Brain Clathrin Complex:

II. Immunofluorescent Correlation and Biochemical Affinity for Actin

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The interaction of clathrin with cytoskeletal proteins was studied cytochemically by immunofluorescent staining and biochemically by the binding of actin to clathrin on the surfaces of polystyrene particles. Using a cytoskeletal-disrupting agent, the linear arrangement of clathrin lattices along actin fibers was altered. As a result of cell retraction, the fluorescent dots of clathrin redistributed, conforming to the new cellular shape. Cytoplasmic areas, largely devoid of fluorescent dots, were observed at the cell’s periphery. In vitro, the native clathrin complex (clathrin plus clathrin-associated proteins (CAPs)) bound up to 1 mol of actin, but when the clathrin polypeptide was separated from accompanying proteins it bound up to 2 mol of actin from solution. It appears that clathrin’s molecular lattices have an affinity for arrays of actin microfilaments, following them closely, and that clathrin lattices display lateral mobility during cytoplasmic reorganization.

KEY WORDS: Clathrin complex; Clathrin-associated proteins (CAPs); Lattice redistribution; Actin stress fibers; Immunofluorescent staining; Actin-clathrin binding.

Introduction

We reported recently that clathrin antibodies purified by affinity chromatography yielded a dotted pattern closely following the arrangement of actin stress fibers (2). Previously, similar dots of fluorescence had been reported by Anderson et al. (1). In our hands, the antigen used to elicit clathrin antibodies consisted of 95% homogeneous clathrin assembled as baskets or cages at pH 6.5. More recently, it was reported that a doublet of polypeptides of ~30,000 daltons associated with clathrin (6,8,10,14,15). Also, it was reported that this doublet of clathrin-associated proteins (CAPs) confers to the clathrin complex its capacity to polymerize into baskets (6,10,14). In addition, we found that the clathrin complex bound actin and α-actinin (13). Coated pits discretely distributed under the membrane are believed to consist of clathrin and they represent an early stage in the formation of endocytotic-coated vesicles (1).

We report here that the 180 kilodalton polypeptide of clathrin separated from CAPs retained its capacity to bind actin in vitro and that an antibody forming immunoprecipitates with clathrin’s 180 kilodalton polypeptide produced a linear, fluorescent-dotted pattern. Responding to a challenge by a cytoskeletal-disrupting agent, chlorpromazine, these clathrin lattices redistributed during cytoplasmic retraction.

Materials and Methods

Protein purification. Clathrin was prepared from bovine brain cortex. Its separation from membrane fragments was performed by column chromatography on Sepharose-4B using a 2 M urea Tris-HCl buffer, pH 7.5 (3,13). Purity of clathrin was assessed by sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis and estimated by gel densitometry to be over 95% homogeneous in the 180 kilodalton protein species. The remaining ~30 kilodalton polypeptides, CAPs, representing 5% of the total protein content, were removed by controlled proteolysis using chymotrypsin. 1.5 μg/mg of clathrin complex in 0.1 M MES buffer, pH 6.5. The reaction proceeded for 15 min at room temperature and was halted by addition of 1 mM phenylmethylsulfonylfluoride. The reaction product was dialyzed for 16 hr at 4°C against 2 M urea buffer, pH 7.5, then for 16 hr against 0.02 M Tris-HCl buffer, pH 7.5, containing 0.5 mM MgCl₂, 7 mM 2-mercaptoethanol and 0.1% sodium azide. Muscle actin...
was prepared as described elsewhere (12). Protein concentrations were estimated by the method of Lowry et al. (7). Bovine serum albumin was used as the protein standard.

Electron microscopy. Clathrin structures were visualized by negative staining with 1% uranyl acetate using 10–15 μg of clathrin solution placed on a Formvar carbon-coated grid. Samples were examined in a Jeol 100B electron microscope at 80 kV.

Preparation of antibodies. Clathrin assembled as baskets was used to elicit antibodies from rabbits. Immune sera were purified by CNBr-activated Sepharose-4B affinity chromatography. Rabbits inoculated with clathrin complex assembled as baskets produced an antigenic response as measured by immunocytochemical and immunoprecipitin reactions (2). Further characterization of the antibody was performed with double-gel immunodiffusion using 1% agarose. Whole antiserum was allowed to react with the clathrin complex and enzyme-purified clathrin, as also were affinity-purified antibodies.

Binding of actin to clathrin-coated Lytron polystyrene particles (LP). The procedure for coating proteins on surfaces of LP has been detailed elsewhere (9,13). Adsorption of enzyme-purified clathrin to LP was carried out at room temperature using a protein solution of 0.5 mg in a volume of 0.5 ml. The clathrin-coated LP were washed, resuspended, and allowed to bind actin from solution essentially as described previously (13).

Immunofluorescent studies. For immunofluorescent staining the indirect sandwich technique was used in which the antibodies reacting first were anti-clathrin immunoglobulin (Ig) G molecules and second, the goat anti-rabbit IgG conjugated with rhodamine (Miles Laboratories, Indiana).

Cell cultures from a human fibroblast cell line (ATCC CCL 186 [American Type Culture Collection Catalog of Staining II]) were maintained in basal medium (Eagle’s) with 10% fetal calf serum. The protocol rendering optimal immunofluorescent staining used fixation with 3.8% paraformaldehyde in phosphate-buffered saline, pH 7.4, for 15 min, followed by membrane permeabilization with 0.1% Triton X-100 for 15 min (2). Cells were postfixed after antibody incubations in 5% acetic acid (in ethanol) and mounted in 50% glycerine in phosphate-buffered saline, pH 8.

Cells treated with micromolar concentrations of chlorpromazine were allowed to react for 1 hr, rinsed, fixed, permeabilized, treated with the antibodies, and examined with phase-contrast optics and epifluorescent illumination for rhodamine under a Zeiss Universal microscope using a 63 × objective.

Results

Clathrin Purification

The ultrastructural morphology of the crude coated vesicle fraction that served as the source for clathrin extraction and purification is illustrated in Figure 1a. Coated vesicles and clathrin baskets are prominent, accompanied by membrane fragments and resealed vesicles of varied sizes. Clathrin emerged from the Sepharose-4B chromatographic column devoid of membrane fragments. Most membrane originating...
from the vesicle pellet emerged as a large peak in the void volume (Figure 1b). The second peak displayed a major polypeptide band on SDS-slab polyacrylamide gels with an electrophoretic mobility of 180 kilodaltons. Two different loads of a typical protein preparation are illustrated in Figure 2. Clathrin's 180 kilodalton polypeptide represents approximately 90% of stain density with the remaining 10% divided among three protein species tentatively identified by their electrophoretic mobilities as tubulin, 55 kilodaltons; actin, 43 kilodaltons; and a polypeptide doublet of ~30 kilodaltons. Rechromatography of this fraction removed the 55 and 43 kilodalton bands, leaving the ~30 kilodalton doublet, the CAPs, attached to clathrin (Figure 2). Removal of these CAPs was accomplished by a brief chymotryptic treatment. Clathrin resisted proteolytic cleavage by chymotrypsin at pH 6.5 (Figure 2, lane 1; Figure 3, lanes 2–5). Clathrin-associated proteins were cleaved rapidly under these conditions, giving rise to a homogeneous protein preparation as judged by gel electrophoresis.

Figure 2. SDS-polyacrylamide slab gel electrophoresis. A continuous gradient of 7.5–15% polyacrylamide was used to establish the polypeptide composition of clathrin preparations. Lane 1: clathrin after chymotrypsin treatment (110 μg). Lanes 2 and 3: clathrin eluted from a Sepharose-4B column after concentration by ammonium sulfate, 80 and 150 μg, respectively.

Figure 3. SDS-slab gel electrophoresis. Conditions are similar to those described in legend for Figure 2, Lane 1: Clathrin (100 μg) prepared by rechromatography on Sepharose-4B. Lanes 2–4: clathrin, 10, 20, and 40 μg, respectively, obtained from the clathrin preparation shown in lane 1 after chymotrypsin treatment.

Figure 4. Electron micrograph of negatively stained clathrin structures. A preparation of clathrin (polypeptide composition shown in Figure 3, lane 1) assembled cage- or basket-like structures simply by adjusting pH from 7.5 to 6.3. In assembled form, the protein was treated briefly with chymotrypsin. Cages shown remained assembled after enzyme treatment. Protein composition after enzyme treatment is illustrated in Figure 2, lane 1 and Figure 3, lanes 2–5. Original magnification ×350,000. Bar = 0.035 μ.

Figure 5. Partially polymerized clathrin molecules (trimers) formed by chymotrypsin-treated clathrin. Baskets were absent. Original magnification ×120,000. Bar = 0.1 μ.
phoresis. For the chymotryptic treatment, clathrin had to be assembled into cages or baskets. Seen by electron microscopy, clathrin's state of polymerization at pH 6.5 remained unassembled into cages or baskets. Seen by electron microscopy, clathrin's state of polymerization at pH 6.5 remained unassembled into cages or baskets. Over 90% of clathrin was involved in this state of assembly as determined by the amount of protein pelleted by high-speed centrifugation. After enzyme treatment, clathrin cages could be depolymerized at pH 7.5 in a Tris-HCl buffer containing 2 M urea. After dialysis to remove the urea, clathrin did not assemble typical cages at pH 6.5. Instead, partially assembled lattices formed that did not sediment by high-speed centrifugation (Figure 5).

**Immunological Studies/Antibody Characterization**

Antibodies elicited by clathrin assembled as baskets were purified by affinity chromatography using clathrin-coupled CNBr-Sepharose-4B. Details of the procedures used for purification and characterization of the IgG molecules were presented in a previous report (2). However, in view of clathrin's higher degree of homogeneity, the anti-clathrin antibodies were characterized further. Figure 6 illustrates an immunodiffusion plate wherein clathrin antibodies reacted producing an immunoprecipitin line against native clathrin. The precipitin line fused with another produced by enzyme-treated clathrin. No immunoprecipitin lines formed against tubulin or actin, indicating that the IgG molecules had reacted only with antigenic determinants of the clathrin polypeptide. To ascertain whether other antibodies were present and reacting with clathrin's copurifying proteins, the IgG solution was absorbed for 2 hr at 37°C followed by overnight incubation at 4°C with enzyme-treated clathrin. After this treatment, the IgG supernatant solution was tested against native clathrin. No immunoprecipitin reaction was observed.

Figure 6. Double-gel immunodiffusion in 0.85% agarose. Center well contains antibodies to clathrin elicited by clathrin shown in Figure 3, lane 1. Well #1: enzyme-treated clathrin shown in Figure 3, lanes 2–5; Well #2: clathrin shown in Figure 2, lanes 2 and 3; Well #3: brain tubulin; Well #4: muscle actin.

**Immunofluorescent Staining/Relationship of Clathrin with Actin Fibers**

Clathrin appeared as fluorescent dots in cells. Of the various fixatives and solvents tested, the most intense dotted staining was produced after paraformaldehyde fixation and membrane solubilization with Triton X-100. Failure to permeabilize the plasma membrane resulted in no staining pattern, signifying that clathrin is exclusively a cytoplasmic protein.

Dots always were aligned either longitudinally, parallel to one another, or superimposed upon actin stress fibers (Figure 7a,b). These fibers, labeled with actin antibodies, are shown in Figure 7c and d. Fluorescent actin stress fibers were labeled optimally after a brief acetone treatment. This process denatures and/or solubilizes other cytoplasmic proteins. As a consequence, the actin-staining protocol adversely affected clathrin's fluorescent staining and precluded dual-labeling experiments to visualize actin filaments and clathrin dots simultaneously in the same preparation. Nevertheless, Figure 7a clearly illustrates areas of the cytoplasm in which the fluorescent dots only are seen arranged linearly with actin fibers.

**Binding Affinity of Clathrin for Actin**

The persistent presence of actin accompanying clathrin through various purification steps prompted us to determine if such binding affinity could be measured between purified clathrin and actin. For this, enzyme-treated clathrin was adsorbed on the surfaces of LP (Figure 8a,b). Table 1 shows that when clathrin-coated LP were incubated with G-actin in solution, they bound approximately 2 mol of actin/mol of clathrin subunit (mol wt = 180 kilodaltons). Native clathrin, which contained varied amounts of a 43 kilodalton band (actin, shown in Figure 2, lanes 2 and 3) bound significantly less actin from solution. When clathrin-bound LP were incubated with chlorpromazine (10⁻³–10⁻⁴ M) for 15 min and the free drug removed from the protein-coated LP by centrifugation, the amount of actin bound by these particles was not altered.

**Redistribution of Clathrin during Cell Retraction**

When fibroblasts were incubated with chlorpromazine (10⁻³–10⁻⁴ M) to effect cytoplasmic retraction, distribution of clathrin's fluorescent dots was altered (Figure 9a,b). Instead of the linear arrangement observed in untreated cells, the dots acquired a circular arrangement, conforming to the new cell shape. Areas of peripheral cytoplasm were devoid of lattices, suggesting that retrieval of clathrin had occurred with accumulation of dots in an area surrounding the nucleus. Chlorpromazine-treated fibroblasts with the membrane-permeation step omitted showed no fluorescent staining, indicating that chlorpromazine’s other disrupting effects were not evident at the concentrations used.

**Discussion**

Clathrin was isolated with a high degree of homogeneity following its solubilization from bovine brain cortex subcellular...
fractions enriched with coated vesicles (3,13). The second protein peak eluted by Sepharose-4B column chromatography contained mostly clathrin with small amounts of copurifying polypeptides that migrated on gel electrophoresis, similar to tubulin and actin. These polypeptides separated by rechromatography, except for the ~30 kilodalton doublet that remained bound to clathrin. These CAPs were susceptible to proteolysis under controlled conditions where clathrin ap-

peared refractive. Limited proteolysis was advantageous for removing not only CAPs but also actin and tubulin when present. Clathrin's 180 kilodalton polypeptide showed no degradation products, as evidenced by gel electrophoretic analysis using decreasing concentrations of protein. Thus, the difference between native and enzyme-treated clathrins resided in their relative capacities for reassembling baskets, suggesting that intact CAPs possess modulatory characteristics enabling clathrin to form baskets.

The ratio of clathrin to CAPs is approximately 3:1. Since clathrin forms a triplet unit when depolymerized (6,15), it is possible that CAPs may be present at the point where clathrin subunits join together to form the trimeric complex. Our results show that clathrin baskets remained assembled after removal of the CAPs, implying that intact CAPs are not essential for maintaining the structural integrity of baskets. The manner by which CAPs affect clathrin-clathrin interaction may derive from their being positioned at the vertex, thereby altering the plane of individual clathrin monomers and allowing the interlocking of trimers. This model precludes the need for CAPs to be positioned at each end of the clathrin polypeptide to mediate their binding of clathrin trimers.

Figure 8. (a) Electron micrograph of a negatively stained preparation of LP coated with enzyme-treated clathrin. These clathrin-coated LP were used to bind actin molecules from solution. Original magnification ×300,000. Bar = 0.05 μ. (b) Plain LP before coating with clathrin. Original magnification ×120,000. Bar = 0.1 μ.
Table 1. Binding of actin by clathrin-coated Lytron particles

<table>
<thead>
<tr>
<th>Protein used</th>
<th>1st Protein bound* µg bound</th>
<th>2nd Protein bound µg actin</th>
<th>Molar ratios actin/clathrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native clathrin</td>
<td>150</td>
<td>40</td>
<td>~1</td>
</tr>
<tr>
<td>Enzyme-purified clathrin</td>
<td>180</td>
<td>100</td>
<td>~2</td>
</tr>
</tbody>
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*The first protein layer was made using LP and clathrin. Clathrin-coated LP were suspended in a solution of muscle G-actin and the particles sedimented by low-speed centrifugation. The amount of protein bound was determined by the amount of protein remaining in the supernatant before and after adsorption (4, 13).

The persistent presence of actin in clathrin preparations suggests it may play a role in clathrin functions. The actin-to-clathrin molar binding ratio was 2:1, as determined on the surfaces of clathrin-coated LP. Purified native clathrin revealed about 1 mol of bound actin per mol of clathrin, by gel densitometry; moreover, it bound 1 mol of G-actin from solution while enzyme-treated clathrin bound 2 mol of actin. Thus, the possibility exists that a certain amount of actin (~1 mol) may be lost during clathrin purification. Two binding sites for actin—one of higher, one of lower affinity—may exist in clathrin. Clathrin-associated proteins are not required for actin/clathrin binding. In support of this, it was reported that coated vesicles are attached through their coat lattices to actin filaments decorated with heavy meromyosin (11). Under the membrane, clathrin lattices were seen attached to actin's double-headed strands by short lateral projections of unknown composition (5).

Further evidence of clathrin's affinity for actin was obtained from our cytological labeling of both proteins in cells. Immunofluorescent patterns for each protein are unique in that actin has been visualized forming stress fibers, while clathrin appeared as dots (2) in accordance with its ability to form lattices apposing the plasma membrane on its cytoplasmic side (5). In vitro, the affinity of clathrin for actin does not enhance polymerization of its molecules into their characteristic ultrastructure, inasmuch as actin does not require clathrin to form filaments nor does clathrin require actin to assemble into baskets. Their interaction, therefore, may be expressed in vivo by the observed correlation and parallelism of the distribution of actin fibers when clathrin is bound to the lipid membrane.

In our work, chlorpromazine was used as a nonspecific effector because of its ability to inhibit both actin polymerization (4) and receptor-mediated endocytosis without affecting the binding affinity of clathrin for actin or the ability of clathrin to form lattices at the levels used. At the concentrations of chlorpromazine applied to the fibroblasts in culture, no membrane damage was observed. This became evident when the permeation step was omitted (with Triton X-100) and no fluorescence could be detected either with antibodies to clathrin or with unrelated antibodies. In this instance, anti-fibronectin stained the extracellular matrix but failed to label cytoplasmic fibronecin.

The exact molecular nature of events that occur during cytoplasmic retraction and retrieval of clathrin lattices or their redistribution undoubtedly deserve further study. At present, we have observed that neither chlorpromazine nor cytoplasmic retraction diminish either the number or the staining intensity of clathrin lattices in the fibroblast. The stability of these lattices appears different from other cytoskeletal structures, i.e., actin filaments or microtubules that are depolymerized and disassembled rapidly by the cell. The clathrin lattices, however, seemed to have been preserved and retrieved laterally with the cytoplasm during retraction (Figure 10).

Figure 9. (a) Indirect immunofluorescent staining with clathrin antibodies. Human fibroblasts were incubated with 10–100 µM chlorpromazine for 1 hr at room temperature. Clathrin lattices redistributed into concentric patterns. Original magnification ×1,900. Bar = 0.5 µ. (b) Phase contrast micrograph of the cell shown in a, illustrating the morphological change undergone by the cell. Bar = 0.5 µ.

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Finally, our observations suggest that the clathrin lattices fulfill an important role for the cell and that they are preserved to stabilize the membrane lipid bilayer in the absence of a fully assembled cytoskeleton.

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Literature Cited