Location and distribution of the light chains in clathrin trimers

(monoclonal antibody/single-molecule immunoelectron microscopy/coated vesicle)

T. KIRCHHAUSEN*, S. C. HARRISON*, P. PARHAM†, AND F. M. BRODSKY†

*Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, Massachusetts 02138; and †Department of Structural Biology, Stanford University School of Medicine, Stanford, California 94305

ABSTRACT Mouse monoclonal antibodies have been made that are specific for one of the light chains (LC-A) of calf brain clathrin. The determinant they recognize has been mapped by immunoelectron microscopy and shown to lie near the center of a clathrin trimer. Quantitative immunoprecipitation experiments with one of the monoclonal antibodies suggests that, despite the overall 1:2 stoichiometry, the light chain composition of an individual clathrin trimer is nearly random.

Clathrin isolated from coated vesicles of bovine brain is a trimer of three heavy chains and three light chains (1). The heavy chains have a molecular weight of 180,000; the light chains are of two species—LC-A, M_r 36,000; LC-B, M_r 33,000—in a ratio (mol/mol) of 1:2 (1–3). Each heavy chain has a single closely associated light chain (1). Purified trimers can reassemble *in vitro* into coat-like structures referred to as cages (1, 3–7).

Clathrin trimers have been visualized in the electron microscope by rotary shadowing (3) and by negative staining (1, 7). Their unusual tribrachial shape (see Fig. 4) makes immunolabeling and single-molecule electron microscopy useful for exploring the localization of different components. The spatial arrangement of ribosomal proteins has been determined with this technique by using affinity-purified polyclonal antibodies bound to specific surface sites (8, 9). The use of monoclonal antibodies as a structural probe has the advantage that they recognize a unique antigenic region. The possibility for spurious crossreactions and nonspecific binding is thus greatly reduced. We have generated mouse monoclonal antibodies against purified clathrin and mapped the location of one of the antigenic regions. We show here that three monoclonal antibodies specifically recognizing light chain LC-A bind at a single site located at the proximal end of a clathrin arm.

We have also used one of these monoclonal antibodies to examine the distribution of light chains LC-A and LC-B in the population of trimers. The 1:2 ratio of these species would be consistent with a distribution of one LC-A and two LC-B on every trimer (note that this could only be true if the 1:2 ratio were precise); with a distribution into two classes of trimers in a 1:2 ratio (mol/mol), each with three copies of a single species of light chain; or with a more random distribution with trimers having all possible LC-A/LC-B ratios from 3:0 to 0:3. Immunoprecipitation of clathrin with a monoclonal antibody against LC-A gives results consistent only with the third possibility: that is, with a nearly random distribution of light chains.

METHODS

Clathrin Purification. Coated vesicles were isolated from calf brains (1), and the clathrin was solubilized by incubation overnight at 4°C in 0.8–1.0 M Tris·HCl, pH 7.0/1 mM EGTA/ 0.2 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride (PhMeSO₂F)/0.02% NaN₃. After dilution with 1 vol of water, lipid vesicles and other membrane residues were removed by two centrifugations (41,000 rpm, 60 min, Beckman type 42 rotor). Clathrin was precipitated from the supernatant by addition of ammonium sulfate to a final concentration of 50% saturated, collected by centrifugation, and redissolved in the same concentrated Tris buffer. The remaining ammonium sulfate was removed by dialysis against 0.5 M Tris·HCl pH 7.0/1 mM EGTA/0.2 mM dithiothreitol/0.5 mM PhMeSO₂F/0.02% NaN₃. The final gel filtration purification was carried out as described (1).

Immunization and Production of Anticlathrin Monoclonal Antibodies. Female BALB/c mice were immunized with purified clathrin. An initial subcutaneous injection of 50 µg complete in Freund's adjuvant was followed by subcutaneous injections of 50 µg and 100 µg in incomplete Freund's adjuvant 5 and 11 wk later and by intraperitoneal injection of 50 µg in incomplete Freund's adjuvant 15 wk later (7 days before fusion). On days 4, 3, and 2 before fusion, one mouse received 50–100 µg of clathrin, both intravenously and intraperitoneally (10). Anticlathrin antibodies were present 6.5 wk after immunization in the sera of all mice, at titers of 1:640 when tested by radioimmunoassay (RIA). Spleen cells from one mouse were fused with 10⁷ SP2/O-Ag14 myeloma cells in 50% polyethylene glycol 1500/RPMI 1640 medium (11). Supernatants from wells containing hybridomas were tested by RIA (see below). Cells from positive wells were grown in mass culture for 2 months, and four cultures retaining anticlathrin activity were cloned by using a fluorescence-activated cell sorter (12). Of the cloned lines, CVC.1, CVC.6, and CVC.7 produced IgG1, IgG1, and IgG2a, respectively, as shown by binding of ¹²⁵I-labeled anti-allotype monoclonal antibodies 20-8.3 and 20-9.10 (13). The other cloned line produced IgM, which was difficult to characterize because of high background binding. IgG-producing lines were passaged as ascites tumors in BALB/c mice, and 100–150 ml of ascites was obtained for each clone. IgG was purified from each pool of ascites as described (14). Initial ammonium sulfate precipitates were purified on Sephadex G-150 or G-200 to give 80–90% pure preparations of immunoglobulin.

Cell Culture. The SP2/O-Ag14 myeloma line was passaged in RPMI 1640 medium/10% fetal calf serum containing penicillin and streptomycin (100 units/ml each) and 0.1 mM 8-aza-guanine (left out two passages before fusion) (15). Hybridoma cells were grown in RPMI 1640 medium/10% fetal calf serum supplemented with hypoxanthine/aminopterin/thymidine. Fused myeloma and spleen cells were seeded in 24-well plates (Linbro) with irradiated (3,000 rads; 1 rad = 0.01 gray) human fibroblasts as a feeder layer. Feeders were also used in early

Abbreviations: LC-A and LC-B, light chains of clathrin; RIA, radioimmunoassay; Tris/NaCl, 10 mM Tris·HCl/0.9% NaCl, pH 7.5; PhMeSO₂F, phenylmethylsulfonyl fluoride.

passages of the hybridomas (16). Cells were cloned in 96-well microtiter plates (Falcon) with mouse peritoneal cells (10^6 per plate) as feeders (17). Hybridoma cells were passaged directly from hypoxanthine/aminopterin/thymidine-containing medium into BALB/c mice primed with 0.5 ml of Pristane by intraperitoneal injection of 5×10^6 cells per mouse. Ascites fluid was collected by peritoneal puncture into heparin (10 units/ml) and centrifuged at 1,500 rpm for 10 min. The supernatant was stored frozen in 0.02% NaN_3 at $-20°C$ and the cells were injected into additional mice.

Antibody Assays. *Iodination.* ^{125}I-Labeled rabbit anti-mouse IgG F(ab')$_2$ fragments and ^{125}I-labeled clathrin were prepared using chloramine-T (18). Clathrin was dialyzed into phosphate-buffered saline before iodination. ^{125}I-Labeled goat anti-mouse IgG F(ab')$_2$ fragments were prepared with Iodo-Gen (Pierce). ^{125}I-Labeled 20-8.3 and ^{125}I-labeled 20-9.10 monoclonal anti-mouse IgG allotype were a gift of L. A. Herzenberg (13).

Indirect RIA. RIA was carried out as in ref. 19. The initial clathrin concentration was 20–30 $\mu g/ml$.

Electrophoretic blotting. Clathrin and coated vesicle proteins were separated by 10% NaDodSO$_4$/polyacrylamide gel electrophoresis (20). The gel was cut into strips, and proteins were transferred by electrophoresis to nitrocellulose paper (0.45-μm pore size) in 20% methanol/25 mM Tris·HCl/192 mM glycine, pH 8.3 (21). One paper was stained in Amido black, and the others were incubated for 1 hr at 37°C in 10 mM Tris·HCl/0.9% NaCl, pH 7.4 (Tris/NaCl)/3% bovine serum albumin and then for 5 hr at room temperature with monoclonal antibody at 9 $\mu g/ml$ in Tris/NaCl/3% bovine serum albumin/5% heat-inactivated rabbit serum. Papers were washed with 50-ml portions of Tris/NaCl, 0.5 M NaCl/Tris/NaCl, 0.1% Triton X-100/Tris/NaCl, Tris/NaCl, and 1% bovine serum albumin/Tris/NaCl, labeled with ^{125}I-RAM (10^6 cpm, 100 ng/ml) for 3 hr at room temperature, washed five times in 0.1% Triton X-100/Tris/NaCl, dried, and exposed to Kodak XAR-5 film.

Immunoprecipitation. Clathrin (25 μg in 5–15 μl) was incubated with CVC.7 (1 μl at 20 mg/ml) at 4°C for 2 to 3 hr and then 15 μl of goat anti-mouse IgG (10 mg/ml, Boehringer-Mannheim) was added. After 1–1.5 hr at 4°C, 130 μl of buffer was added and the mixture was centrifuged in an Airfuge (Beckman) for 20 min at room temperature and 100,000 rpm. Immunoprecipitated complexes and the remaining supernatant were assayed by NaDodSO$_4$/polyacrylamide gel electrophoresis (20). Immediately before the reaction with the monoclonal antibody, aggregates and self-assembled cages were removed by centrifugation in the Airfuge. Buffers used were 50 mM phosphate, pH 7.8/100 mM triethanolamine, pH 7.8, or 500 mM Tris·HCl, pH 7.2/1 mM EDTA/0.5 mM PhMeSO$_2$F/0.02% NaN$_3$.

Electron microscopy. Single molecules were visualized by rotary shadowing (22). Samples at 40–80 nmol of protein in 45% glycerol/50 mM Tris·HCl, pH 7.4, were sprayed onto freshly cleaved mica, dried at reduced pressure, and rotary shadowed with Pt at a glancing angle of 7.5°. Preparations were examined at 80 kV in a JEOL 100 CXII.

Clathrin–antibody complexes. We have used two methods to prepare specimens for electron microscopy. In one approach, clathrin at ≈2.0 mg/ml and antibody are incubated together at 2–5:1 (mol/mol), and then diluted 1:50 with 45% glycerol/50 mM Tris·HCl, pH 7.4 (4°C), dispersed, and shadowed directly. In the other, a large excess of antibody is used, and free antibody is removed by rapid gel filtration through a small column of Ultrogel ACA-22 (LKB) equilibrated with 50 mM Tris·HCl, pH 7.4/1 mM EGTA in a 1-ml graduated pipette. Drops that appear after ≈15 min, corresponding to the void volume, are diluted 1:1 with 90% glycerol/100 mM Tris·HCl, pH 7.4, and

visualized as described. It is necessary to work quickly to avoid dissociation of the complex.

RESULTS

Monoclonal Antibodies to Clathrin. Four hybridoma cell lines were obtained producing monoclonal antibodies specific for native clathrin. The three IgG-producing lines—CVC.1, CVC.6, and CVC.7—were used in this study.

Elastase treatment selectively degrades light chains (1), and an indication that antigenic sites for all three monoclonal IgG are on light chains came from the observation that mild elastase proteolysis of clathrin strongly reduced antibody binding. The NaDodSO$_4$/polyacrylamide gel electrophoresis analysis of a series of elastase digests of clathrin and the corresponding binding characteristics for CVC.7 are shown in Fig. 1. Similar results were obtained with CVC.1 and CVC.6 (data not shown). Electrophoretic blotting gives more direct evidence that all three antibodies recognize a site on LC-A (Fig. 2). There is strong labeling of the M_r 36,000 band (LC-A) and faint labeling of an additional band, just below LC-B. We believe that this latter band represents a proteolytic cleavage product of one or both light chains, since its strength increases with time of storage of any clathrin preparation.

Localization of the Antigenic Region of LC-A Along an Arm of the Clathrin Pinwheel. Electron microscopy of rotary-shadowed complexes of clathrin trimers and anti-LC-A monoclonal antibodies locates the determinant at the proximal end of a clathrin arm. A field of clathrin–CVC.1 complex is shown in Fig. 3A. The sample was prepared by directly shadowing a mixture containing a 2-molar excess of clathrin. The antibody can in some cases be recognized as a trilobal structure attached to the arm, in others simply by its overall dimensions. Free antibodies provide control images (open arrow). The arms of native clathrin have relatively uniform thickness, facilitating identification of the antibody. Clear images were scored according to the location of the bound antibody (Table 1). Some images
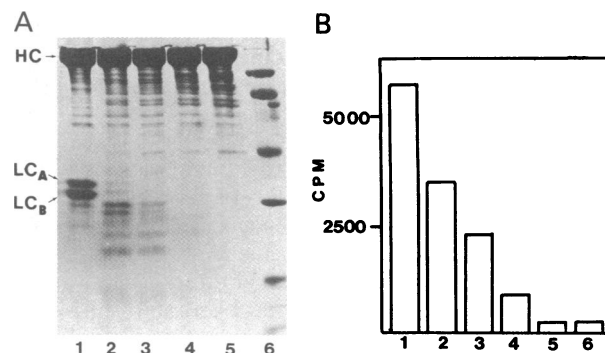

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis and RIA of elastase-treated clathrin. Aliquots (200 μl) of clathrin (0.29 mg/ml) were incubated for 6 hr at room temperature with 2–12 μl of elastase (Worthington) containing 0.06, 0.12, 0.25, and 0.45 μg of elastase (samples 2–5, respectively). Proteolysis was stopped with 2 μl of 50 mM PhMeSO$_2$F (in ethanol). (*A*) NaDodSO$_4$ (10%)/polyacrylamide gel electrophoresis (20) of overloaded samples (25 μg) of native clathrin (lane 1) and elastase-digested clathrin (lanes 2–5). The heavy chain (HC) and light chains LC-A and LC-B of brain clathrin migrate with apparent molecular weights of 180,000, 36,000, and 33,000, respectively. Elastase preferentially degrades the light chains. Standards (lane 6) were phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme (Bio-Rad Laboratories). (*B*) RIA of clathrin (sample 1), elastase-digested clathrin (samples 2–5), and background control (sample 6, no clathrin). Anticlathrin monoclonal antibody CVC.7 and ^{125}I-labeled goat anti-mouse IgG F(ab')$_2$ were used as first and second antibody, respectively.
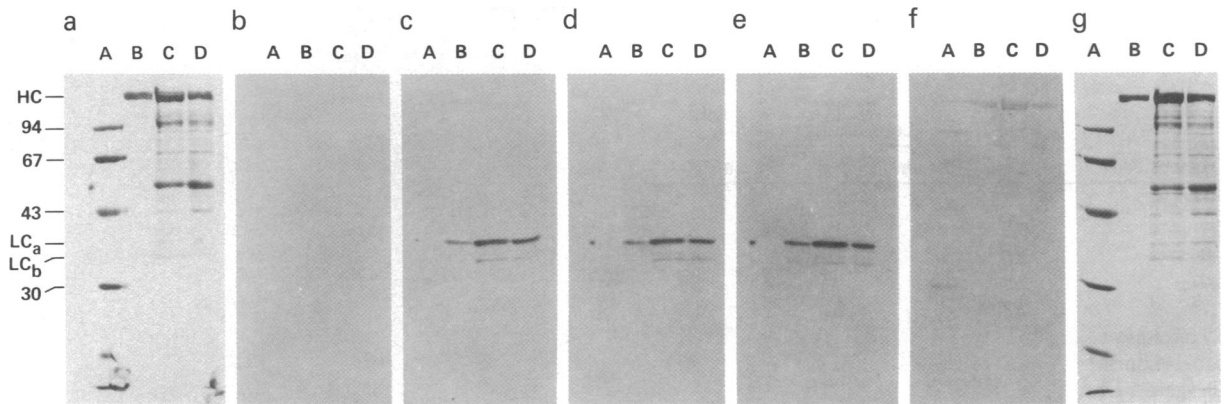
FIG. 2. Electrophoretic blots of pure clathrin and proteins from coated vesicles probed with anticlathrin monoclonal antibodies. (*a*) Amido black-stained nitrocellulose paper (post-transfer). (*g*) Coomassie brilliant blue-stained gel strip (pretransfer) from the same experiment. (*c–e*) Blots with anticlathrin monoclonal antibodies CVC.1, CVC.6, and CVC.7. (*b* and *f*) Blots with MB40.5 and W6/32, control antibodies of the same isotypes against the human histocompatibility antigens HLA-A,B,C (23, 24). Lanes: A, standards (see Fig. 1A); B, purified clathrin (2 μg per lane); C and D, proteins from coated vesicles (≈2.6 and 1.3 μg of clathrin per lane) from the 10% sucrose step of the second gradient and the 5% sucrose step of the first gradient of the purification procedure (1).

appear to be ternary complexes of two clathrin molecules linked near their centers by an IgG (Fig. 3, indicated by stars). A number of trimers appear to have more than one antibody bound at their center, but unambiguous identification of the antibodies is difficult.

The presence of a large molar excess of IgG in the initial incubation can be used to enhance the binding to antigenic sites.

The high background of free IgG can be reduced by introducing a rapid gel filtration step to separate antigen–antibody complexes from unbound antibody. Fig. 3B shows a field of clathrin complexed with CVC.7. The antibody again appears always to bind near the center of a trimer (about 30% of the clathrin molecules scored, Table 1).

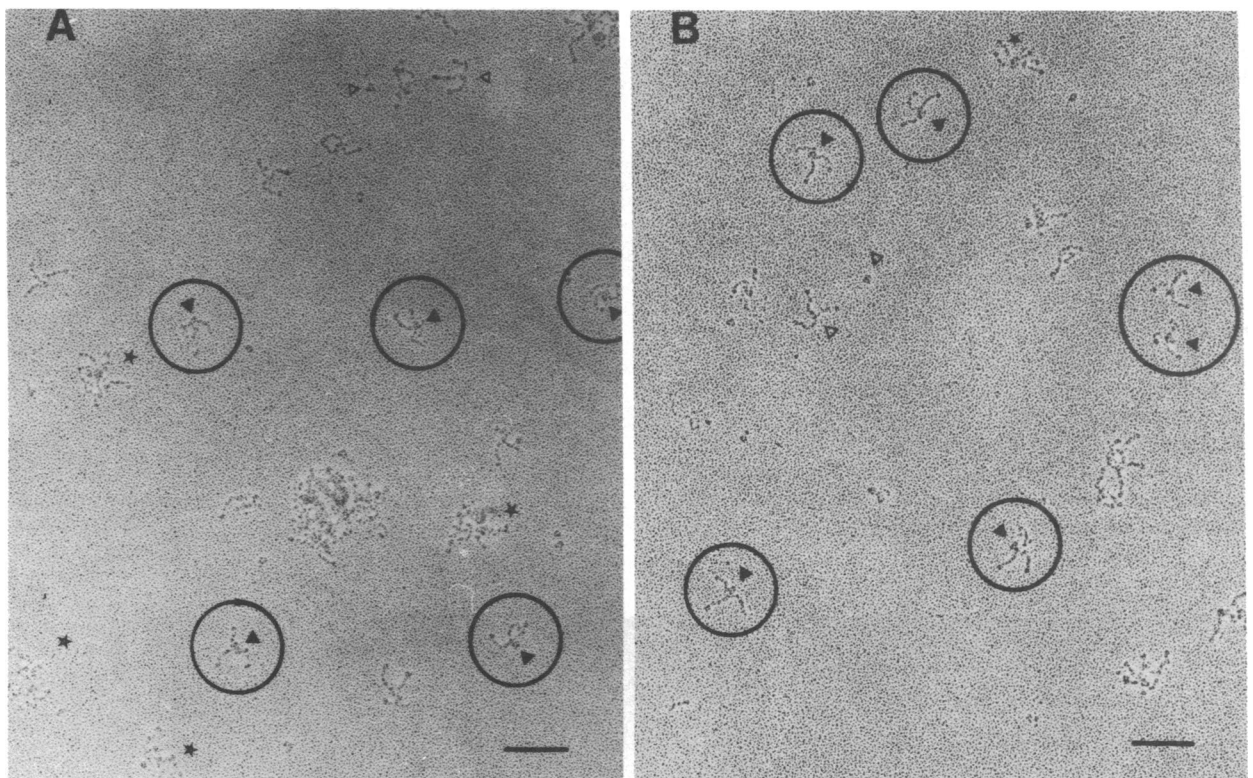A gallery of images of clathrin complexed with each of the

FIG. 3. Electron microscopy of complexes of clathrin trimers and anticlathrin monoclonal antibodies to light chain LC-A. (*A*) Clathrin trimers (10 μl, 2.1 mg/ml) were incubated with CVC.1 (1 μl, 0.9 mg/ml) for 10 min at 4°C and prepared for rotary shadowing. Clathrin trimers, each with a monoclonal antibody molecule (arrow) near the center of the trimer, are circled. Uninterpretable images of immunoaggregates can also be seen (star). Free clathrin and IgG molecules are indicated by empty arrows. The low frequency of free monoclonal antibodies adsorbed to the mica rules out the possibility that the images are of antibodies lying close to clathrin trimers by pure chance. (*B*) A mixture of 20 μl of clathrin (2.1 mg/ml) and 20 μl of CVC.7 (2.8 mg/ml) was incubated for 12 hr at 4°C. Free IgG was removed by gel filtration, and the complex was visualized by rotary shadowing. Clathrin trimers with antibodies at the center (arrow) are circled. Note that this procedure generates images with fewer free antibodies and a relatively higher frequency of clathrin–antibody complexes adsorbed to the mica surface. Free clathrin and IgG molecules are indicated by empty arrows. A complex of two clathrin molecules joined by a monoclonal antibody is indicated by a star. (Bars = 1,000 Å.)

Table 1. Position of binding of monoclonal antibody molecules to single clathrin trimers

Type of antibody	Conc., mg/ml	Localization			Trimers without bound antibody
		Center	Elbow	Tip	
Against LC-A					
CVC.1	0.9	23	1	4	67
CVC.6	1.6	12	1	5	41
CVC.7	2.8	37	0	2	59
CVC.7*	2.8	115	20	2	188
CVC.7/elastase-treated clathrin[†]	2.8	3	0	5	22
Controls against HLA					
MB40.5	1.8	2	4	3	41
W6/32	1.3	2	1	6	36
206	2.5	3	3	27	119

Unambiguous images were scored as indicated. Photographs were examined "blind" (i.e., without knowing the source of the antibody). Unless otherwise indicated, micrographs scored were from specimens prepared by incubation of 10 μl of clathrin (2.1 mg/ml in 50 mM Tris·HCl, pH 7.4/1 mM EGTA/0.02% NaN$_3$) with 1 μl of monoclonal antibody phosphate-buffered saline at 4°C for ≈15 min and then preparing the sample directly for microscopy. Conc., concentration.
* Clathrin was incubated overnight with a 5-fold molar excess of antibody and prior to visualization the free antibody was separated by gel filtration. (See Fig. 3.)
† Elastase-treated clathrin (10 μl, 2.1 mg/ml) was incubated with 1 μl of CVC.7 (2.8 mg/ml) at 4°C for ≈15 min and the mixture was prepared directly for microscopy.

three antibodies studied is shown in Fig. 4. In all cases, the LC-A determinant appears to be close to the trimer center.

The specificity of the interaction between clathrin and the monoclonal antibodies was checked by electron microscopy of clathrin with control monoclonal antibodies of the same isotype but directed against the HLA antigen. An example (Fig. 5 and Table 1) shows that most clathrin trimers are free but that occasional attached antibody can be seen. One of the antibodies we used (no. 206) tends to bind to the distal end of clathrin arms (Table 1). The specificity was also checked by microscopy of elastase-treated clathrin incubated with CVC.7 (Table 1).

Distribution of Light Chains LC-A and LC-B in the Population of Clathrin Trimers. The NaDodSO$_4$/polyacrylamide gel electrophoresis analysis of the quantitative immunoprecipitation of complexes of clathrin trimers and CVC.7 is shown in Fig. 6. About 60% of the clathrin heavy chain is precipitated (lane 6). This amount, estimated in several independent experiments, is consistent with the expected fraction of trimers that, on a random model, have one or more copies of LC-A.
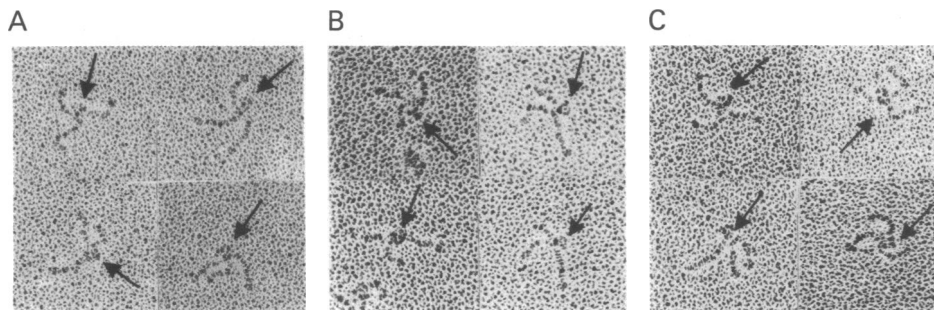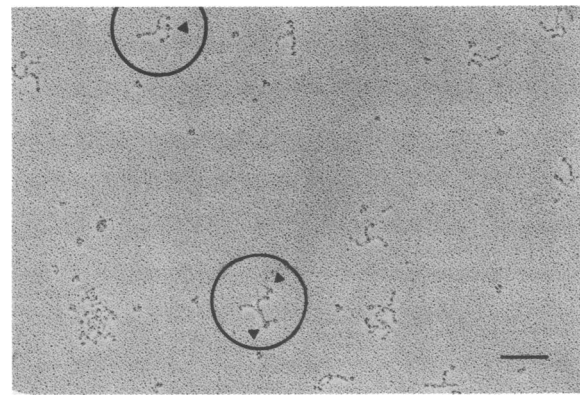

FIG. 5. Electron microscopy of clathrin trimers incubated with a monoclonal antibody not specific to clathrin. Clathrin (10 μl, 2.1 mg/ml) was mixed with anti-HLA monoclonal antibody Wb/32 (1 μl, 1.3 mg/ml) and rotary shadowed. Most of the clathrin trimers are free, but occasional molecules (circles) show bound antibodies at various sites. (Bar = 1,000 Å.)

Moreover, while LC-A is absent from trimers that remain in the supernatant (lane 5), the pellet has a LC-A/LC-B ratio of approximately 1:1 (lane 6). If the two species of light chains are distributed randomly on heavy chain arms, there will be four classes of trimers and they will have LC-A/LC-B ratios of 3:0, 2:1, 1:2, and 0:3. The population of trimers in each class can be calculated from the binomial distribution as $r^3:3r^2:3r:1$, where r is the overall LC-A/LC-B ratio in the sample. Since in our clathrin preparations, $r = 1/2$, the expected fractions are 0.04, 0.22, 0.44, and 0.30, respectively. The first three classes are the only ones expected to precipitate with monoclonal antibody to LC-A. The precipitated clathrin, 70% of the total, should contain LC-A and LC-B in molar ratio of 1:1.1, in agreement with results in Fig. 6. Alternative models for light-chain distribution are ruled out. If every trimer contained one LC-A and two LC-B, the light chain ratio would not be different in the immunoprecipitate, which should contain most if not all the clathrin. The results likewise rule out two distinct populations of clathrin trimers, each with only LC-A or only LC-B, because this model predicts that only one-third of the clathrin will complex with anti-LC-A and that no LC-B will appear in the precipitate. We therefore conclude that light chains LC-A and LC-B are distributed in a close to random fashion.

DISCUSSION

The three IgG monoclonal antibodies we have studied selectively recognize a determinant on LC-A, the M_r 36,000 light

FIG. 4. Selected views of complexes of clathrin trimers and anti-LC-A monoclonal antibodies CVC.1 (*A*), CVC.6 (*B*), and CVC.7 (*C*). Complexes are indicated by arrows. These images were generated with different clathrin preparations using direct shadowing of the mixtures (Fig. 3*A*). (Bar = 1,000 Å.)
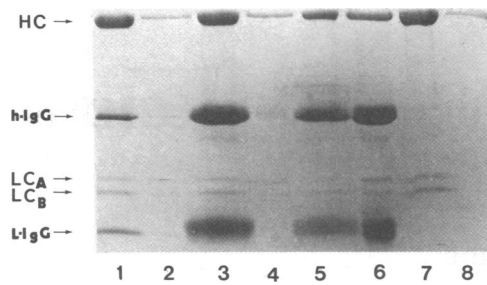
FIG. 6. NaDodSO$_4$ (12%)/polyacrylamide gel electrophoresis of immunoprecipitated clathrin. Clathrin was allowed to react with CVC.7 anti-clathrin LC-A monoclonal antibody, and the product was precipitated with goat anti-mouse IgG. The upper 50 μl of supernatant (from a total volume of 150 μl) was carefully removed, mixed with 50 μl of sample buffer (20), and used for electrophoresis. The corresponding pellet was suspended in 150 μl of sample buffer and 50 μl was applied to the gel. Lanes 1, 3, 5, and 7 are derived from supernatants and lanes 2, 4, 6, and 8 are derived from pellets. More than half of the clathrin heavy chains have been precipitated (lane 6) along with both light chains. Clathrin in the supernatant (lane 5) contains only LC-B. Lanes: 1 and 2, clathrin/CVC.7; 3 and 4, clathrin/goat anti-mouse IgG; 5 and 6, clathrin/CVC.7/goat anti-mouse IgG; 7 and 8, clathrin alone. HC, heavy chain of clathrin; LC-A and LC-B, light chains of clathrin; H-IgG and L-IgG, heavy and light chains of immunoglobulins.

chain of bovine brain clathrin. The determinant is located near the proximal end of an arm. We emphasize that the micrographs give information only about the determinant recognized, not about the entire light chain. If LC-A has a relatively globular structure, the complete polypeptide will be localized in this region. A much more extended structure could span part or most of an arm. Images of unidirectionally shadowed light chains suggest that they are somewhat elongated molecules, 50–100 Å long (unpublished data). Since each heavy chain has one and only one bound light chain (1), symmetry arguments suggest that LC-B will bind at positions homologous to those occupied by LC-A. An alternative approach to light chain localization has been taken by Ungewickell *et al.* (25), who have reconstituted clathrin from separated light and heavy chains after modification of isolated light chains with biotin. They find that ferritin-conjugated avidin then labels the center of trimers.

What is the function of clathrin light chains? Chemical cross-linking experiments give no evidence for inter-light chain contact (1). Thus, despite their central location, light chains do not appear to contribute to interactions between arms within a trimer. Other possible functions include mediation of interaction with cytoplasmic or membrane-associated structures and stabilization of assembly.

The interdigitated arrangement of trimer arms in cages (1, 7) appears not to prevent immunoaggregation by CVC.7 of coated vesicles or *in vitro*-assembled clathrin cages, as judged by electron microscopy of negatively stained samples (unpublished observation). This accessibility of the light chain determinant in the assembled structure suggests that it lies on the surface of a coated vesicle and that it could make contact with other cytoplasmic molecules.

The apparently random distribution of the two light chain species is puzzling and difficult to reconcile with a regulatory role in assembly. A possibility we cannot at present rule out is that an originally homogeneous distribution (1:2 on all trimers) has been randomized in the course of purification. A step at

which this might occur is the dissociation of trimers from coated vesicles. Nonetheless, the purified clathrin is competent to reassemble into well-defined closed cages. Further understanding must await determination of structural relationships between LC-A and LC-B.

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