Clathrin Assembly Involves a Light Chain-binding Region

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Abstract. Two regions on the clathrin heavy chain that are involved in triskelion interactions during assembly have been localized on the triskelion structure. These regions were previously identified with anti–heavy chain monoclonal antibodies X19 and X35, which disrupt clathrin assembly (Blank, G. S., and F. M. Brodsky, 1986, *EMBO (Eur. Mol. Biol. Organ.) J.*, 5:2087–2095). Antibody-binding sites were determined based on their reactivity with truncated triskelions, and were mapped to an 8-kD region in the middle of the proximal portion of the triskelion arm (X19) and a 6-kD region at the triskelion elbow (X35). The elbow site implicated in triskelion assembly was also shown to be included within a heavy chain region involved in binding the light chains and to constitute part of the light chain-binding site. We postulate that this region of the heavy chain binds to the interaction site identified on the light chains that has homology to intermediate filament proteins (Brodsky, F. M., C. J. Galloway, G. S. Blank, A. P. Jackson, H.-F. Seow, K. Drickamer, and P. Parham, 1987, *Nature (Lond.)*, 326:203–205). These findings suggest the existence of a heavy chain site, near the triskelion elbow, which is involved in both intramolecular and intermolecular interactions during clathrin assembly.

D URING the process of receptor-mediated endocytosis, the plasma membrane of a cell invaginates to form a coated pit, which subsequently pinches off into the cytoplasm to become a coated vesicle (11). The coat of these organelles is formed by polymerization of the protein clathrin on the cytoplasmic face of the invaginating membrane (29). Clathrin assembled on coated pits and coated vesicles forms a lattice-like, polyhedral structure composed of hexagons and pentagons (8, 12, 15). Purified clathrin will also self-assemble in vitro under the appropriate ionic conditions (pH 6.0–6.5), forming "baskets" with a structure similar to that seen on the surface of coated vesicles (16, 26, 34, 44). Depolymerized clathrin exists in the form of a triskelion which is composed of three identical heavy chains (180 kD) and three light chains of two types, LC_a and LC_b (30–40 kD) (9, 17, 39). Analysis of the triskelion shape and dimensions has generated a working model of how these triskelions associate during assembly (9, 13, 18). Based on stoichiometric and electron microscopic data, it is apparent that the vertex of one triskelion is located at each vertex formed between hexagonal and pentagonal faces of assembled clathrin, and each arm spans two edges. This arrangement indicates that the edge of each pentagon or hexagon is composed of arms from four different triskelions, implying extensive lateral interactions between the triskelion arms.

The clathrin light chains have been localized to the vertex of the triskelion (19, 40, 42), and antibody binding data sug-

gests they span the entire proximal portion of the arm, from the vertex to the elbow (38). Consistent with this extended interaction, the light chains have a region of homology with intermediate filament proteins which is involved in their binding to clathrin heavy chains (4, 14, 20). The role of the clathrin light chain subunits in triskelion assembly is not yet completely defined. The light chains have biochemical properties expected of regulatory proteins that include tissue polymorphism (6, 28), differential phosphorylation (1, 33, 41), and calcium–calmodulin binding (22, 23, 25). They are also the recognition site for a protein which causes clathrin depolymerization in an ATP-dependent reaction (31). Whether their presence is required for assembly is unclear. Removal of the light chains from triskelions by proteolysis renders the triskelions assembly-incompetent (17). However, separation of the subunits by chromatography in chaotropic salts yields heavy chain triskelions which can assemble into clathrin baskets (43). Although incomplete light chain dissociation has been demonstrated in this latter procedure (31), and the efficiency of heavy chain reassembly has not been characterized, these data indicate that the clathrin heavy chain provides the structural backbone for assembled clathrin. This is confirmed by the fact that mild proteolysis of clathrin baskets removes the light chains without disrupting the basket structure (42).

Consistent with a prominent role for heavy chain interactions during clathrin assembly, we have identified three anti–heavy chain monoclonal antibodies (mAbs) that inhibit assembly and induce formation of abnormal clathrin structures (2). We have shown previously that these antibodies re-

act with two distinct epitopes on the clathrin heavy chain which seem to be critical for arm–arm interactions during assembly. In the studies described in this paper, sequential proteolysis has been used to map these epitopes with respect to their physical position along the triskelion arm. One epitope is included in an 8-kD segment of the heavy chain at the midpoint of the proximal portion of the arm. The second epitope localizes to a 6-kD segment at the triskelion elbow. This second site is also demonstrated to be involved in heavy chain binding of clathrin light chains, defining a heavy chain region which mediates both intramolecular and intermolecular interactions.

Materials and Methods

Clathrin Preparation and Proteolysis

Clathrin was purified from bovine brain-coated vesicles as described by Blank and Brodsky (2), and modified from Pearse and Robinson (30).

Conditions were established to optimize proteolysis of terminal domain from the triskelion core (18, 32). Clathrin was assembled into baskets by overnight dialysis (4°C) against 20 mM 2-(N-morpholino)ethane sulfonic acid (MES), 2 mM $CaCl_2$, pH 6.2. L-1-p-tosylamino-2-phenylethyl chloromethyl ketone (TPCK)-trypsin (Worthington Biochemical Corp., Freehold, NJ) was added to clathrin baskets at trypsin/clathrin ratios of 1:100 and 1:200, and the solution was incubated at 37°C. Samples were removed after 0, 0.5, 1, 2, 3, and 4 h, and the digestion stopped by the addition of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) in 2X molar excess to trypsin. Digestion products were analyzed by SDS-PAGE. Digestion conditions were chosen which totally eliminated the 180-kD heavy chain yet gave the greatest yield of 100-120 kD core material. The digestion was then repeated using 6–8 mg of clathrin. After stopping the reaction, baskets were depolymerized by slowly raising the pH to 7.4 with 0.1 N NaOH. Remaining intact baskets and aggregates were removed by centrifugation at 30,000 g for 10 min, then proteolyzed clathrin was separated into fragments by size exclusion high pressure liquid chromatography (HPLC).

HPLC fractions from the above preparation containing core (100-120 kD) were pooled and subjected to additional proteolytic digestion. TPCK-trypsin was added to give a trypsin ratio of 1:150, and the solution incubated at 37°C for 5, 10, 20, and 50 min. The reaction was stopped by addition of 2X molar excess soybean trypsin inhibitor to trypsin. These digests were centrifuged at 30,000 g for 10 min, and analyzed by size exclusion HPLC as described below.

HPLC

Clathrin digests were analyzed and purified by size exclusion HPLC using a model 334 system with a Spherogel TSK-3000SW 0.75 × 30-cm column (Beckman Instruments, Inc., Palo Alto, CA). Clathrin fragments were loaded and eluted at 0.2 ml/min in 50 mM ammonium bicarbonate, pH 7.4. 1-min fractions were collected, so that the fraction number corresponds to the elution time. The column was calibrated using the above conditions with BioRad Laboratories gel filtration standards which include thyroglobulin, 670 kD; IgG, 158 kD; ovalbumin, 44 kD; and myoglobin, 17 kD.

Electrophoresis

SDS-PAGE was by the method of Laemmli (21). Low molecular mass marker proteins were from Pharmacia Fine Chemicals, Piscataway, NJ, and include phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (44 kD), soybean trypsin inhibitor (20 kD), and α-lactalbumin (14.4 kD). High molecular mass marker proteins were from BioRad Laboratories, Richmond, CA, and include myosin (200 kD), β-galactosidase (116 kD), phosphorylase b, bovine serum albumin, and ovalbumin.

MAb Reactivity

Production of anti–clathrin mAbs X19, X22, X35, and control antibody anti-Leu 10 has been previously described (2, 3, 7). Anti-Leu 10 reacts with HLA-DQ cell surface antigens and does not react with clathrin (3, 7). MAbs were purified from ascites fluid, as described (27).

Binding of mAbs to clathrin fragments was determined by indirect radio-

immunoassay (3). 20-µl samples of HPLC fractions were applied to wells of polyvinyl chloride microtiter plates and incubated for 90 min at room temperature. After washing and blocking with 0.5% bovine serum albumin in phosphate buffer, pH 7.4 (PBS/BSA), 50 µl of mAb (5 µg/ml) was added to each well and incubated for 90 min at 4°C. The wells were washed with PBS/BSA and 20 µl ^{125}I-F(ab')$_2$ of rabbit anti–mouse immunoglobulin (^{125}I-RAM) containing 300,000 cpm was added per well. This was incubated for 60 min at 4°C. The wells were washed and the bound radioactivity was counted.

Western blot analysis was carried out, as previously described (19), with the following modifications. Protein samples were run in three identical sets on SDS-PAGE and transferred electrophoretically to nitrocellulose at 20 W for 1 h. The nitrocellulose was then stained with Amido Black and cut into three panels for incubation with mAbs X22 or anti-Leu 10, or retained for total protein staining. Anti-Leu 10 was used as a control for nonspecific binding. For antibody binding, two panels were treated with Tris-buffered saline (TBS,[1] 10 mM Tris, 150 mM NaCl, pH 7.4, 0.1% Triton X-100, 0.02% SDS) containing 3% BSA and 0.1% normal goat serum for 1 h at 37°C. MAbs were then added in the above solution at a concentration of 5 µg/ml and incubated overnight at room temperature. The panels were then washed five times with TBS and treated with horseradish peroxidase-conjugated goat anti–mouse immunoglobulin in TBS plus 3% BSA and 0.1% normal goat serum for 2 h at room temperature. The panels were then washed five times with TBS and five times with TBS without detergent. Peroxidase was detected by incubation in substrate 3'-3'-diaminobenzidine tetrahydrochloride (50 µg/ml in TBS without detergent) followed by addition of 30% hydrogen peroxide to a final concentration of 0.01%.

Light Chain Binding Studies

Clathrin light chains were purified from bovine brain-coated vesicles by heat treatment (5, 23) and DEAE chromatography (38) as previously described (4). They were then iodinated using Enzymobeads (BioRad Laboratories) with 1 mCi ^{125}I-NaI per 25 µg. Iodinated light chains were tested for binding to preparations of clathrin heavy chain, triskelion core, and HPLC fractions containing truncated triskelions by direct radioimmunoassay (RIA) (4). When binding to heavy chain fragments was tested, saturating concentrations of iodinated light chains were used. When the effects of anti–heavy chain mAbs on light chain–heavy chain association were tested, heavy chains were preincubated with dilutions of mAbs for 30 min before adding iodinated light chains. Heavy chains were produced by limited proteolysis of triskelions under conditions which removed the light chains but did not cleave the heavy chains (4, 17). Fab fragments used in these studies were produced from purified mAbs, as described (2, 27).

Results

We have previously found three mAbs binding to two distinct antigenic sites on the heavy chain, which are involved in clathrin assembly (2). To determine the position of these epitopes with respect to the triskelion form, clathrin was proteolyzed and the resulting fragments were analyzed for their ability to bind mAbs. These truncated triskelions were also tested for binding of clathrin light chains. Binding activity was then correlated with fragment size to determine the relative positions of mAb-binding sites along the heavy chain, and the region of light chain interaction.

Generation of Truncated Triskelions

Limited proteolysis of assembled clathrin baskets has been shown to remove light chains and to fragment the heavy chain into a triskelion core composed of three 100-120 kD arms, liberating globular terminal domains (40–60 kD) (18, 32, 40). Separation of clathrin heavy chain into these two relatively large fragments allowed initial localization of mAb-binding sites and provided starting material for further proteolysis. To determine optimal conditions for generation of the triskelion core, clathrin baskets were digested for varying

1. Abbreviations used in this paper: RAM, rabbit anti–mouse (immunoglobulin G); TBS, Tris-buffered saline.
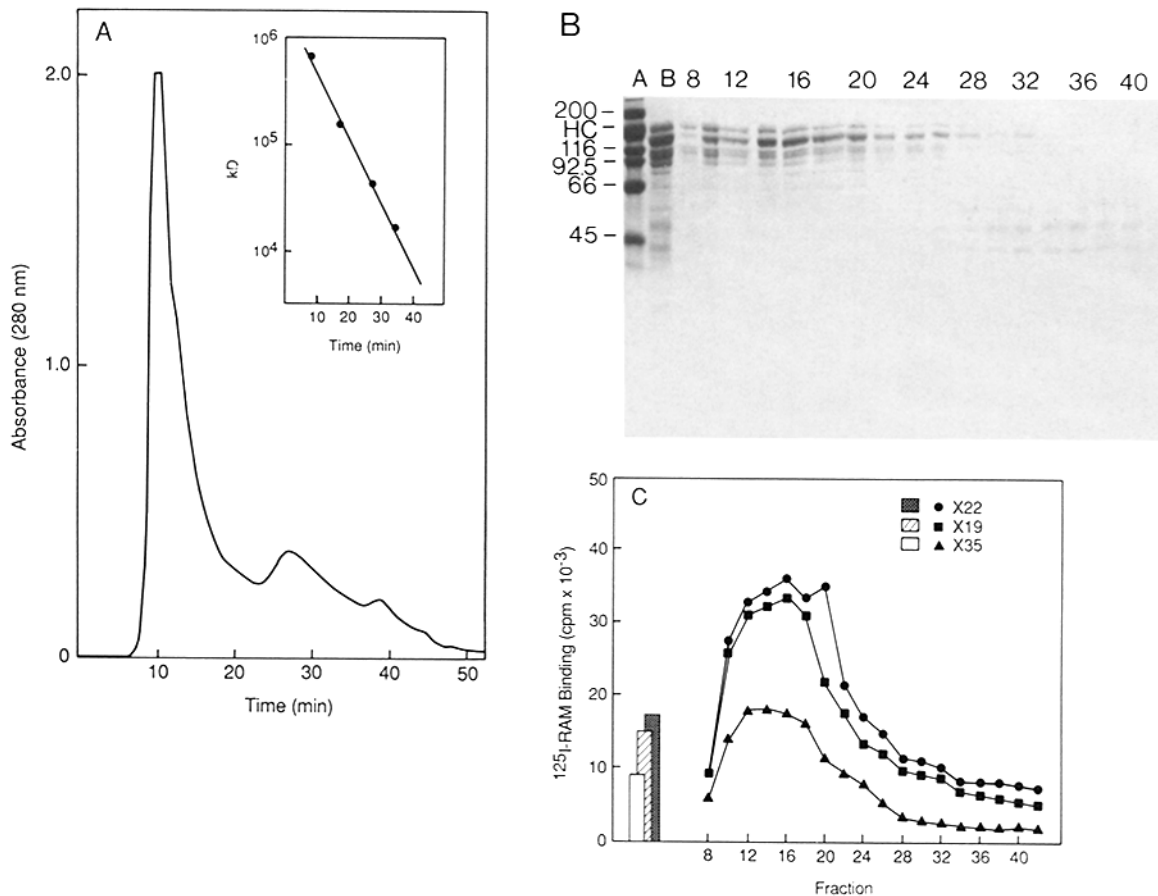
Figure 1. Purification of triskelion core fragments. (*A*) HPLC separation of triskelion fragments produced by trypsin digestion of clathrin baskets. Clathrin baskets (6 mg) were digested with trypsin at a ratio of 1:200 and depolymerized, and fragments were separated by size exclusion HPLC as described in Materials and Methods. (*Inset*) Gel filtration standards were chromatographed under the same conditions used for digested clathrin. The elution times of these standards were plotted versus molecular mass. (*B*) SDS-PAGE analysis of HPLC fractions. 1-min fractions were collected during the HPLC separation of digested clathrin shown in *A*. Elution time in minutes corresponds to fraction number (lanes labeled *8–40*). Lane *A*, high molecular mass marker proteins plus clathrin heavy chain (*HC*); lane *B*, digested clathrin prior to HPLC separation. (*C*) MAb binding to HPLC fractions. Binding of mAbs X22 (●), X19 (■), and X35 (▲) to clathrin fragments in fractions 8–42 (*A* and *B* above) was determined by indirect, solid-phase RIA. The bars represent mAb binding to undigested clathrin. MAb binding was detected with iodinated F(ab′)₂ of rabbit anti–mouse immunoglobulin (^{125}I-RAM).

times using two different concentrations of trypsin. Based on this trial proteolysis, clathrin baskets were digested under conditions which maximized the yield of 100–120-kD fragments and elimination of the 180-kD heavy chain. The digestion products were separated by size exclusion HPLC under nondenaturing conditions. An initial peak eluted at 12 min, which corresponds to a molecular mass of ∼360 kD. A second, much smaller peak eluted at 27 min and corresponds to a molecular mass of ∼45 kD (Fig. 1 *A*). When these fractions were analyzed by SDS-PAGE, the high molecular mass peak consisted mainly of 100–120-kD fragments with some undigested 180-kD heavy chain (Fig. 1 *B*). These fragments were still in triskelion form and their elution position from HPLC corresponded to three times the molecular mass of the denatured fragments in SDS-PAGE. The second peak consisted of two fragments of 43 and 55 kD derived from the terminal domain. The presence of triskelion core and terminal domain was confirmed by electron microscopy of rotary-shadowed samples from HPLC fractions (18, 37, 39).

To generate smaller fragments for localization of mAb-

binding sites, HPLC fractions containing 100–120-kD fragments were pooled and further digested with trypsin for increasing times (5, 10, 20, and 50 min). These digests were then separated by size exclusion HPLC. The 5-min digest eluted as a single, relatively broad peak, from 12 to 16 min. With increased digestion times, the major elution peak became smaller and shifted towards the right with the appearance of several smaller peaks in the trailing shoulder. The elution position of the major peak at all four digest times was consistent with that of an increasingly smaller triskelion form. This indicated that truncated triskelions were being generated by cleavage from the distal ends of the triskelion arms. To determine the size of the arms, HPLC fractions from each time point were analyzed by SDS-PAGE (Fig. 2). This analysis demonstrated that cleavage occurred at discrete sites producing arms of 90, 62, 56, 46, and 38 kD. With longer digestion time there was an increase of the lower molecular mass species corresponding to disappearance of the higher molecular mass fragments. The smallest truncated triskelion isolated by size exclusion HPLC was composed of
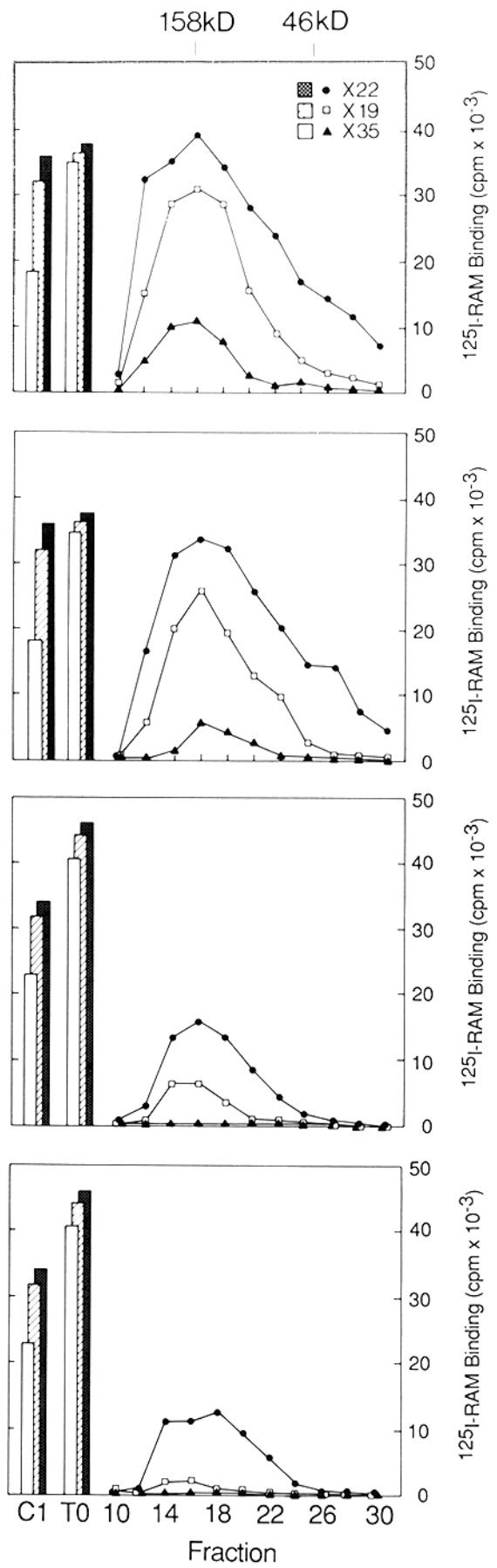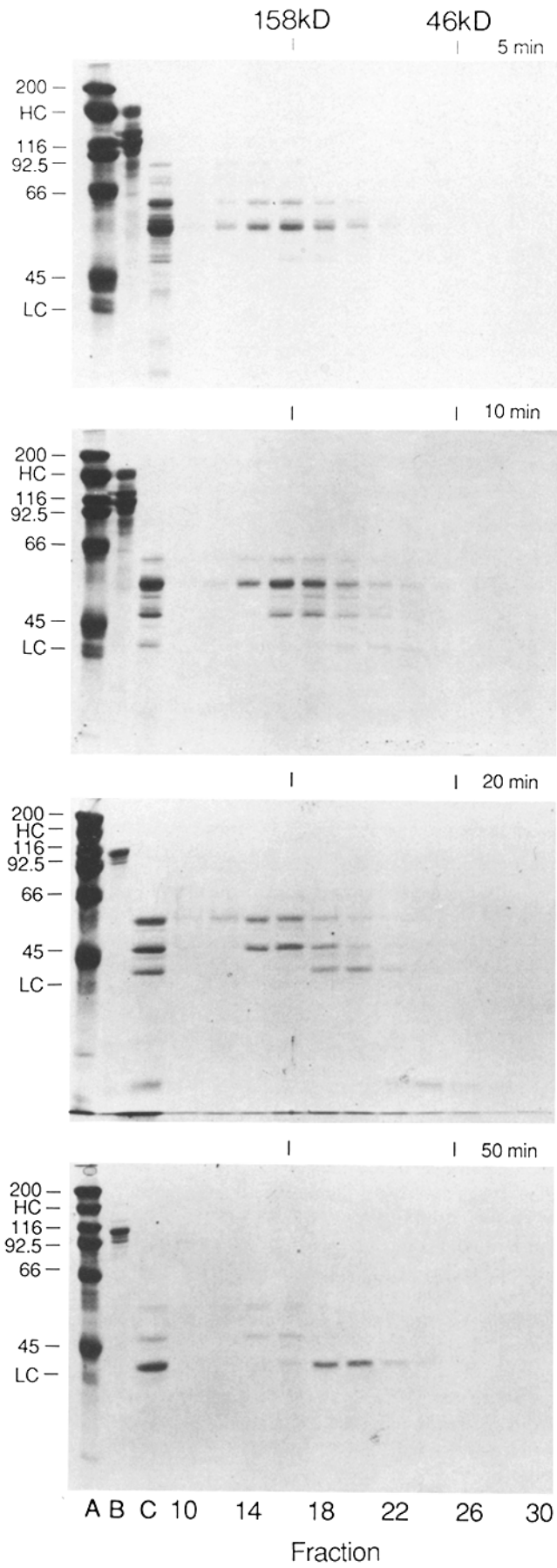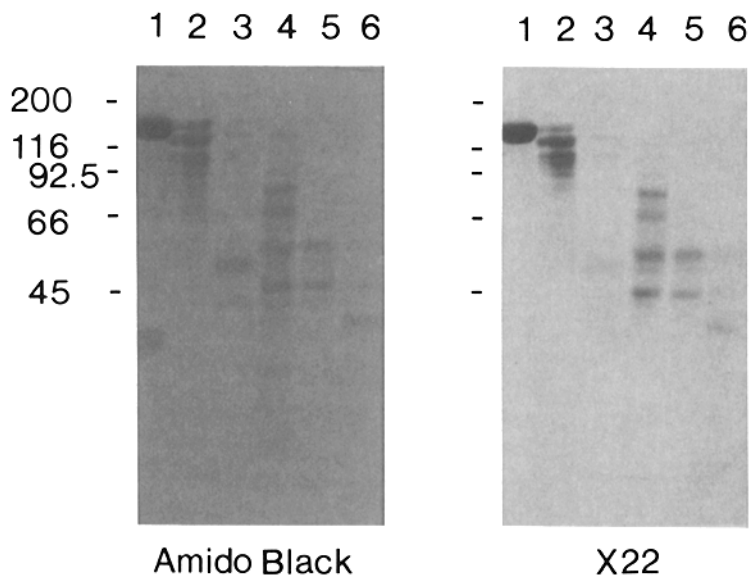
Figure 3. X22 binding to clathrin and clathrin fragments detected by Western blot. Protein samples were applied to a polyacrylamide gel in three identical sets. After SDS-PAGE and electrophoretic transfer to nitrocellulose, one set of samples was stained with Amido Black to detect total protein. The other set of samples was tested for reactivity with mAb X22 and a control mAb as described in Materials and Methods. Lane *1*, clathrin; lane *2*, triskelion core (Fig. 1 *B*, lane *10*); lane *3*, terminal domain (Fig. 1 *B*, lane *38*); lanes *4*, *5*, *6*, fragments of triskelion core from HPLC separations of timed digests shown in Fig. 2: respectively, fraction 12 (5-min digest), fraction 18 (20-min digest), fraction 20 (50-min digest). Migration positions of molecular mass marker proteins are indicated along the left edges. No binding was detected when samples were reacted with control antibody.

38-kD heavy chain fragments and had a molecular mass of 114 kD.

Localization of Antigenic Sites Involved in Assembly

To localize functional sites on the clathrin triskelion, anti-clathrin mAbs were tested for binding to truncated triskelions using an indirect solid-phase RIA. The antibodies tested (X19, X22, and X35) represent three types of antibodies that have different effects on clathrin assembly (2). X19 and X35 inhibit assembly, producing loose aggregates of triskelions or abnormally assembled structures, respectively. X22 has no detectable effect on clathrin assembly. Competition studies have indicated that these three mAbs bind to different sites on the clathrin triskelion. MAbs were initially tested for their ability to bind to either triskelion core or terminal domain. As seen in Fig. 1 *C*, these three mAbs reacted with HPLC fractions containing triskelion core.

For further localization of mAb-binding sites along the triskelion core fragment, secondary tryptic digests of the core were separated by HPLC and fractions analyzed for antibody binding by solid-phase RIA (Fig. 2). The position of mAb-binding sites on the triskelion was determined by correlating binding activity with the fragment sizes present in each fraction. Because X35 binding activity was lost first, the X35 site must lie farthest from the vertex along the heavy chain. In the 5-min digest, X35 binding was associated with fractions containing the 90, 62, and 56 kD fragments. Analyzing the 10-min digest where the 90-kD fragment is absent indicates that X35 binding activity correlates with the 62-kD

fragment, but not with the 56-kD fragment. In addition, there is no X35 reactivity with the 20- or 50-min digests in which the 56-kD fragment is the largest fragment present. Thus, X35 binds within the 6-kD portion of the heavy chain between the 56- and 62-kD fragments. Kirchhausen and Harrison (18) have estimated the mass per unit length of the proximal segment of the triskelion arm to be 4.2 kD/nm. Using this value, X35 binds to a region 13–15 nm from the vertex along the proximal segment.

X19 reactivity is considerably reduced between 20- and 50-min digestion, localizing the X19-binding site closer to the triskelion vertex than the X35-binding site. X19 binding is associated with the 56- and 46-kD fragments in the 20-min digest, but does not correlate with the presence of the 38-kD fragment. The 50-min digest demonstrates that X19 binds to fractions containing the 46- and 38-kD fragments, but does not react with the major peak of the 38-kD fragment. Thus, the X19-binding site lies between the cleavage sites producing the 46- and 38-kD fragments. This localizes the X19 site to a region of 8-kD, spanning 9–11 nm from the vertex.

As a reference point, an anti–heavy chain mAb (X22) which does not affect assembly (2) was also tested for reactivity with truncated triskelions. As measured by RIA in Fig. 2, X22 reacts with all the fragments generated by trypsin digestion of triskelion core. It was possible to confirm this reactivity by Western blotting with X22, in that it is the only antibody of the three which binds to denatured clathrin heavy chain (3) (Fig. 3). As predicted by RIA, X22 binds in a Western blot to triskelion core and its fragments of 90–38 kD (lanes *4–6*), but does not react with terminal domain (lane

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Figure 2. Analysis of timed trypsin digests of triskelion core. Fractions containing triskelion core (trimerized 100–120-kD fragments), from the separation shown in Fig. 1, were pooled and subjected to further trypsin digestion for 5, 10, 20, and 50 min. Digests were then separated by HPLC as described in Fig. 1. HPLC fractions were analyzed by SDS-PAGE and indirect, solid-phase RIA. Molecular mass markers across the top of the panels (158 kD, 46 kD) indicate their time of elution during HPLC with respect to the fraction numbers below. (*Left*) SDS-PAGE panels: lane *A*, molecular mass markers plus clathrin (indicated along left side—*HC*, clathrin heavy chain; *LC*, clathrin light chains); lane *B*, triskelion core before additional digestion; lane *C*, timed digest of triskelion core before HPLC separation. HPLC fraction numbers at the bottom correspond to their time of elution. (*Right*) RIA binding panels: binding of mAbs X22 (●), X19 (□), and X35 (▲). The bars represent mAb binding to undigested clathrin (*Cl*) or triskelion core (*TO*). MAb binding was measured by indirect, solid-phase RIA using ^{125}I-RAM.
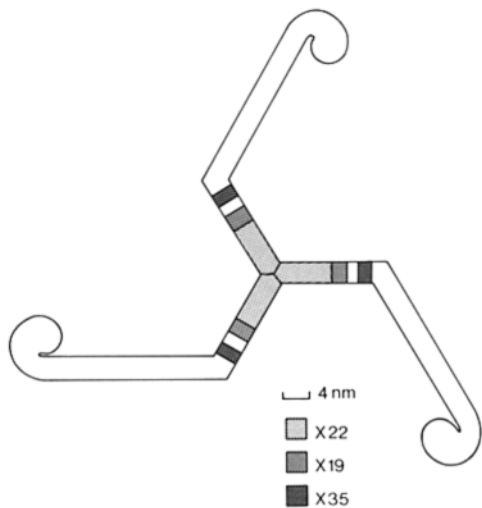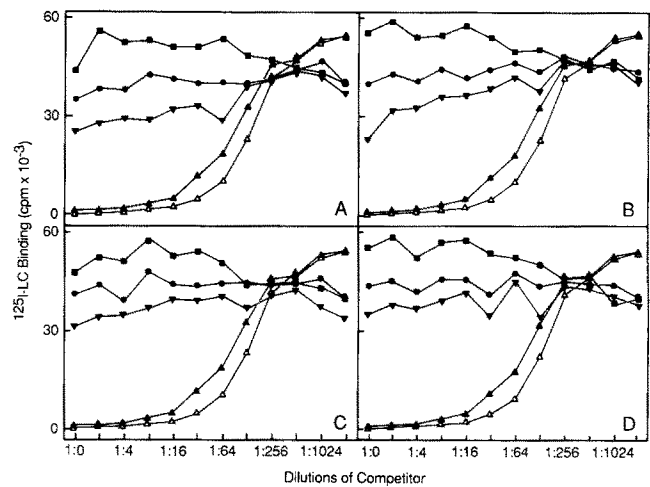
Figure 4. Diagram of a clathrin triskelion based on the work of Crowther and Pearse (9), and Kirchhausen and Harrison (18) showing the binding sites for each of the mAbs. The X22, X19, and X35 epitopes were localized based on antibody binding to truncated triskelions and mapped on the triskelion, using an estimate of 4.2 kD/nm (18).

Figure 5. MAb effects on light chain–heavy chain reassociation. Iodinated light chains were tested for binding to clathrin heavy chains in the presence of mAbs and unlabeled light chains. (*A* and *C*) ^{125}I-LC$_a$ binding to heavy chain; (*B* and *D*) ^{125}I-LC$_b$ binding to heavy chain. Before adding iodinated light chains, increasing dilutions of IgG were preincubated with the heavy chain in *A* and *B*. Fab fragments were used for preincubation in *C* and *D*. MAbs tested in both IgG and Fab form were X19 (■), X22 (●), and X35 (▼). Unlabeled LC$_a$ (△) and LC$_b$ (▲) were used as control inhibitors.

3). Blotting of X22 also confirmed that the 90-, 62-, 56-, 46-, and 38-kD fragments are all generated by progressive digestion of the triskelion core. We have been unable to generate a truncated triskelion with fragments smaller than 38 kD to establish the location of the X22 site more precisely. Thus, X22 binds somewhere between the vertex and 9 nm along the region of the arm proximal to the vertex, using the estimate of 4.2 kD/nm (18). Measurements of negatively stained triskelions have indicated that the proximal region of the arm extends 16 ± 1 nm from the vertex to the elbow, with the distal region of the arm having an approximate length (elbow to terminal domain) of 26 nm (9, 13, 18). This localizes the binding sites of all three mAbs to the proximal arm of the clathrin heavy chain, as shown in Fig. 4. The binding sites, proceeding from the vertex in order are X22, X19, and X35, with the X35 site on the proximal portion of the triskelion elbow.

An Assembly Interaction Site Is Involved in Light Chain Binding

In addition to localizing the mAb-binding sites, truncated triskelions were used to analyze the region of light chain

binding. Purified, iodinated clathrin light chains (LC$_a$ or LC$_b$) were tested for their ability to bind to HPLC fractions containing triskelion core (100–120-kD arms) and fragments of core (56- and 46-kD arms). These fractions were selected from HPLC separations of trypsin digests of clathrin, such as the ones shown in Figs. 1 and 2. In order to compare binding between samples that varied in protein concentration, X22 binding was measured and used to normalize the radioactivity bound, because X22 reacts with all the fragments tested. Initially, several dilutions of each fraction containing heavy chain fragments were tested to be sure that antibody binding and light chain binding were measured under saturating conditions. The results listed in Table I were calculated from binding to a triskelion core preparation, plated at ~25 µg/ml, and binding to HPLC fractions containing lower concentration of protein. X22 binding to triskelion core was set at 100% for each experiment so that the relative concentrations of heavy chain fragments in the fractions could be determined using X22 binding. LC$_a$ and LC$_b$ both bind to triskelion core. Their lack of reactivity with the trun-

*Table I. Light Chain and MAb Binding to Truncated Triskelions**

Heavy chain fragments in triskelion form	MAb binding		Light chain binding	
	X22	X35	LC$_a$	LC$_b$
kD			%	
100–120 (core)	100	41.0 ± 5.0	30.5 ± 7.3	55.5 ± 16.5
56	54.5 ± 17.7	2.1 ± 0.9	3.2 ± 2.2	5.4 ± 5.0
46 + 56	71.0 ± 31.5	2.2 ± 0.8	3.4 ± 2.7	5.1 ± 4.1
38	30.0 ± 15.9	2.2 ± 0.8	2.8 ± 1.9	3.3 ± 2.7

*MAbs (X22 and X35) and iodinated clathrin light chains (LC$_a$ and LC$_b$) were tested for binding to HPLC fractions containing triskelion core and truncated triskelions. Binding was measured by indirect and direct RIA under saturating conditions. Results are reported as a percentage of X22 binding to triskelion core. Results shown are the average of three experiments testing fragments from two separate digests. Separate binding experiments using nonsaturating conditions confirmed X19 reactivity with the 46-kD, 56-kD, and core fragments.

cated triskelions far exceeds the drop in binding expected from the reduction in fragment concentration demonstrated by X22 binding. This indicates that a critical region involved in light chain–heavy chain interaction is present in the triskelion core distal to the 56-kD cleavage site. As expected from the mapping studies described above, this distal part of the triskelion core contains the X35 binding site but not the X19 or X22 sites.

It has been previously demonstrated that X35 binds with greater avidity to clathrin heavy chain when the light chains have been dissociated than when the light chains are attached (2). This suggested that X35 recognizes a heavy chain site involved in light chain binding and correlates with the location of the X35 site within the distal part of the triskelion core, which contains a light chain interaction region. The role of the X35 site in light chain binding was further investigated by antibody blocking studies (Fig. 5). We have previously shown that light chain–heavy chain interactions can be studied by binding iodinated light chains to heavy chains which have been stripped of light chains by proteolysis (4). When clathrin heavy chains were preincubated with X35 IgG, reassociation of iodinated LC_a or LC_b with the heavy chain was slightly inhibited (~25% at maximum IgG concentrations). In contrast, preincubation with X22 IgG had no effect on light chain reassociation, whereas preincubation with X19 IgG slightly enhanced the degree of light chain binding. These results suggest that X35 and X19 binding influence light chain association with heavy chain, and that the X35 site may be directly involved in this association. When this experiment was repeated using Fab fragments, the relative effects of each antibody were similar but only marginally significant. This can be attributed to the decreased avidity of the univalent Fab fragment in comparison to whole IgG and its inability to compete effectively with light chain binding. Considered together, the IgG inhibition studies and enhanced avidity of X35 for free heavy chain suggest the X35 site constitutes part of the light chain-binding region localized to the distal part of the triskelion core.

Discussion

The experiments described above have localized two regions on the clathrin heavy chain which are involved in clathrin–clathrin interactions during assembly (2). These two regions were initially identified in previous studies using mAbs X19 and X35, which interfere with clathrin assembly (2). In the studies reported here, the X19 site has been mapped to an 8-kD sequence in the center of the proximal portion of the triskelion arm. The X35 site has been mapped to a 6-kD sequence on the proximal side of the elbow bend. We also demonstrate that the X35 site maps within a region on the heavy chain which is involved in light chain binding and constitutes part of the light chain-binding site.

These functional sites were localized on the clathrin triskelion by testing truncated heavy chain triskelions for binding of mAbs or iodinated light chains. Truncated heavy chain triskelions were generated by mild trypsin treatment of clathrin triskelion fragments and were composed of heavy chain fragments of 90, 62, 56, 46, and 38 kD which remained joined at the triskelion vertex. The trimeric nature of these structures indicates that <25% of the 180-kD heavy chain is needed to maintain the triskelion structure. A Western blot with the X22 mAb, which reacted with all the truncated

triskelions, confirmed that they were composed of fragments which were derived from progressive cleavage from the distal ends of the triskelion arms. This allowed orientation of the antibody- and light chain-binding sites, based on progressive loss of binding activity. The approximate physical locations of the cleavage sites on the truncated triskelion were determined using an estimate of 4.2 kD/nm, proposed by Kirchhausen and Harrison (18) from earlier proteolytic studies involving larger heavy chain fragments (Fig. 4). Thus, the antibody- and light chain-binding sites were assigned to regions on the triskelion flanked by proteolytic cleavage sites, defining reactive and nonreactive fragments.

Colocalization of the X35 mAb binding site and a region of interaction with clathrin light chains has been suggested by earlier data and supported by the experiments described above. We previously showed that X35, which interferes dramatically with clathrin assembly, binds preferentially to clathrin heavy chains when the light chains are removed (2). We have demonstrated here that X35 binding also has a slight inhibitory effect on light chain reassociation with heavy chain. Furthermore, proteolysis of the clathrin heavy chain, resulting in loss of the X35-binding site, coincidentally removes the ability of truncated heavy chain triskelions to reassociate with clathrin light chains. In conjunction with data localizing light chain binding to the clathrin vertex and the proximal portion of the triskelion arm (19, 38, 40, 42, 43), these results indicate that light chain–heavy chain interactions extend minimally from the vertex to a region of strong interaction near the elbow. As might be expected, the X19 mAb, which binds to an 8-kD region in the center of the proximal portion of the triskelion arm, also has an effect on light chain–heavy chain reassociation. In this case, it slightly enhances light chain binding, possibly by inducing the heavy chain into a favorable conformation inasmuch as X19 binds equally well to free and light chain-associated heavy (2). Finally, the X22 mAb which binds the heavy chain closer to the triskelion vertex, has no effect on light chain binding. It is possible that the light chain–heavy chain interactions in this region are less strong because this region contains the tissue-specific polymorphic sequences of the light chains (4).

The locations of the binding sites of the X19 and X35 mAbs and the extent of light chain interactions can now be considered with regard to their respective roles in clathrin assembly. Our previous results showed that X19 Fab fragments prevent recognizable triskelion–triskelion interactions and only allow small clumps to aggregate (2). As shown in Fig. 6 B, the X19-binding site is situated such that Fab binding would disrupt the proximal portion of the triskelion arm from participating in either distal–proximal or proximal–proximal interactions and thereby prevent both nucleation and further polymerization from taking place. On the other hand, binding of X35 Fab fragments would not interfere as much with interactions between arms allowing some nucleation to take place (Fig. 6 C). However, the presence of the Fab fragments would cause steric problems at the polyhedron vertices where a triskelion vertex must associate with three adjacent elbows during polymerization. It is also possible that X35 binding perturbs the triskelion structure at the elbow by interfering with light chain interactions in that region. Such perturbation could be responsible for the unusual angles formed by triskelions in the structures induced by coassembly with X35 fragments (Fig. 6 C). Similar triskeli-

A. Assembly

B. X19 Inhibition

C. X35 Inhibition
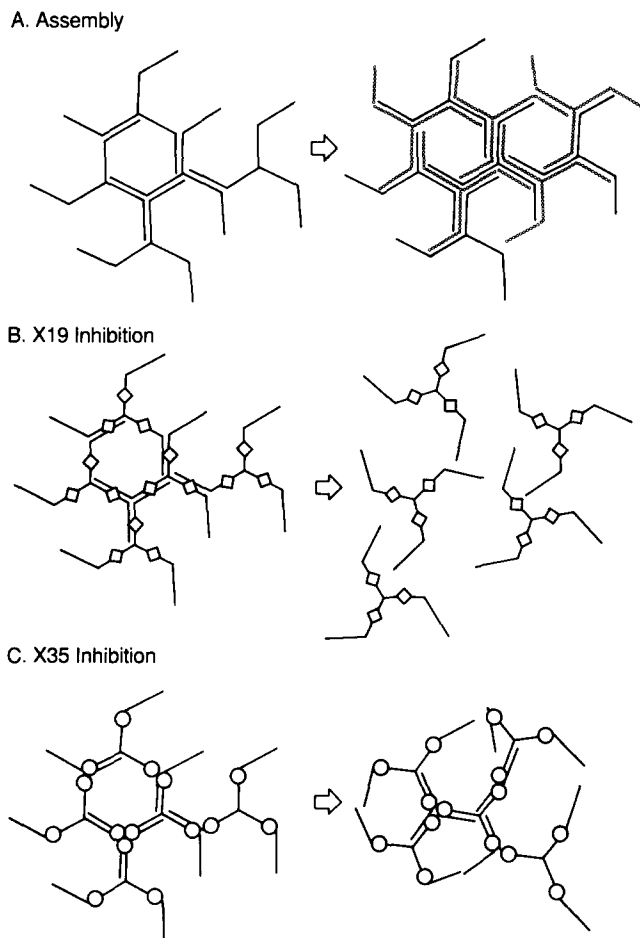
Figure 6. Model for the effect of mAbs on clathrin assembly. (*A*) Normal assembly: the triskelion interactions diagrammed here are based on models proposed by Crowther and Pearse (9), and Kirchhausen and Harrison (18). This diagram indicates the two types of triskelion interactions that occur during assembly involving either vertex–distal arm contacts (*left*) or the vertex–elbow contacts shown by addition of a second layer of triskelions (*right*). This model does not attempt to indicate the interweaving of arms which is observed in situ (9, 13, 18). The terminal domains which project toward the center of an assembled basket (13, 42) have been omitted for clarity. (*B*) X19 inhibition: the squares represent X19 Fab binding to triskelions (approximately to scale). Their location at the center of the proximal portion of the arm would disrupt proximal–distal and proximal–proximal arm interactions that occur in both types of triskelion interactions shown in *A*. This is a likely explanation for the lack of assembly nucleation seen in the presence of X19 Fab which only allows formation of loose triskelion aggregates (2). (*C*) X35 inhibition: the circles represent X35 Fab binding to triskelions (approximately to scale). Electron micrographs of clathrin assembled in the presence of X35 Fab indicate that some nucleation can occur (2). However, it is likely that polymerization is blocked by the inability of additional triskelions to make vertex–elbow contacts due to the presence of X35 Fab fragments, which may also disrupt the angles formed by free arms. The disrupted structure shown is based on electron micrographs of clathrin assembled in the presence of X35 Fab fragments (2).

on interactions have been reported by Sorger et al. (35) who have established conditions for forming cubes of clathrin. Their results confirm that such interactions are structurally permissible. However, they are also formed under disruptive conditions.

The implication of a light chain-binding region in triskelion interactions during assembly indicates a possible structural role for the light chains, as well as a role for the light chain binding site. The fact that the heavy chain–light chain interactions are strong near the elbow suggest the light chain may play a role in stabilizing the angle at the elbow. We, in collaboration with Jackson et al. (14), have recently demonstrated that the region of light chains that interacts with the heavy chain has homology to an α-helical domain which is conserved between intermediate filament proteins, nuclear lamins, and neurofilament subunits and participates in a coiled-coil helix structure in these proteins (4, 10, 24, 36). In an independent analysis of the clathrin light chain sequences based on observation of heptad repeats, Kirchhausen et al. (20) suggest that the light chains may form a coiled-coiled α-helix with the heavy chain. These data suggest that the light chain-binding site at the heavy chain elbow might have corresponding secondary structure such that it could bind light chains. Involvement of this light chain-binding site in triskelion assembly suggests further homology with the intermediate filament family of proteins in which assembly has been shown to result from interaction between coiled-coil subunits (36). Sequence analysis of peptides reactive with the X35 mAb, which defines the heavy chain site involved in intramolecular and intermolecular binding, should elucidate the nature of the structural interactions involved.

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