LC_a$ or $LC_b$. Proteins were transferred to nitrocellulose that was then probed with anti-$LC_a$ or anti-$LC_b$ mAb plus iodinated rabbit anti-mouse IgG and exposed to film. Densitometry revealed a standard curve for the purified light chains and allowed determination of the quantities of light chain in unknown samples. Most of the tissues contained an $LC_a/LC_b$ ratio of approximately 1:1 (Table I). However, both brain cortex and adrenal medulla were found to have more $LC_b$ than $LC_a$ suggesting a correlation between regulated secretion and $LC_b$ 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 $LC_a$ divided by total $LC_b$, cellular variation in ratios is obscured in these numbers.

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 I). For rat and mouse cell lines, light chain ratios were estimated by immunoprecipitation of clathrin from [$^{35}$S]cysteine-labeled cells (Fig. 2). This second approach was implemented because the anti-$LC_b$ antibody (LCB.1) used for immunoblotting analysis does not react with rat or mouse $LC_b$. LCB.1 recognizes residues 1-19 at the amino terminus of $LC_b$ (S. Acton, unpublished results) that are identical in human and bovine $LC_b$ and differ by one amino acid in rat $LC_b$ (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 de-

*Table I. Clathrin Light Chain Ratios in Tissues and Cell Lines*

| Sample | $LC_a/LC_b$ (ng/ng)* |
|---|---|
| Tissue | |
| 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) |
| Cell line | |
| 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) |

* The sample standard deviation of the ratios is given along with the number of samples tested (in parentheses).

termined by both methods for the human cell line LB indicates that the relative labeling of immunoprecipitated light chains with $[^{35}S]$cysteine (Fig. 2) qualitatively reflects the light chain ratio measured by immunoblotting (Table I). 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).

Cell lines with a regulated secretory pathway were found to have a higher proportion of $LC_b$ than other lines (Table I; Fig. 2). Supe T, PC12, and AtT20 are lines that maintain a regulated secretory pathway, while MDBK, LB, and EBTr are lines that 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 $LC_a$ (~5:1 $LC_a/LC_b$ ratio) while the Supe T cells have more $LC_b$ than $LC_a$ (0.83:1 $LC_a/LC_b$ ratio). The intensely predominant labeling of $LC_b$ in AtT20 and PC12 cells also indicates increased $LC_b$ expression in cells capable of regulated secretion. AtT20 cells are mouse pituitary tumor cells that secrete ACTH in response to 8-bromo-cAMP (Moore et al., 1983). PC12 cells are rat adrenal pheochromocytoma cells that secrete norepinephrine in response to various secretagogues (Greene and Rein, 1977). In the cell lines that do not maintain a regulated secretory pathway (MDBK, LB, and EBTr) $LC_a$ expression predominates. Considering the light chain ratios determined for 13 tissues and cell lines, the five samples with predominant expression of $LC_b$ were those capable of regulated secretion.

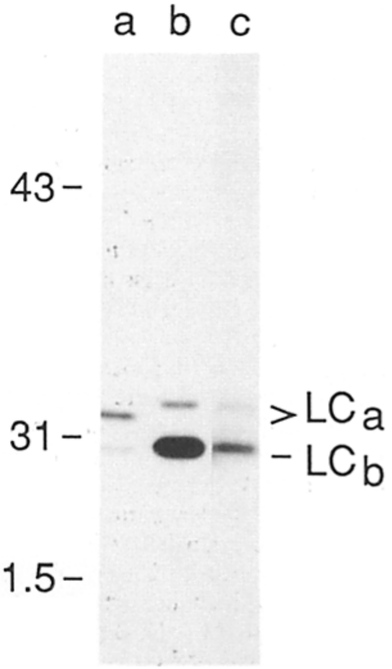

*Figure 2.* Comparison of light chain ratios in LB, PC12, and AtT20 cells. Cells were labeled for 10 h with $[^{35}S]$cysteine and clathrin was immunoprecipitated from cell lysate with anticlathrin heavy chain antibody (X22). Immunoprecipitated light chains were isolated by boiling and analyzed by SDS-polyacrylamide (10%) gel electrophoresis. (Lane *a*) LB cells (these cells have a 5:1 $LC_a/LC_b$ ratio); (lane *b*) PC12 cells; (lane *c*) AtT20 cells.

### *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 $LC_a/LC_b$ ratios were chosen for analysis. Clathrin from a postnuclear supernatant of tissue or cell homogenate was separated into assembled and unassembled fractions by centrifugation at 100,000 *g*. The pellet (assembled clathrin) and supernatant (triskelions) 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 $LC_a/LC_b$ ratio. In this tissue, the same light chain ratio was found in the total, assembled and unassembled clathrin fractions (Table II) indicating no preferential incorporation of light chains into assembled structures. The percentage of assembled $LC_a$ and $LC_b$ 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

*Table II. Ratios of Light Chains in Assembled and Unassembled Clathrin**

| | Sample | |
|---|---|---|
| | Brain cortex | LB |
| $LC_a/LC_b$ (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) |
| $\%LC_a$ assembled** | 88% ± 1% (3) | 70% ± 6% (4) |
| $\%LC_b$ assembled | 87% ± 1% (3) | 79% ± 4% (4) |

* 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 nanograms.

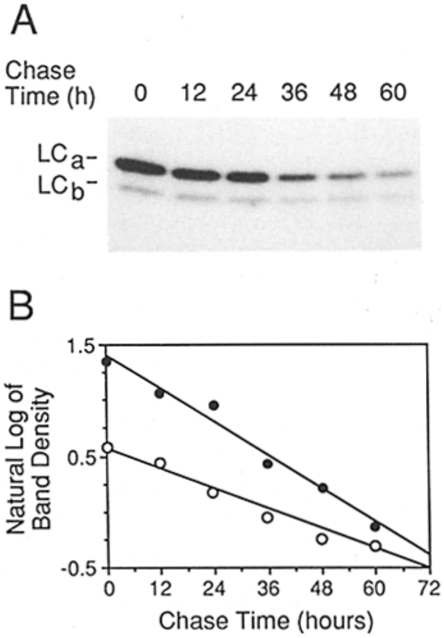

*Figure 3.* Half-life determination for clathrin light chains. (*A*) Autoradiogram of light chains labeled with [$^{35}$S]methionine for 10 min and chased with cold methionine for the indicated time periods. Light chains were quantitatively immunoprecipitated with a mixture of anti-$LC_a$ and anti-$LC_b$ antibodies (X16/X43); (*B*) Natural log of the light chain band density versus chase time. The densities of the bands in *A* were determined and plotted according to the first order rate equation $\ln [P]_t/[P]_0 = -kt$ where $[P]_t$ is band density at time $t$ and k is the rate constant of degradation. The half-life is calculated as $t_{1/2} = \ln 2/k$. (●) $LC_a$ degradation where the line can be defined as $y = 1.402 - 0.025x$ with a correlation coefficient of 0.99. This line showed a half-life of 27 h for $LC_a$. (○) $LC_b$ degradation where the line can be defined as $y = 0.574 - 0.016x$ with a correlation coefficient of 0.99. This line showed a half-life of 44 h for $LC_b$. Additional experiments demonstrated an average half-life of 24 h for $LC_a$ and 45 h for $LC_b$.

was assembled. LB cells were selected to represent cells containing predominantly $LC_a$. Similar to brain, most of the light chains in these cells were in the assembled pool (Table II). Therefore, it appears that the ratio of $LC_a$ to $LC_b$ in a cell does not confer a specific level of assembly. Much more of the clathrin was assembled in these B lymphoblastoid cells (70–79% assembled) 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 use 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 $LC_a$ was maintained in both pools.

### Clathrin Turnover

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 calculated. LB cells were pulse labeled with [$^{35}$S]methionine followed by a chase after 10 min. A mixture of monoclonal antibodies specific for $LC_a$ and $LC_b$ were used for quantitative isolation of total cellular $LC_a$ and $LC_b$ at various times after chase medium was added. Samples were analyzed by SDS-PAGE and autoradiography to determine the levels of labeled light chain remaining at each time point (Fig. 3). The half-life of the heavy chain was established from separate quantitative immunoprecipitations using the X32 monoclonal antibody, specific for clathrin heavy chain (Fig. 4). 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 heavy chain in the 10-min pulse label period. The half-lives of $LC_a$, $LC_b$, and the clathrin heavy chain were determined to be 24, 45, and 50 h, respectively. Thus, $LC_a$ is turned over independently of the other subunits, and therefore triskelions are not turned over as a unit. The lengths of the half-lives for the clathrin subunits are about average 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

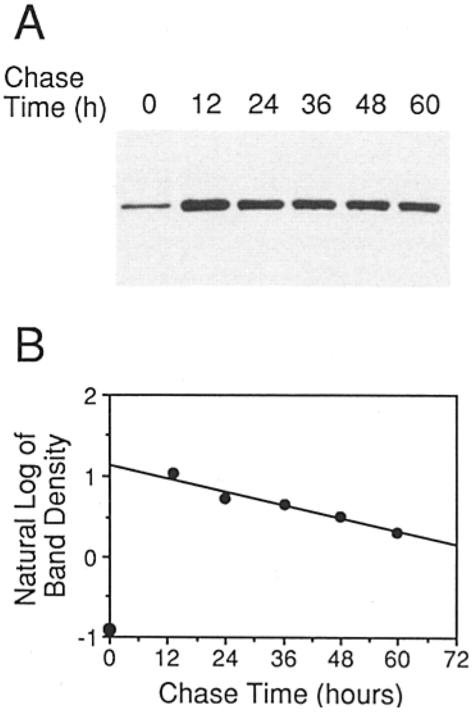

*Figure 4.* Half-life determination for clathrin heavy chain. (*A*) Autoradiogram of the clathrin heavy chain labeled with [$^{35}$S]methionine for 10 min and chased with cold methionine for the indicated time periods. Heavy chains were quantitatively immunoprecipitated with X32 mAb. (*B*) Natural log of the heavy chain band density versus chase time. The half-life was calculated as described in Fig. 3. The obtained line can be defined as $y = 1.1344 - 0.0137x$ with a correlation coefficient of 0.986. This line showed a half-life of 50 h for the heavy chain. The zero time point falls well below the line and was not used to determine the half-life (see text).

clathrin subunits have half-lives on the order of 1-2 d, clathrin activity is most likely regulated by other means.

## Discussion

### *Prevalence of $LC_b$ Correlates with the Regulated Secretory Pathway*

Expression patterns of clathrin light chains, $LC_a$ and $LC_b$, were investigated to determine their differential influence on clathrin function. Correlation between an increased proportion of $LC_b$ 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 $LC_b$. 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 $LC_b$, all cells examined express both $LC_a$ and $LC_b$ and the intracellular distribution of $LC_b$ looks similar to that of $LC_a$ (Puszkin et al., 1989). $LC_b$ specific antibodies stain the Golgi complex and periphery of many different cell types (S. Acton, unpublished results). Given its ubiquitous distribution, $LC_b$ 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 that is also used to a small capacity in nonsecretory cells, explaining why all cells have some $LC_b$. 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 $LC_b$ and $LC_a$ is that $LC_b$ is readily phosphorylated in vitro (Usami et al., 1985) and in vivo (Bar-Zvi et al., 1988). Phosphorylation of $LC_b$ could provide a signal influencing regulation and recruitment of triskelions for coated pit formation. Indeed, there is potential for coordinate $LC_b$ phosphorylation/dephosphorylation 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 that has been shown to phosphorylate $LC_b$ (Bar-Zvi and Branton, 1986). The phosphorylation acceptor site (Hill et al., 1988) is located in the part of $LC_b$ most different from $LC_a$ and is included in the epitope recognized by monoclonal antibody LCB.1 (residues 1-19). 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 $LC_b$ and the predominant expression of $LC_b$ in specialized secretory cells suggests that the $LC_b$ light chain may have evolved to fulfill regulatory functions different from those of $LC_a$. Recent data demonstrating preferential stimulation of the 70-kD coated vesicle uncoating protein by $LC_a$ as compared to $LC_b$ 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 $LC_a$ and $LC_b$, or yeast does not have all the clathrin using 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 it appears to have 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.

### *Turnover and Assembly of Clathrin Subunits*

Measurement of clathrin subunit half-lives demonstrated that the two light chains have different fates after synthesis, but their turnover is too slow to regulate the availability of assembly competent clathrin. Light chain expression is most likely regulated at the level of transcription, since the relative amounts of $LC_a$ and $LC_b$ mRNA correspond approximately to ratios of light chain protein observed in both bovine brain and human B cell lines (A. Jackson, personal communication).

The half-life of $LC_a$ was calculated to be 24 h. A free pool of $LC_a$ (unassociated with heavy chain) in these cells was previously shown to have a half-life of ~30 min (Brodsky, 1985*b*). 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 (not shown). The lack of a biphasic curve in Fig. 3 *B* indicates that the free pool was either relatively very small or was not present in this study. Thus, the half-life of 24 h is that of heavy chain–associated $LC_a$. The half-life of $LC_b$ was determined to be ~45 h, which is close to twice that of $LC_a$. Thus, $LC_b$ must be less susceptible to degradation within the cell. $LC_b$ also degrades more slowly than $LC_a$ in vitro after long term storage and upon incubation with proteases (not shown). An additional factor that may contribute to the longer half-life of $LC_b$ is its apparent higher affinity for the heavy chain. Clathrin extracted with thiocyanate retains $LC_b$ almost exclusively (Schmid et al., 1984). In addition, $LC_b$ competes more effectively with iodinated light chains for binding sites on heavy chain than does $LC_a$ (Brodsky et al., 1987). Binding to the heavy chain may stabilize the light chains and reduce their susceptibility

*Table III. Theoretical Ratios of Triskelion Types in Different Cells**

| Triskelion type** | Brain | Supe T | LB |
|---|---|---|---|
| AAA | 2% | 9% | 58% |
| AAB | 14% | 34% | 35% |
| ABB | 42% | 41% | 7% |
| BBB | 42% | 16% | <1% |

* The theoretical percentage of each type of triskelion has been calculated for brain, Supe T, and LB cells using the light chain ratio from Table I. 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 $r = LC_a/LC_b$.

** Each triskelion type is represented by three capital letters indicating the number of $LC_a$ and $LC_b$ polypeptides on that triskelion.

to proteolytic enzymes. Clathrin heavy chain was found to have a half-life of 50 h, long enough to keep the light chains bound until they are degraded. This finding correlates with previous data that showed the light chains do not alternate between a bound and free state (Brodsky, 1985*b*). Assuming one round of endocytosis takes 1 min (Pearse and Bretscher, 1981) half of the heavy chains will undergo at least 3,000 rounds of endocytosis before they are degraded. Our data explain earlier results that showed endocytosis continues despite protein synthesis inhibition (Goldstein et al., 1979).

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. However, the light chain ratios observed in assembled clathrin still maintain a predominance of $LC_a$ 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 (AAA, AAB, ABB, BBB) 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 (Table III). Cells with a light chain ratio of ~1:1, such as Supe T cells, would produce all four types of triskelions in significant quantities and the majority would contain both $LC_a$ and $LC_b$. 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, <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 $LC_a$ polypeptide and almost all of the brain triskelions will have at least one $LC_b$. Since regulated secretory cells have a predominance of $LC_b$, most of the triskelions in these cells could potentially be regulated by the phosphorylation/dephosphorylation of $LC_b$.

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