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Clathrin Structure Characterized with Monoclonal Antibodies.
II. Identification of In Vivo Forms of Clathrin

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ABSTRACT Clathrin was isolated from detergent-solubilized, biosynthetically radiolabeled cells by immunoprecipitation with anti-clathrin monoclonal antibodies. Immunoprecipitates obtained after pulse-chase labeling demonstrated that after biosynthesis the LC_a light chain of clathrin could be found either complexed to heavy chain or in a free pool (not associated with heavy chain) which decreased steadily over time. More than half of the free LC_a disappeared within the first hour after biosynthesis, but some was still detectable after several hours. Incorporation of clathrin LC_a light chain and heavy chain into coated vesicles was coordinate and increased up to 4 h after biosynthesis. Comparison of these kinetics suggested that once incorporated into coated vesicles, LC_a and heavy chain did not dissociate, even during depolymerization of the vesicle. There was also little apparent degradation of clathrin found in coated vesicles for up to 22 h after biosynthesis. Immunoprecipitation with anti-clathrin monoclonal antibodies was carried out after fractionation of continuously radiolabeled cell lysates using two different sizing columns. These experiments indicated that the triskelion form of clathrin that has been isolated from coated vesicles in vitro also exists in vivo. They also confirmed the existence of a transient but detectable pool of newly synthesized free LC_a light chain.

Coated pits and coated vesicles are the structures that result from membrane invagination during endocytosis and intracellular membrane translocation (1). To form these organelles, the protein clathrin polymerizes at the membrane surface (2). After formation of an endocytic coated vesicle, the clathrin depolymerizes within a few minutes, and the resulting uncoated vesicle (endosome) is acidified (3–5). This sequence of events suggests that within a cell there must be a pool of depolymerized clathrin in equilibrium with polymerized clathrin. The amount of depolymerized clathrin detectable in the cytoplasm seems to vary depending on the cell type examined. It has been demonstrated that in some cell types up to 70% of the clathrin in the cell is not membrane associated (6). However, in other cell types large patches of plasma membrane-associated clathrin have been observed (2, 7), and it has been proposed that the clathrin subunits rearrange on the membrane to form coated pits and initiate internalization (2).

One question raised by these data is, What is the molecular form of the clathrin subunits involved in the polymerization–depolymerization cycle in vivo? Clathrin is composed of a heavy chain of 180 kD and two light chains, LC_a and LC_b, of 32–38 kD (8–10). It has been shown that the molecular unit of clathrin that is stripped off coated vesicles under nonphysiological conditions is a "triskelion" consisting of three clathrin heavy chains and three light chains (9–11). These structures apparently have a random distribution of the two forms of clathrin light chain (12). Thus far, no evidence has been presented to demonstrate that disassembled clathrin in vivo also has this structure. The possibility remains that free pools of clathrin heavy chain or light chains or even heavy chain–light chain monomers may exist in vivo, and that these regulate coated vesicle assembly directly or indirectly by regulating triskelion assembly.

In this study, the form of in vivo clathrin has been examined. The monoclonal anti-clathrin antibodies described in the companion article have been used to immunoprecipitate biosynthetically radiolabeled clathrin present in cells in tissue culture (13). Pulse-chase labeling was used to examine assembly of clathrin subunits after biosynthesis. Fractionation of

labeled cell lysate was used to determine the size of the clathrin molecules involved in steady state polymerization and depolymerization. These experiments demonstrated the existence of a transient pool of free LC_a light chain, after biosynthesis, as well as the existence of clathrin triskelions in vivo.

MATERIALS AND METHODS

Cell Lines, Media, and Reagents

The LB cell line, an Epstein Barr virus–transformed human B cell line, was a gift from V. Engelhard (University of Virginia, Charlottesville). The BL-3 cell line, grown from bovine leukemia cells, was a gift from G. Theilen (University of California, Davis). These cells were grown in RPMI 1640 with 10% fetal calf serum and no antibiotics. For biosynthetic radiolabeling, amino acid–free media were prepared from the RPMI-1640 Select-Amine kit (No. 300-7402, Gibco Laboratories, Grand Island, NY) and used with 5% dialyzed fetal calf serum. The Anti-Leu 4, Anti-Leu 10, and anti-Igh5b monoclonal antibodies, used as control antibodies of the IgG_1 isotype, were obtained in purified form from Becton Dickinson Immunocytometry Systems. Their respective target antigens are a human T cell surface glycoprotein, a human B cell surface glycoprotein (HLA-DQ), and an allotypic determinant of mouse IgD (14–16). For immune complex formation, sheep anti-mouse IgG (Cappel 0211-1744, Cappel Laboratories, Cochranville, PA) was used. All other antibodies were purified from mouse ascites fluid as previously described (17). Fixed *Staphylococcus aureus* (Staph A)[1] bacteria were from The Enzyme Center (Boston, MA).

Biosynthetic Radiolabeling

CONTINUOUS LABELING (18): Lymphoid cell line cells were washed once in RPMI 1640 medium without the amino acid used for labeling. They were resuspended at 2×10^6 cells/ml in fresh amino acid free–medium and incubated for 30 min at 37°C, and then the radioactive amino acid at 1 mCi per 2×10^7 cells was added. [^{35}S]Methionine (>800 Ci/mmol, No. NEG-009T, New England Nuclear, Boston, MA), [^{3}H]leucine (120–190 Ci/mmol, TRK.683, Amersham Corp., Arlington Heights, IL) or [^{3}H]lysine (80–110 Ci/mmol, NET-376, New England Nuclear) were used. After 4 h of incubation at 37°C, cells were washed once in cold phosphate-buffered saline, pH 7.4, and then resuspended at 2×10^7 cells/ml in buffer I (10 mM Tris, pH 7.3, 1 mM $MgCl_2$, 0.5% Nonidet P-40 [NP40], 0.005% phenylmethylsulfonyl fluoride) (19), buffer II (10 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP40, 0.005% phenylmethylsulfonyl fluoride), or buffer III (10 mM 2-(*N*-morpholino)ethane sulfonic acid, pH 6.2, 200 mM NaCl, 2.5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5% NP40, 0.005% phenylmethylsulfonyl fluoride). After 30 min at 4°C, solubilized cells were microfuged for 15 min, and the pellet was discarded.

PULSE-CHASE LABELING (20): Lymphoid cell line cells (4×10^7) were washed once in methionine-free medium, resuspended at 4×10^6/ml in fresh methionine–free medium, and incubated at 37°C for 30–60 min. Cells were then centrifuged, resuspended at 4×10^7 cells/ml in methionine-free medium, and 2 mCi [^{35}S]methionine was added. After 10 min at 37°C, chase medium (RPMI-1640, 10% fetal calf serum, 75 μg/ml L-methionine) was added, and 4×10^6 cells were removed for solubilization at designated chase times, with the culture kept at 37°C. Cells were solubilized as above, without washing, in either buffer I or buffer II. Results obtained with both solubilization buffers were identical.

TRICHLOROACETIC ACID (TCA) PRECIPITATION: The amount of radioactivity incorporated into protein during biosynthetic labeling was determined by precipitation of an aliquot of cell lysate with 10% TCA. Precipitates were resuspended in solubilization buffer and counted in Aquasol (New England Nuclear) in a scintillation counter. This procedure was used to monitor the pulse-chase labeling and confirm that no further radioactivity was incorporated into protein after the chase medium was added.

Immunoprecipitation

STAPH A METHOD (19): Aliquots of cell lysate were preincubated with normal rabbit serum and Staph A, which was removed by centrifugation. Pretreated lysate was then incubated with specific MAb for 1 h at 4°C. Rabbit anti-mouse immunoglobulin serum and Staph A were used to isolate antibody antigen complexes. Buffers, washing procedures, and sample elution were the same as those described previously (18, 19).

IMMUNE COMPLEX METHOD (21, 22): Immune complexes were formed with 200 μg purified monoclonal antibody and 300 μl sheep anti-mouse immunoglobulin (specific antibody at 7 mg/ml) and washed in 50 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% NP40 (complex buffer). Aliquots of cell lysate were preincubated with immune complexes formed from a control antibody (anti-Igh5b). These were removed by centrifugation, and specific immune complexes were added. After incubation for 1 h at 4°C, lysate containing specific immune complexes was layered on a step gradient that consisted, from bottom to top, of 1 ml 40% sucrose, 10 mM Tris, pH 8.0, 1 ml 30% sucrose, 0.2% NP40, 10 mM Tris, pH 8.0, 1 ml 20% sucrose, 0.5 M NaCl, 0.2% NP40, 10 mM Tris, pH 8.0, and 1 ml 10% sucrose, 0.5% NP40, 10 mM Tris, pH 8.0, and centrifuged at 2,000 *g* in a swinging bucket rotor. The pelleted complexes were washed once in complex buffer and then dissolved in the sample buffer used for electrophoresis.

Electrophoresis and Autoradiography

SDS PAGE was carried out according to the method of Laemmli (23). All gels were 10% acrylamide. Gels were stained with Coomassie Brilliant Blue R and, after destaining (13), treated with Enlightening (New England Nuclear). Gels were dried and exposed to XAR-5 Kodak film using an intensifying screen (Dupont Lightening Plus, DuPont Photo Products Division-X-Ray, Burbank, CA). Molecular weight markers (Pharmacia Fine Chemicals, Piscataway, NJ) used were phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD).

Column Chromatography

Columns (0.9 cm × 30 cm) of Ultrogel AcA 22 (LKB Instruments Inc., Gaithersburg, MD) or Sephacryl S-1000 (Pharmacia Fine Chemicals) were equilibrated in buffer II without NP40 for calibration. Protein standards used for calibration were molecular weight markers (Bio-Rad No. 151-901 at 3.6 mg/ml, Bio-Rad Laboratories, Richmond, CA), clathrin triskelions at 200 μg/ml, and coated vesicles at ~2 mg/ml in column buffer with 0.5 mg/ml blue dextran. A 1-ml sample of each protein standard was applied to each column, and 1-ml fractions were collected. The columns were then equilibrated in buffer II with 1 mg/ml ovalbumin and used repeatedly for fractionation of 1 ml lysate from 2×10^7 cells labeled with [^{35}S]methionine for 4 h. The lysate applied to the column was preincubated with normal rabbit serum and Staph A or with control immune complex. The 1-ml fractions collected were also preincubated with normal rabbit serum and Staph A or control immune complex and then divided into two aliquots for immunoprecipitation with specific and control monoclonal antibodies. For the AcA 22 column runs, the fraction containing the peak of blue dextran was noted, and the preceding fraction was counted as fraction 1. For the S-1000 column separations of radioactive cell lysate, 5 μl of each fraction was suspended in Aquasol and counted in a scintillation counter. The first fraction containing radioactivity was counted as fraction 1. This corresponded to fraction 1 of the calibration runs based on the position of blue dextran, the number of fractions collected after the sample was loaded, and the bed volume of the column. The clathrin triskelions and coated vesicles used for calibration were purified from bovine brain as described by Pearse and Robinson (24).

RESULTS

Isolation of Biosynthetically Radiolabeled Clathrin

To determine the feasibility of studying in vivo forms of clathrin, three MAbs were tested for their ability to immunoprecipitate clathrin from detergent-solubilized, biosynthetically radiolabeled cells. The specificities of the antibodies used in this study are listed in Table I. A human B cell line (LB) was grown for 4 h in the presence of [^{35}S]methionine or [^{3}H]lysine in appropriately depleted tissue culture media. These cells were solubilized in a detergent-containing buffer (buffer I), commonly used for solubilizing biosynthetically labeled cell surface glycoproteins. Fig. 1 shows the antigens immunoprecipitated from [^{35}S]methionine LB cell lysate using the X16, X19, and X22 MAbs and Staph A. Results from the [^{3}H]lysine-labeled cells were identical, as were results using

[1] *Abbreviations used in this paper*: MAb, monoclonal antibody; NP40, Nonidet P-40; Staph A, fixed *Staphylococcus aureus* bacteria.

TABLE I. *Monoclonal Antibodies Used in this Study*

MAb	Clathrin binding specificity*			Species specificity		
	Subunit	Triskelions	Coated vesicles	Bovine	Human	Mouse
CVC.6	+ (LC_a)	+	+	+	–	NT
X16	+ (LC_a)	–	–	+	+	+
X19	+ (heavy chain)	+	+	+	+	NT
X22	+ (heavy chain)	+	+	+	+	NT

* MAb reactivity summarized here is based on specificity in immunoprecipitation or immunoaffinity experiments. Reactivity is indicated by +. Lack of reactivity is indicated by –. NT, not tested.

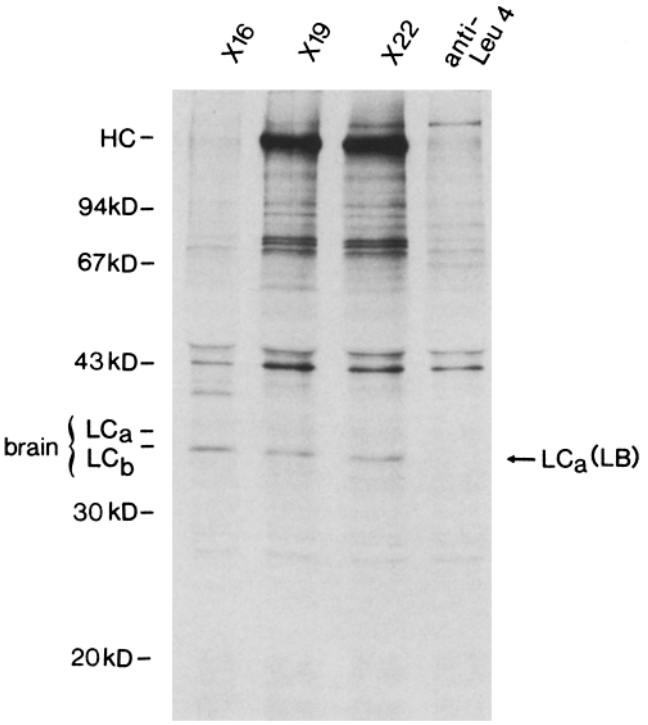

FIGURE 1 Clathrin isolated by immunoprecipitation from an ^{35}S-labeled human B cell line (LB). Anti-clathrin MAbs X16, X19, and X22, and control MAb anti-Leu 4 were combined with aliquots of NP40-solubilized LB cells, labeled for 4 h with [^{35}S]methionine. The antibody-antigen complexes were isolated with rabbit anti-mouse immunoglobulin serum and Staph A, analyzed by SDS PAGE, and the antigen was visualized by autoradiography. Each lane shows the antigen immunoprecipitated by the antibody indicated at the top of the autoradiograph. The specificities of the antibodies are listed in Materials and Methods and Table I. The migration distance of standard proteins of known molecular weight and human brain clathrin polypeptides are indicated at the left of the autoradiograph. The position of LC_a light chain isolated from LB cells is indicated at the right.

solubilization buffers II and III, designed to stabilize triskelions and coated vesicles, respectively. The X16 antibody, which immunoprecipitates free LC_a but not intact clathrin triskelions, isolated LC_a from the cell lysate. This immunoprecipitated LC_a was not associated with clathrin heavy chain. Note that the molecular weight of the immunoprecipitated lymphoid LC_a is lower than that of human brain clathrin LC_a, used as a molecular weight marker, as would be expected for nonbrain clathrin (25). The fainter band of ~40 kD also seen in the X16 immunoprecipitate was not a consistent component in repeated immunoprecipitations, and its significance is unclear. The clathrin immunoprecipitated by anti-heavy chain antibodies X19 and X22 appeared to contain heavy chain and LC_a light chain, but a band at the expected molecular weight for LC_b light chain was barely detectable. To investigate this result, X19 and X22 were used to immunoprecipitate clathrin from cells labeled with [^3H]lysine or [^3H]leucine for 3–5 h or from cells labeled with [^{35}S]methionine for as long as 24 h. In some of these immunoprecipitates the LC_b band was more clearly visible than in the examples shown in Fig. 1, but it was always lighter than the LC_a band. Its presence did not correlate with the amino acid used for labeling, the length of the labeling period, or whether the cell lysate had been frozen before immunoprecipitation, which might have caused preferential degradation. The X19 and X22 immunoprecipitates also contained a series of polypeptides from 60–150 kD, some of which are presumably clathrin- or coated vesicle–associated proteins. Others correspond to bands seen in the immunoprecipitate obtained with a control antibody of the same isotype, Anti-Leu 4.

This initial experiment established that anti-clathrin MAbs could be used to immunoprecipitate biosynthetically radiolabeled clathrin from cell lysate to study its form in vivo. The pulse-chase experiments described below were carried out to determine whether the free LC_a immunoprecipitated by the X16 antibody represents a diminishing pool of newly synthesized LC_a or is derived from a regenerating stable pool of free LC_a. Experiments on gel filtration of cell lysate were then carried out to determine the size of the heavy chain-LC_a complex immunoprecipitated by X19 and X22 and to see if a separate pool of free LC_a was immunoprecipitated by X16.

Fate of Clathrin Polypeptides After Biosynthesis

The stability of the intracellular pool of LC_a detected by the X16 MAb was determined by immunoprecipitation after a pulse-chase labeling. Cells were labeled for 10 min with [^{35}S]methionine and then diluted in medium containing an excess of unlabeled methionine (chase medium). Aliquots of cells were solubilized at the time of dilution and at specific intervals thereafter. The X16 (anti-LC_a), X22 (anti–heavy chain) and control MAbs were combined with these lysates, and immunoprecipitated antigen was analyzed by SDS PAGE and autoradiography. This experiment was carried out several times using the human B cell line, LB, and the bovine leukemia line, BL-3. The results were the same for both cell lines, and representative experiments are shown in Fig. 2. The X16 and anti-Leu 10 immunoprecipitates were obtained using preformed immune complexes according to the method of van Agthoven et al. (21). The X22 and anti-Leu 4 immunoprecipitates were isolated with Staph A. The first method produced less background immunoprecipitation, as indicated by the arrows at the right of the anti-Leu 10 and anti-Leu 4

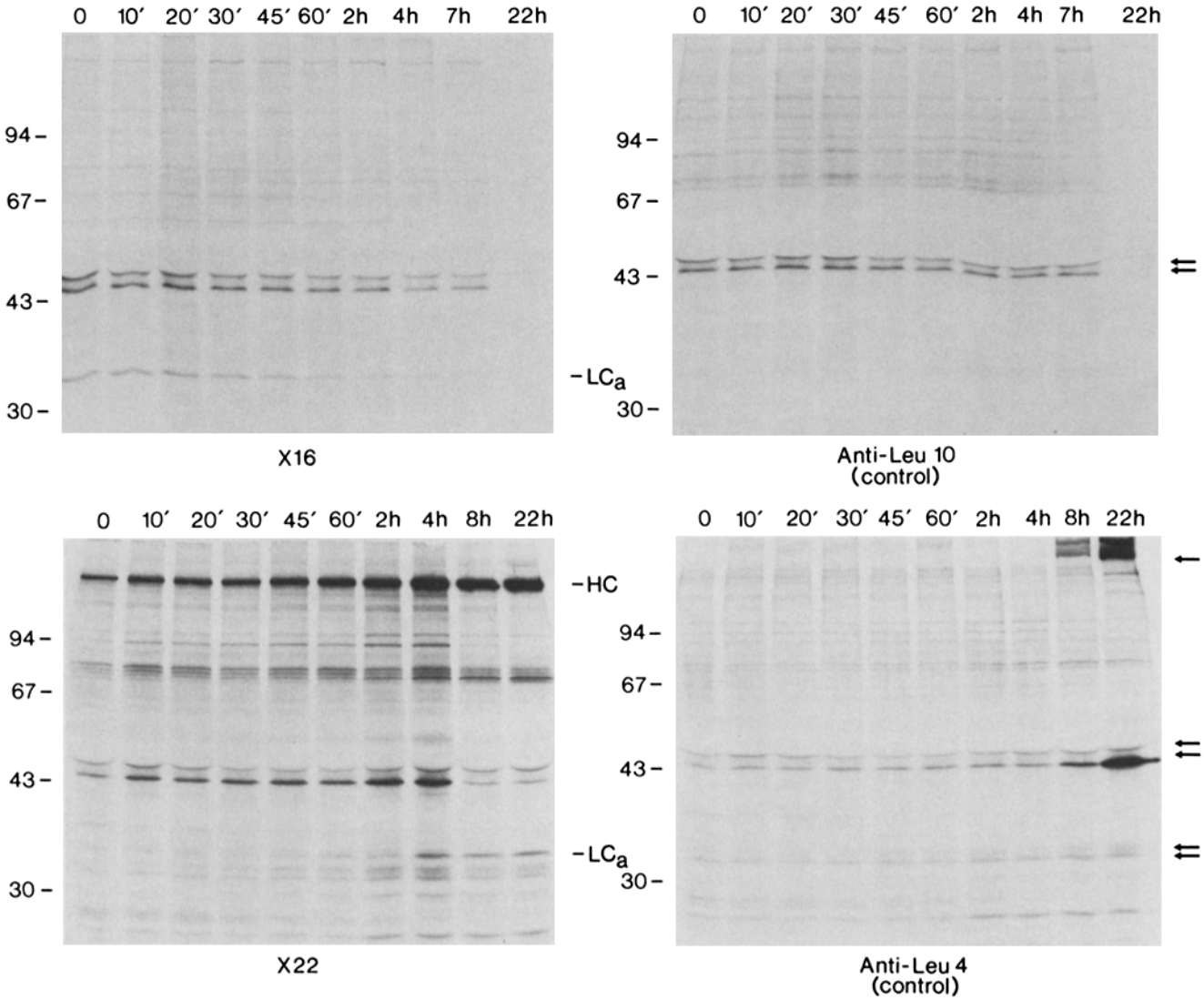

FIGURE 2 Clathrin biosynthesis in BL-3 and LB cells analyzed by pulse-chase labeling and immunoprecipitation. Cells were labeled for 10 min with [^{35}S]methionine. Then chase medium, containing excess unlabeled methionine, was added at time 0. The MAbs indicated below each panel were used to immunoprecipitate antigen from aliquots of cells solubilized at the chase times indicated at the top of each panel. The prime symbol stands for minutes. Each panel shows an autoradiograph of SDS PAGE analysis of the immunoprecipitates. At the left of each panel are the migration positions of the molecular weight markers used. At the right of the X16 and X22 panels the immunoprecipitated clathrin components are indicated. *HC*, heavy chain. At the right of the Anti-Leu 10 and Anti-Leu 4 panels the background bands are indicated. The X16 and anti-Leu 10 immunoprecipitates are from pulse-labeled BL-3 cells and were obtained by the immune complex method. The X22 and anti-Leu 4 immunoprecipitates are from pulse-labeled LB cells and were obtained by the Staph A method.

autoradiographs. However, the second method gave more efficient results with the X22 antibody. The position of the LC_a polypeptides immunoprecipitated by X16 is indicated at the right of the autoradiograph. The amount of free LC_a immunoprecipitated began to decrease by 30 min, and gradual reduction continued for several hours. By 1 h of chase time, less than half of the LC_a detected immediately after the pulse labeling was present. However, some free LC_a could still be immunoprecipitated 4 h after biosynthesis, and it was just barely detectable for as long as 7 h after chase medium was added. Additional experiments showed that it completely disappeared by 8 h. The amount of clathrin heavy chain immunoprecipitated by X22 increased up to 4 h after chase medium was added and then leveled off. The amount of coprecipitated LC_a increased with the same kinetics. Although some newly synthesized LC_a could be found in a free pool after biosynthesis, a faint band at the molecular weight of LC_a could be seen associated with heavy chain in the X22 immunoprecipitates, within 10 min after synthesis. The presence of LC_a associated with heavy chain at the time chase medium was added was confirmed by longer exposure of the X22 autoradiograph.

Sizing Clathrin Molecules in Cell Lysates

To determine the size of clathrin molecules present in vivo, columns (0.9 × 30 cm) composed of gel filtration media Ultrogel AcA 22 and Sephacryl S-1000 were used. These columns were calibrated with known molecular weight markers, with clathrin triskelions purified from bovine brain, and with bovine brain coated vesicles, which were applied to the

columns in the volume to be used for the test sample. After calibration, radiolabeled cell lysates were applied to the columns and fractions were collected. The MAbs X22 (anti–heavy chain), CVC.6 (anti-LC_a in triskelions), and X16 (anti–free LC_a) were used to immunoprecipitate clathrin from these fractions and thereby determine the elution position of in vivo clathrin. X22 and CVC.6 were chosen because they reacted with clathrin in triskelion and coated vesicle form when tested as immunoaffinity columns. X16 was used to confirm the existence of a free pool of the light chain. The bovine lymphoid cell line, BL-3, was used for radiolabeled lysate preparation because the CVC.6 antibody, which immunoprecipitates LC_a associated with clathrin heavy chain, reacts only with bovine clathrin. In a typical experiment, lysate was applied to a column and the fractions collected were immunoprecipitated with a specific antibody and a control antibody. The fractions analyzed in the experiments described below are all numbered starting with the void volume and therefore represent equivalent elution positions off of each column.

Fig. 3 shows results obtained with the AcA 22 sizing column. The calibration profile, seen in Fig. 3*A*, shows resolution of molecular weight markers in the 17 to 670 kD range. Calibration with purified clathrin triskelions and coated vesicles showed that both species co-eluted in the void volume with blue dextran, even though the buffer was at pH 8.0 and contained chelating agents to stabilize the triskelion form (12). Lysate was applied to the column, and fractions from

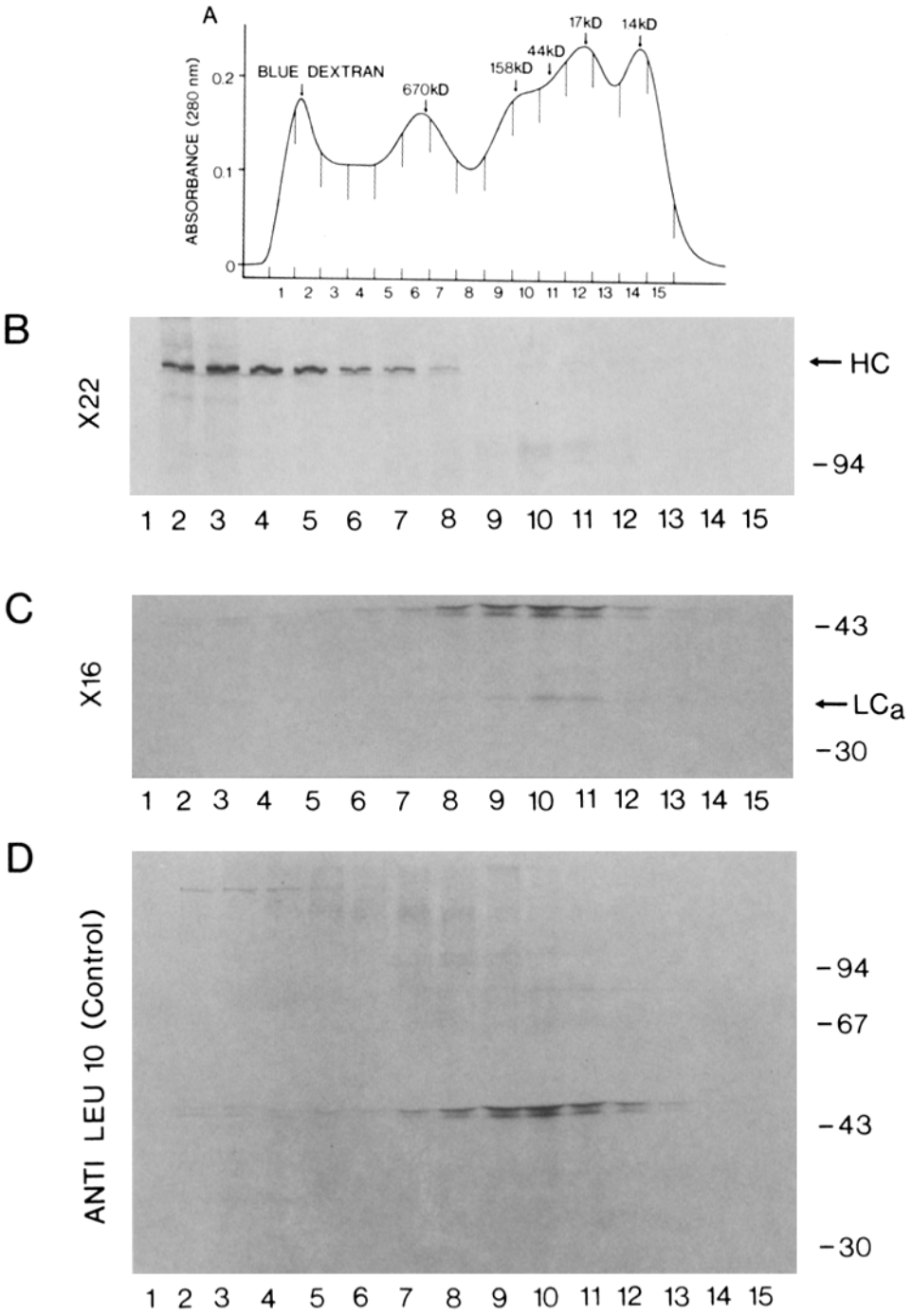

FIGURE 3 The size of in vivo clathrin examined by AcA 22 fractionation of radiolabeled cell lysate. Protein standards of known molecular weight were separated on a column (0.9 × 30 cm) of Ultrogel AcA 22, and 15 1-ml fractions were collected. The absorbance profile of the column eluate is shown in *A*. The elution position of each protein was confirmed by SDS PAGE analysis. The proteins applied to the column were thyroglobulin (670 kD), IgG (158 kD), ovalbumin (44 kD), myoglobin (17 kD), and vitamin B-12 (1.4 kD). Purified clathrin triskelions and coated vesicles eluted in the void volume (not shown). Cell lysate (1 ml) prepared from [^{35}S]-methionine-labeled BL-3 cells (2 × 10^7) was fractionated on the same Ultrogel AcA 22 column, and corresponding fractions were collected. These fractions were divided into two aliquots and used for immunoprecipitation with anti-clathrin and control MAb anti-Leu 10. Immunoprecipitates were analyzed by SDS PAGE and autoradiography. *B* shows the clathrin heavy chain (*HC*) region of the autoradiograph of X22 immunoprecipitates. *C* shows the LC_a light chain region of the autoradiograph of X16 immunoprecipitates. *D* shows the region of the autoradiograph of anti-Leu 10 immunoprecipitates corresponding to *B* and *C*. Immunoprecipitates shown in *C* and *D* were obtained from the same fractions. The control immunoprecipitates for the results in *D* were identical to those shown in *D*. The migration of molecular weight markers is indicated at the right of each panel.

one run were immunoprecipitated with X22 (Fig. 3*B*) and anti-Leu 10. Fractions from a second run were immunoprecipitated with CVC.6 and anti-Leu 10. A third run was immunoprecipitated with X16 (Fig. 3*C*) and anti-Leu 10 (Fig. 3*D*). Since the anti-Leu 10 results were similar, only one example is shown. The CVC.6 and X22 antibodies both immunoprecipitated clathrin (heavy chain and LC_a light chain) from the fractions containing blue dextran. The peak trailed into the column, but there was no additional peak of heavy chain clathrin precipitated at a lower molecular weight than triskelions. The X16 MAb immunoprecipitated free LC_a light chain in a separate peak of lower molecular weight than triskelions.

Since purified coated vesicles eluted off the AcA 22 column at the same position as clathrin triskelions, it was of interest to repeat these experiments on a column that would distinguish between triskelions and coated vesicles. The S-1000 column was used for this purpose. Calibration of this column is shown in Fig. 4. The peak of triskelion elution was contained in fractions 10–12 (Fig. 4*B*), and the peak of coated vesicle elution was located in fractions 6–8 (Fig. 4*C*). Analysis of immunoprecipitates from two lysate runs on the S-1000 column (Fig. 5) shows that the peak of clathrin isolated by the CVC.6 antibody (Fig. 5*A*) spanned the positions of elution of the coated vesicle and triskelion peaks. The clathrin peak isolated by X22 (Fig. 5*B*) overlapped primarily with the coated vesicle peak. Again, the clathrin immunoprecipitated by both antibodies (CVC.6 and X22) contained heavy chain and LC_a light chain. Control autoradiographs (not shown) of immunoprecipitates obtained with anti-Leu 10 from these fractions had essentially no bands in the heavy chain region.

DISCUSSION

In the experiments described above, anti-clathrin MAbs have been used to immunoprecipitate clathrin directly from cell

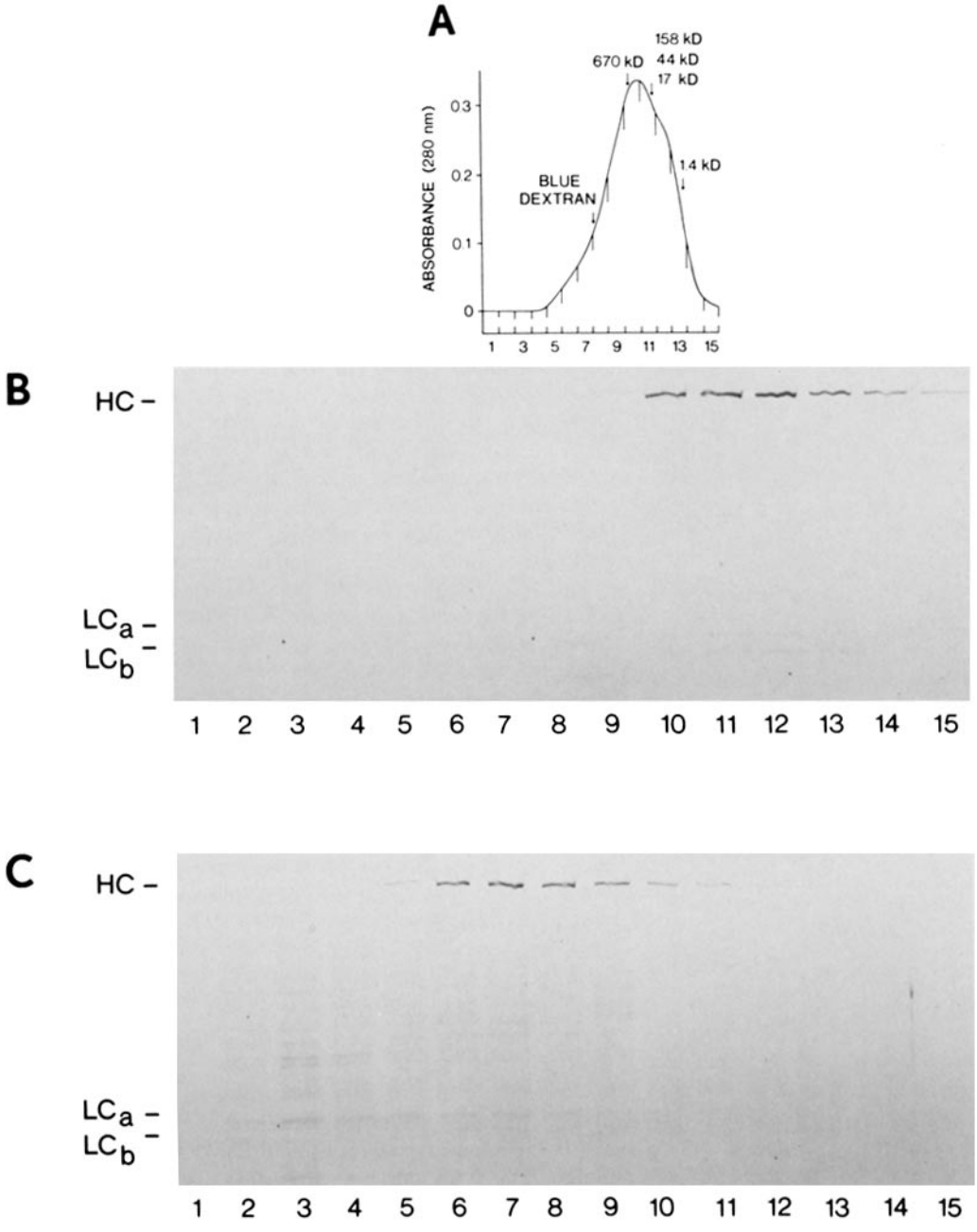

FIGURE 4 S-1000 fractionation of (*A*) standard proteins, (*B*) clathrin triskelions, and (*C*) coated vesicles. Protein standards of known molecular weight were separated on a column (0.9 × 30 cm) of Sephacryl S-1000, and 15 1-ml fractions were collected. The absorbance profile of the column eluate is shown in *A*. The proteins applied to the column were the same as described in the legend to Fig. 3. Their elution position was confirmed by SDS PAGE. Clathrin triskelions (*B*) and coated vesicles (*C*), purified from bovine brain, were applied to the same Sephacryl S-1000 column. Corresponding fractions were collected, and a 20-μl sample of each fraction was analyzed by SDS PAGE. Gels were stained with Coomassie Blue and photographed. The migration positions of the clathrin heavy chain (*HC*) and light chains (LC_a and LC_b) are indicated at the left of *B* and *C*.

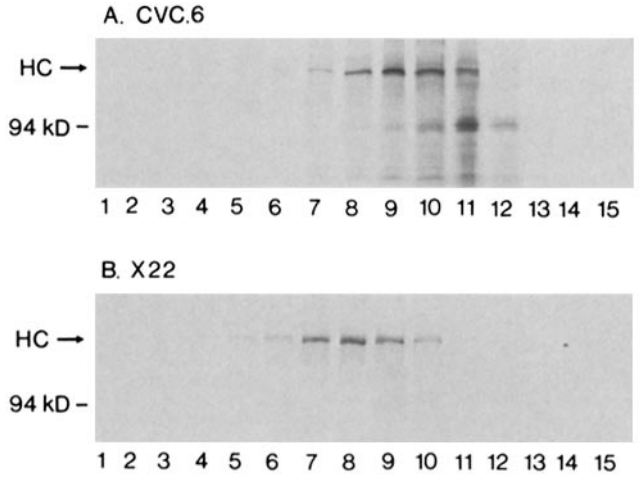

FIGURE 5 The size of in vivo clathrin examined by S-1000 fractionation of radiolabeled cell lysate. Cell lysate (1 ml) prepared from [^{35}S]methionine-labeled BL-3 cells (2×10^7) was fractionated on the Sephacryl S-1000 column described in Fig. 4, and corresponding fractions were collected. These fractions were divided into two aliquots and used for immunoprecipitation with anti-clathrin and control MAb anti-Leu 10. Immunoprecipitates were analyzed by SDS PAGE and autoradiography. *A* shows the region of the autoradiograph of the CVC.6 immunoprecipitates that contain the clathrin heavy chain (*HC*). *B* shows the corresponding region of the autoradiograph of X22 immunoprecipitates. These immunoprecipitates were obtained from different sets of fractions collected after identical lysate fractionations. The bands shown are unique as compared with those of autoradiographs of anti-Leu 10 immunoprecipitates from the same fractions (not shown). The migration position of the molecular weight marker, phosphorylase b (94 kD), is shown at the left of both panels.

lysates to examine the forms of clathrin that exist in vivo. Using anti-clathrin heavy chain MAbs (X19 and X22) and an MAb (X16) that immunoprecipitates LC_a light chain only if it is not associated with heavy chain, it was demonstrated that the LC_a light chain can be found associated with heavy chain or in free form. To determine the size of the immunoprecipitated structures and the kinetics of their intracellular existence, immunoprecipitation was carried out after cell lysate fractionation by column chromatography or after pulse-chase labeling of the cells used to prepare lysates. Lysate fractionation on a column of Ultrogel AcA 22 showed that clathrin heavy chain associated with LC_a light chain localized to the void volume, as determined by immunoprecipitation with anti-heavy chain MAb X22 and CVC.6, which preferentially immunoprecipitates LC_a in association with heavy chain. Both antibodies reacted exclusively with clathrin in this peak. Calibration with purified triskelions and coated vesicles showed that both structures eluted in the void volume, demonstrating that triskelions that are ~645 kD (9, 10) eluted at a higher apparent molecular weight, perhaps as a result of their unusual geometry. The X16 MAb (anti-free LC_a) isolated LC_a light chain from a separate peak, eluting at lower molecular weight. This elution zone corresponded to fractionation of the 44–158-kD protein standards, which is higher than would be expected for a single free light chain. However it has been shown previously that on a Spherogel TSK 3000 column, free clathrin light chains run with an apparent molecular weight of 90 kD (26). They appear to have similar properties in this system. Lysate from cells labeled continuously for 4 h was then applied to a Sephacryl S-1000 column, which resolves purified coated vesicles from triskelions, running at a position corresponding to their expected molecular weight on this particular column. The X22 MAb immunoprecipitated clathrin primarily from the elution position of coated vesicles. The CVC.6 MAb isolated clathrin, which comigrates with both coated vesicles and triskelions. Within the limit of the resolution of the AcA 22 and S-1000 columns, and the specificities of the antibodies used, no other peaks containing clathrin heavy chain in association with light chain were identified. These results indicate that the triskelion and coated vesicle forms identified for depolymerized and polymerized clathrin in vitro also exist in vivo. The results obtained with X16 suggest that, in addition, a pool of free LC_a light chain can be found in vivo.

One result that is still unexplained is the predominance of the LC_a light chain seen in immunoprecipitates obtained with the anti-heavy chain MAbs, X19 and X22. This is unexpected because the light chains of bovine brain clathrin purified in vitro apparently have a random distribution in their association with heavy chain in triskelions (12). A simple explanation is that lymphoid cell clathrin might contain predominantly LC_a light chain and that the 1:2 ratio of LC_a/LC_b light chain, found in brain tissue, is not typical of other tissues (9, 10). However, the variation in amounts of immunoprecipitated LC_b suggests that an additional factor may influence the LC_a/LC_b ratio in immunoprecipitates. One possibility is that triskelions and even coated vesicles in vivo may have a nonrandom distribution of the two light chains. More experiments on light chain quantitation in different tissues and the development of an anti-LC_b MAb are needed to clarify the question of light chain distribution in vivo.

Immunoprecipitation after pulse-chase labeling showed that the amount of free LC_a immunoprecipitated by the X16 MAb decreased gradually after biosynthesis but could be detected for as long as 7 h. Within 1 h after biosynthesis the amount of free LC_a detected was less than half of that detected just after pulse labeling. Parallel experiments with X22 showed that labeled clathrin heavy chain and light chain were increasingly incorporated into coated vesicles for up to 4 h. After 4 h, the amount of labeled clathrin in coated vesicles dropped slightly and then remained constant for at least 22 h. This indicates there is little turnover of the heavy chain and light chain involved in coated vesicle formation within a 22-h period. The fact that the heavy chain and associated light chain appear in coated vesicles with similar kinetics suggests that they are either incorporated simultaneously or become associated with each other as triskelions before assembly. Since the rate of free LC_a disappearance was more rapid than that of coated vesicle formation, light chain and heavy chain probably form triskelions before assembly occurs. Comparing the kinetics of reduction of the free LC_a pool with those of coated vesicle formation also suggests that the association of LC_a with heavy chain is irreversible once the complex has been incorporated into a coated vesicle. This implies that LC_a remains associated with heavy chain when coated vesicles depolymerize.

The kinetics of clathrin biosynthesis determined above were found to be equivalent in lymphoid cell lines derived from a bovine leukemia cell and an Epstein Barr virus–transformed human B cell. Although, it is possible that the kinetics of clathrin biosynthesis may vary among cell types, it is still

interesting to compare these results with the biosynthesis of other proteins composed of multiple subunits. Class I histocompatibility antigen heavy chain and β_2-microglobulin light chain associate within 15 min after biosynthesis, and this association is required for expression on the cell surface (20, 27). Vimentin, which is initially synthesized as a soluble cytoplasmic precursor, becomes completely associated with the cytoskeleton by polymerization into intermediate filaments within 2 h, and the soluble form has a half life of 7 min (28). Most newly synthesized α-spectrin is found in a cytoplasmic pool; either the pool rapidly associates with the β-spectrin in the cytoskeleton, or the excess is degraded with a half-life of 45–60 min (29, 30). It is possible that, similar to α-spectrin, LC_a is synthesized in excess of heavy chain, and reduction of the free pool detected by X16 indicates the degradation rate of LC_a that has not associated with heavy chain. It is also possible that the reduction of free LC_a directly reflects the rate at which LC_a becomes associated with heavy chain and is therefore no longer detectable with the X16 MAb. These two explanations will be resolved when methods for quantitive immunoprecipitation have been worked out. Further experiments will also be required to determine the kinetics of light chain–heavy chain association before coated vesicle formation, and whether a pool of heavy chain monomers or trimers (31) exists that might interact with the free light chain pool. Such investigations will become possible as more monoclonal antibodies with specificity for clathrin in different structural forms are produced.

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