Stable Clathrin: Uncoating Protein (*hsc70*) Complexes in Intact Neurons and Their Axonal Transport

Mark M. Black,¹ Matthew H. Chestnut,² Irene T. Pleasure,^{2,3} and James H. Keen^{2,3}

¹Department of Anatomy and Cell Biology, ²Fels Institute for Cancer Research and Molecular Biology, and ³Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

We have studied the organization of clathrin during its transport in axons. Using immunoprecipitation techniques we have confirmed earlier findings that clathrin is transported as part of slow component b, but we also detect small amounts of clathrin in fast component. As fast component is known to correspond to the transport of membranous material, including coated vesicle membrane components, our findings suggest that some clathrin in axons undergoes transport in the form of coated membranes and that a portion of the clathrin delivered to axons and axon terminals arrives by way of fast component. The organizational form of clathrin in slow component b (SCb) was examined in more detail, as it is thought to represent a non-membrane-associated species, is relatively long-lived, and at any instant represents the major transport species in axons. We used nondenaturing immunoprecipitation methods with stringent wash procedures to identify other SCb proteins that interact with clathrin. The immunoprecipitates contained major labeled bands that corresponded to clathrin heavy and light chains, along with a prominent 70-kDa band and several minor bands that ranged in apparent *M_r* from 70,000 to 150,000; the 70-kDa band was shown to be the ATP-dependent uncoating protein by two-dimensional gel electrophoresis. A very similar profile of polypeptides was also immunoprecipitated from extracts of cultured neurons. The results from a variety of control immunoprecipitations, including the use of antisera preadsorbed with purified clathrin trimers or clathrin light chains, indicate that coprecipitation of clathrin and uncoating protein with the other 70,000–150,000-Da polypeptides from SCb reflects specific interactions. Including exogenous uncoating protein in the lysis buffer had no detectable effect on the levels of endogenous uncoating protein recovered in the immunoprecipitates, indicating that complexes of clathrin, uncoating protein, and the other coimmunoprecipitating SCb proteins existed in the intact neurons prior to lysis. Finally, a specific and functional association is further supported by the release of uncoating protein, but not the other 70,000–150,000-Da polypeptides, from the immunoprecipitated complexes on the addition of ATP. Collectively, these

observations provide the first direct evidence of interaction between clathrin and uncoating protein in intact cells, lend strong support to the concept that uncoating protein plays an intimate role in clathrin dynamics within cells, and reveal a family of 70,000–150,000-Da polypeptides that form a stable nonmembranous association with clathrin in intact cells.

Studies of clathrin organization in intact cells have revealed assembled and unassembled forms that correspond to a membrane-associated coat structure and to a nonmembranous, apparently soluble cytoplasmic form, respectively (Louvard et al., 1983; Brodsky, 1985; Goud et al., 1985). In the intact cell clathrin is believed to cycle repeatedly between these forms, as, for example, in the uncoating and reformation of coated pits and vesicles at the plasma membrane during the process of receptor-mediated endocytosis (Goldstein et al., 1985).

The assembly of clathrin in cells is thought to be regulated at least in part by one or more of the assembly proteins (APs) because of the potent coat assembly activity of APs under *in vitro* conditions (Keen, 1990a,b). This view is reinforced by the colocalization of the APs with assembled clathrin *in situ* (Robinson and Pearse, 1986; Robinson, 1987; Ahle et al., 1988). Because the clathrin lattice of coated membranes is quite stable under physiological conditions of pH and ionic strength, it has also been suggested that the uncoating of such membranes is an active process (Patzner et al., 1982; Rothman and Schmid, 1986). An ATP-dependent uncoating protein (UP) has been identified and extensive studies with purified proteins have demonstrated its ability to disassemble the clathrin lattice of coated vesicles under *in vitro* conditions (Schmid and Rothman, 1985a,b; Schmid et al., 1985; Rothman and Schmid, 1986). The UP is identical to one of the constitutively synthesized 70-kDa heat shock “cognate” proteins, termed *hsc70* (Ungewickell, 1985; Chappell et al., 1986). Genetic and biochemical studies have provided evidence that members of this family of proteins, and the related 78-kDa homolog in the endoplasmic reticulum, are involved in protein unfolding and refolding events of both native and aberrant proteins (Gething et al., 1986; Chirico et al., 1988; Deshaies et al., 1988; Zimmermann et al., 1988; Hurtley et al., 1989; Pelham, 1989). These observations, the presence of substantial amounts of UP relative to clathrin in many cells (Schlossman et al., 1984; Davis and Bennett, 1985; Davis et al., 1986; Giebel et al., 1988; Perkins et al., 1990) and the quite different response of UP and clathrin to heat stress (Ungewickell, 1985), have led to questioning of the view that uncoating of clathrin-coated membranes is a major, or possibly even a significant, function of the UP/*hsc70* in cells. In short, there has

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Correspondence should be addressed to Dr. Mark M. Black, Department of Anatomy and Cell Biology, Temple University School of Medicine, Philadelphia, PA 19140.

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been no direct evidence of either transient or long-term interactions between clathrin and UP in intact cells.

Neurons provide a useful model system to characterize cytoplasmic forms of clathrin and UP. Neurons synthesize relatively high levels of clathrin, much of which is conveyed from the cell body into the axon and axon terminal by the process of axonal transport (Garner and Lasek, 1981). Proteins undergo axonal transport at discrete rates in axons (reviewed in Grafstein and Forman, 1980). Three major rate classes of axonal transport have been identified and termed fast component (FC), slow component b (SCb), and slow component a (SCa). FC has an average transport rate of 250–400 mm/d and corresponds to the transport of membranous structures. SCa and SCb are transported much more slowly than FC and they represent the movement of distinct groups of cytoplasmic proteins. SCa has a transport rate of 0.2–1.5 mm/d and corresponds primarily to the transport of microtubule and neurofilament proteins (Lasek et al., 1984). SCb has a transport rate of 2–5 mm/d. SCb contains hundreds of distinct proteins, including glycolytic enzymes, actin, myosin, calmodulin, synapsin, and clathrin (Willard, 1977; Black and Lasek, 1979; Brady and Lasek, 1981; Brady et al., 1981; Garner and Lasek, 1981; Baitinger and Willard, 1987; Oblinger et al., 1988); clathrin represents $\approx 1\%$ of the total material of SCb (Garner and Lasek, 1981). As SCb does not contain detectable intrinsic membrane components, the transport form of SCb clathrin is most likely nonmembranous. In typical axons, which have lengths of millimeters to centimeters, it takes days to weeks for SCb clathrin to be transported from the cell body to the axon terminal, where it may function in the membrane recycling processes associated with neurotransmission (Heuser and Reese, 1973). Thus, an apparently nonmembranous transport form of clathrin is a relatively long-lived species in intact neurons, particularly in the axon.

In considering the nature of the transport form of SCb clathrin, it is noteworthy that SCb also contains two families of clathrin-binding proteins, the UP and the AP (Chestnut et al., 1986; de Waegh and Brady, 1989). Cotransport of these proteins provides circumstantial evidence for a long-term association of one or both of these proteins with clathrin during their transport in the axon but the significance of this observation is clearly limited by the enormous compositional complexity of SCb (Black and Lasek, 1980; Garner and Lasek, 1982). In the present studies, we have used nondenaturing immunoprecipitation in an attempt to isolate the transport form of SCb clathrin. Using an antibody specific for clathrin light chain b, we found that a discrete subset of minor SCb proteins was indeed specifically immunoprecipitated with clathrin, and demonstrated that the major coprecipitating protein was the 70-kDa UP. Furthermore, a similar complex containing clathrin and other cellular polypeptides could also be isolated by immunoprecipitation from cultured neurons. These results provide the first definitive demonstration of a stable association of clathrin and UP *in situ*, and raise the possibility that clathrin, UP, and other SCb proteins exist in a novel form that may be specialized, in part, for intracellular transport.

Materials and Methods

Protein preparations. Bovine brain clathrin was prepared by gel filtration with Superose 6B (Pharmacia Fine Chemicals, Piscataway, NJ) as described previously (Keen, 1987). Enriched preparations of clathrin light chains were also prepared from bovine and rat brains as follows. All steps were performed at 0–4°C unless otherwise noted. We homogenized ≈ 10 gm of brain in 2 volumes of buffer A (0.1 M NaMES, pH 6.5, 1.0

mM EGTA, 0.5 mM MgCl₂, 0.02% Na azide, 0.5% Trasylol, and 10 μ g/ml each of leupeptin, pepstatin and antipain) using three strokes of a motorized glass-Teflon homogenizer. The homogenate was centrifuged for 30 min at 14,500 $\times g$, and the supernate recentrifuged for 60 min at 81,000 $\times g$ in a Beckman type 40 rotor. The high speed pellet was resuspended in buffer B (buffer A:1.0 M Tris-HCl, pH 7.0; 1:1), incubated at room temperature for 10 min, and then placed in a boiling water bath for 5 min. The material was then centrifuged for 60 min at 81,000 $\times g$ to obtain a heat-stable supernate which contained predominantly clathrin light chains (Lisanti et al., 1982; Fig. 1). Initially, ATP-dependent uncoating protein was the generous gift of Dr. Cliff Steer and was subsequently prepared according to published procedures (Heuser and Steer, 1989). The purified UP used for these experiments was active as judged by its ability to uncoat reassembled coats *in vitro* (data not shown).

Radiolabeling axonally transported proteins. The present studies have examined SCb and FC for clathrin–UP complexes. Labeled proteins of FC or SCb were obtained from optic nerves and tracts of animals killed at 2.25–2.5 hr or 6–7 d postinjection, respectively. Injections were performed on male CD rats weighing 350–550 gm (Charles River Laboratories, Wilmington, MA) anesthetized with 50 mg/kg Nembutal (Abbott Laboratories, North Chicago, IL) administered intraperitoneally. ³⁵S-methionine (Amersham Corp., Arlington Heights, IL), 0.5–1.5 mCi, was lyophilized and redissolved in 5 μ l of deionized water and then injected into the vitreous of the eye. For most experiments, both eyes of each rat were injected with isotope, and the optic nerves were either pooled or analyzed separately for clathrin by immunoprecipitation (see below). In addition, control experiments were carried out in which one eye from each rat was injected with ³⁵Smet and then the optic nerves from the injected and uninjected eyes were assayed for labeled clathrin by immunoprecipitation. In all of these latter experiments, the levels of labeled clathrin and other coimmunoprecipitating proteins in the material from the injected eye greatly exceeded that from the uninjected eye (see Fig. 3, compare lanes 2 and 4 and data not shown). Thus, local incorporation of ³⁵Smet into protein by non-neuronal cells in the optic nerve was not a significant factor in the experiments reported here.

Immunological procedures. A rabbit antisera to bovine brain clathrin trimers (Keen et al., 1981) was used in immunoblotting and immunoprecipitation analyses. For evaluation of clathrin heavy chain proteolysis, a monoclonal anti-bovine brain clathrin heavy chain antibody (C_{Hc} 5.9, Boehringer–Mannheim) was used in immunoblotting analysis. Immunoblotting was carried out as described previously (Peng et al., 1985). Immunoprecipitation was used to assay SCb and FC for clathrin. Optic axons containing labeled SCb or FC were homogenized under denaturing or nondenaturing conditions as indicated. For denaturing conditions, the tissue was homogenized in 1% SDS containing protease inhibitors, boiled for 5 min, clarified by centrifugation, and then diluted with 9 volumes of RIPA buffer [50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, protease inhibitors (the same cocktail as in Buffer A)]. For nondenaturing conditions, the tissue was homogenized in buffer B or in RIPA buffer, and then centrifuged for 30 min at 100,000 $\times g$; $\approx 70\%$ of labeled SCb proteins and $\approx 90\%$ of the labeled FC proteins were solubilized under these nondenaturing conditions. The supernates were then preincubated for 30–60 min at 4°C on a rotator with protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in the appropriate buffer. The protein A-Sepharose was removed by centrifugation, the preadsorbed extract was divided into appropriate numbers of equal-sized aliquots and incubated with either an immunoglobulin fraction of antiserum or non-immune serum; for some experiments, antiserum was preadsorbed with clathrin trimers or clathrin light chains. Immunoglobulin fractions of antiserum and nonimmune serum were used at a dilution of 1:100 (vol/vol). Immunoglobulin fractions of antiserum were preadsorbed with antigen by incubation for 60 min with 0.33 mg clathrin trimers or 1 mg clathrin light chains per milliliter of immunoglobulin fraction. The labeled samples were incubated with antibody for 30–60 min at 4°C and then incubated with 5 mg protein A-Sepharose equilibrated in the appropriate buffer containing 2% BSA, for ≥ 2 hr at 4°C on a rotator. Antibody–antigen complexes bound to the protein A-Sepharose were collected by centrifugation, washed twice with RIPA buffer, spun through a 1-ml cushion of 0.5 M sucrose + 2 M urea in RIPA buffer, and then rinsed sequentially with RIPA + 2 M urea, RIPA without Triton X-100 and SDS, and finally 10 mM phosphate buffer, pH 7.0. Proteins bound to the beads were then solubilized by boiling in electrophoresis sample buffer.

Studies of clathrin–UP complexes in cultured neurons. These analyses

Figure 1. Immunological and biochemical analysis of brain clathrin light chains. *Panel A*, Immunoblotting analysis of clathrin trimers and purified light chains from calf and rat brain. *Lanes 1, 3*, Clathrin trimers and purified light chains, respectively, from calf brain. *Lane 2*, Clathrin light chains from rat brain. A single band is recognized in all three samples; the band in the calf brain samples comigrates precisely with authentic LCb; the band in the rat brain sample migrates slightly slower than that from calf brain. *Panel B*, Coomassie blue-stained gel loaded identically to the blot shown in panel A. *Panels C–E*, Analysis of calf and rat brain light chains by 2-D gel electrophoresis. Shown are reproductions of portions of the Coomassie blue-stained gels containing calf brain light chains (panel C), rat brain light chains (panel D), or mix of calf and rat brain light chains (panel E). In all panels, the *arrows* point to light chain a (LCa) and the *arrowheads* point to light chain b (LCb).

used 10–14-d-old dissociated cultures of rat sympathetic neurons prepared and metabolically labeled as described previously (Peng et al., 1985). These cultures typically consist of >95% neurons. For most experiments, cultured neurons were lysed by homogenization in buffer C (RIPA without SDS), and the resulting extracts were clarified by centrifugation for 10 min at 245,070 g_{max} at 2°C; variations on the lysis procedure are discussed in the Results section. The supernates were then assayed for clathrin by immunoprecipitation as described above, except that buffer C was used in place of RIPA buffer.

Gel electrophoresis. One-dimensional gel electrophoresis in SDS was as described previously (Keen, 1987). Two-dimensional isoelectric focusing $\times$ SDS gel electrophoresis was as described (Keen and Black, 1986) using an ampholine mixture of 0.5% pH 3.5–10 and 1% each of pH 4–6 and pH 5–6. Gels were stained with Coomassie brilliant blue. Labeled proteins were visualized by fluorography using either Enhance (NEN, Boston, MA) or PPO/DMSO (Bonner and Laskey, 1974); processed gels were incubated with preflashed XAR-5 film (Eastman Kodak Co., Rochester, NY) at -70°C . Quantification of radioactivity in selected bands was performed by excising the bands from the gels, dissolving the gel pieces in H_2O_2 , and then measuring the radioactivity by scintillation counting.

Results

Clathrin and uncoating protein of SCb exist as a stable complex

If clathrin and uncoating protein of SCb exist as a stable complex, then it should be possible to coimmunoprecipitate both proteins with a monospecific antibody to clathrin under non-denaturing conditions. We have tested this prediction using a

polyclonal antibody prepared against gel filtration-purified bovine brain clathrin, which consists of a heavy chain plus two light chains designated LCa and LCb. On immunoblotting of bovine brain clathrin trimers or partially purified clathrin light chains from bovine brain or rat brain, the antibody specifically recognized LCb (Fig. 1, panels A,B); a single band was also observed on immunoblotting of whole rat brain SDS extracts (data not shown). We note that the clathrin light chain bands from rat brain were slightly higher in apparent M_r than their counterparts from bovine brain (Fig. 1, panels B–E).

The antibody can immunoprecipitate labeled clathrin from nerve extracts containing labeled SCb proteins. Previous studies have shown that SCb contains both clathrin heavy chain (Garner and Lasek, 1981) and light chains (Gower and Tytell, 1987) and we have confirmed the presence of LCa and LCb in SCb by 2-D gel electrophoresis (Fig. 2B). When optic nerve containing labeled SCb proteins was dissolved in SDS, which fully dissociates and denatures clathrin trimers, and then assayed for clathrin by immunoprecipitation, a single labeled band was precipitated that comigrated with rat brain LCb in 1-D gels (Fig. 2, lane 2) and in 2-D gels (data not shown). The presence of this labeled material in the immunoprecipitates could be blocked completely by preadsorbing the antibody with clathrin trimers (Fig. 2, lane 1). These findings further establish the specificity of this antibody and document that it can be used in axonal transport studies.

Figure 2. Immunoprecipitation of clathrin from SCb. **Panel A**, Immunoprecipitates from optic axons containing labeled SCb proteins that were dissolved in 1% SDS (lanes 1, 2) or RIPA buffer (lanes 3, 4). The precipitates in lanes 1 and 3 were obtained with antiserum preabsorbed with clathrin trimers, whereas those in lanes 2 and 4 were obtained with unabsorbed antiserum. In the sample dissolved in SDS, a single band was precipitated by the unabsorbed antisera but not by the preadsorbed antisera, and this band comigrated with rat LCb. In the sample dissolved in RIPA buffer, clathrin heavy and light chains plus several nonclathrin bands were precipitated by the unabsorbed antisera but not by the preadsorbed antisera. The numbers ($\times 1000$) to the left of panel A indicate molecular weight. The positions of clathrin heavy chain (HC), clathrin light chain b (LCb; arrowheads) and the major 70-kDa coimmunoprecipitating polypeptide are indicated to the right of panel A. **Panel B**, Optic axons containing labeled SCb proteins were dissolved directly in SDS and analyzed by 2-D isoelectric-focusing $\times$ SDS gel electrophoresis and fluorography. Unlabeled rat brain light chains were added to the sample as internal markers. The arrow and arrowhead indicate the positions of the carrier brain LCa (arrow) and LCb (arrowhead), respectively. Labeled proteins of SCb comigrate precisely with carrier rat brain LCa and LCb.

We used nondenaturing immunoprecipitation to look for stable interactions between clathrin and uncoating protein during axonal transport. Optic nerves containing labeled SCb proteins were extracted with either RIPA buffer or buffer B and then probed for clathrin-uncoating protein complexes by immunoprecipitation. Clathrin trimers remain intact under these solution conditions and can be immunoprecipitated by our anti-LCb antibody. The most prominent band in these immunoprecipitates of SCb is the clathrin heavy chain, with an apparent M_r of 180 K, and bands corresponding to LCa and LCb are also present (Fig. 2A, lane 4 and, in a considerably heavier exposure from a replicate experiment, Fig. 3, lane 6). In addition, several other bands were present in the immunoprecipitates. The most prominent of these bands had an apparent M_r of 70,000 with most of the other nonclathrin bands having apparent M_r ranging from 70,000 to 150,000 (Fig. 2A, lane 4 and Fig. 3, lane 6). The same profile of bands was reproducibly immunoprecipitated in replicate experiments and was observed in extracts of nerve prepared in RIPA buffer or in buffer B.

The possibility that these 70,000–150,000-Da polypeptides represent partially degraded clathrin heavy chains immunoprecipitated by the antibody is unlikely for several reasons. First, the antibodies responsible for immunoprecipitation are directed exclusively toward the clathrin light chains (see above). As these are much more proteolytically sensitive than the clathrin heavy chain (Ungewickell and Branton, 1981), complexes with degraded heavy chain would be expected to be devoid of light chain and not recognized by the antibody. Second, the diversity of the 70,000–150,000-Da polypeptides observed in the immunoprecipitates does not correspond to the specific and limited clathrin heavy chain fragments of 100,000–115,000-Da that are generated by a variety of different proteases *in vitro* (Kirchhausen and Harrison, 1984). Finally, as an indistinguishable pattern of 70,000–150,000-Da polypeptides has been observed in immunoprecipitates of cultured rat sympathetic neurons (see below), we directly lysed these cells in RIPA buffer and used an anti-clathrin heavy chain antibody to probe for heavy chain fragments by immunoblotting. In addition to intact heavy chain,

minor species in the 100,000–115,000 Da range were observed, consistent with the pattern expected from *in vitro* proteolysis experiments, but unlike the pattern of the immunoprecipitates obtained with our anti-LCb antibody. Furthermore, incubation of the RIPA lysate for 6 hr to mimic the immunoprecipitation protocol had no detectable effect on the relative levels of intact heavy chain and heavy chain fragments (data not shown), arguing against significant proteolysis in the sample after cell lysis. These observations indicate that proteolyzed clathrin heavy chain does not account for the 70,000–150,000 Da polypeptides observed in our nondenaturing immunoprecipitates.

The presence of nonclathrin bands in the immunoprecipitates obtained with the anti-LCb antibody could reflect nonspecific processes such as adsorption or trapping, the presence of contaminating antibodies, or specific interactions between the clathrin and nonclathrin proteins that survive immunoprecipitation. The possibility that the nonclathrin proteins are nonspecifically precipitated is unlikely for two reasons. First, a very stringent wash procedure, which included washing with RIPA buffer supplemented with 2 M urea (see Materials and Methods), was used in the present experiments. Second, if the presence of the nonclathrin proteins in the immunoprecipitates was due to nonspecific processes, then these proteins should for the most part correspond to major components of SCb. This expectation was not supported by the data. The most heavily labeled SCb proteins range in apparent *M_r* from $\approx 30,000$ to $\approx 70,000$ (Black and Lasek, 1980; Garner and Lasek, 1982; M. M. Black, M. H. Chestnut, I. T. Pleasure, and J. H. Keen, unpublished observations), whereas the most prominent SCb polypeptides that coimmunoprecipitate with clathrin range in apparent *M_r* from 70,000 to 150,000 (Fig. 2*A*, lane 4 and Fig. 3, lane 6). These observations indicate that the coimmunoprecipitating proteins are not major components of SCb and that immunoprecipitation resulted in selective enrichment of a subset of minor SCb proteins.

To evaluate directly the contribution of nonspecific processes to the results, we have carried out immunoprecipitation analyses with antibody preadsorbed with purified unlabeled clathrin. Preadsorption should have minimal effect on nonspecific precipitation because immune complexes of antibody + unlabeled clathrin will be present and precipitated. These complexes should be equivalent to the antibody + labeled clathrin precipitated with nonadsorbed antibody with respect to trapping and nonspecific adsorption of SCb proteins. In addition, preadsorption experiments using purified clathrin provide stringent tests for the presence of contaminating antibodies that could be present in our anti-LCb antisera. Preadsorption of the anti-LCb antibody with either purified clathrin trimers or isolated clathrin light chains blocked not only the immunoprecipitation of labeled clathrin but also the 70-kDa band and most of the other coprecipitating bands (Fig. 2*A*, lane 3 and Fig. 3, lane 5). Similar results were also obtained in other control experiments that used preimmune or various nonimmune antisera. These results provide direct evidence that nonspecific processes and contaminating antibodies are not responsible for the coimmunoprecipitation of nonclathrin proteins with clathrin under the nondenaturing conditions used in these experiments. Thus, we conclude that clathrin heavy and light chains, the 70-kDa band, and a subset of nonclathrin proteins of SCb are specifically immunoprecipitated by the antibody, even though it is specific for LCb. The most likely explanation for the coprecipitation of these proteins with clathrin is that the nonclathrin proteins form

Figure 3. Identification of clathrin in FC and SCb. Optic nerves containing labeled proteins of either FC or SCb were extracted with RIPA buffer and assayed for clathrin by nondenaturing immunoprecipitation as described in Materials and Methods. Extracts of optic nerve containing equal CPM of either FC or SCb were also adjusted to contain equal nerve protein by adding appropriate volumes of identically prepared extracts from unlabeled nerve. The resulting samples, which thus contained the same amount of total radioactivity, the same amount of total nerve protein, and the same total amount of clathrin (i.e., labeled + unlabeled), were then challenged with the anti-clathrin light chain antibody. *Lanes 1–4*, Immunoprecipitates from the uninjected (lanes 1 and 2) or injected (lanes 3 and 4) optic nerve of a rat killed 2.5 hr postinjection; the nerve from the injected eye contains labeled fast component proteins whereas the nerve from the uninjected eye serves as a control for local incorporation due to blood-borne delivery of ^{35}S -L-Met. *Lanes 5 and 6*, Immunoprecipitates from the injected optic nerve of a rat killed 6 d postinjection and thus containing labeled SCb proteins. Immunoprecipitates obtained with unadsorbed antibody are shown in lanes 2, 4, and 6, whereas those obtained with antibody preadsorbed with clathrin trimers are shown in lanes 1, 3, and 5. The positions of molecular weight markers, clathrin heavy and light chains, and the 70-kDa coimmunoprecipitating protein are indicated as in Figure 2.

one or more complexes with clathrin that are precipitated by the antibody. This view is strongly reinforced by the demonstration that the 70-kDa coprecipitating protein is the UP, a known clathrin binding protein (see below).

Clathrin is transported in fast component

Previous studies using 2-D electrophoresis of axonal extracts containing labeled FC proteins have failed to detect clathrin in FC (Gower and Tytell, 1987). As this type of assay is relatively insensitive, we have used immunoprecipitation to reevaluate

FC for the presence of clathrin (Fig. 3). Equal amounts of radioactive FC and SCb were challenged with the anti-LCb antibody (details of the procedure are noted in the figure legend). This experimental design reveals the proportion of FC and SCb comprised by clathrin but does not directly compare the total amounts of clathrin transported in each rate class.

Clathrin is detected in FC, though it comprises much less of this rate class than of SCb (Fig. 3, compare lanes 4 and 6). Nonetheless, these levels were considerably greater than those obtained from controls derived from uninjected eyes (see Fig. 3, lanes 2 and 4, and Materials and Methods), thereby confirming that clathrin is indeed transported in FC. The relative contribution of FC and SCb to the steady state levels of clathrin in the axon and axon terminal is a complex issue. The observations that SCb is labeled to a much greater extent than FC in pulse-labeling experiments (Grafstein and Forman, 1980) and that clathrin comprises a much greater proportion of SCb than of FC (Fig. 3) suggests that much more clathrin is delivered to the axon by SCb than by FC. However, because the transport rate of FC is ≈ 100 -fold greater than SCb, the role of FC in maintaining clathrin levels in the axon and axon terminal may be greater than that suggested solely by considering the amounts of clathrin in FC and SCb.

The immunoprecipitates from FC also differed from those of SCb in another important respect. As seen earlier, the immunoprecipitate from SCb was very heterogeneous, consisting of clathrin heavy and light chains, and the 70-kDa uncoating protein, as well as many other bands. Nonclathrin proteins were also present in the immunoprecipitates from FC, but unlike the situation for SCb, most of these corresponded to major FC proteins (data not shown), thus raising the possibility that their presence in the immunoprecipitates was due to nonspecific processes. This prospect is reinforced by the finding that preadsorption of the antibody with clathrin trimers completely eliminated labeled clathrin from the precipitates but only partially reduced the levels of many of the nonclathrin proteins (Fig. 3, lanes 3 and 4 and data not shown). This contrasts with the result obtained with SCb, in which preadsorption much more completely eliminated the nonclathrin proteins from the precipitates (Fig. 3, lanes 5 and 6).

The 70-kDa band in immunoprecipitates from SCb obtained with the anti-LCb antibody is the clathrin uncoating protein

Two observations suggested that the 70-kDa band of SCb that coprecipitated with clathrin was the UP. First, its apparent M_r corresponds to that of the ATP-dependent UP (Schlossman et al., 1984). Second, during the course of these studies, transport of the UP in SCb was reported (de Waegh and Brady, 1989). To determine whether the 70-kDa coprecipitating protein was indeed the UP, immunoprecipitates from nerve extracts containing labeled SCb proteins were obtained with the anti-LCb antibody and coelectrophoresed with authentic UP (generously provided by Dr. C. Steer) on 2-D gels. Autoradiographic exposures of the gels revealed a single labeled spot in the molecular weight range of $\approx 70,000$, and this spot comigrated precisely with the most acidic variant of the UP (Fig. 4). Labeled species comigrating with other, more basic, variants of the UP preparation were not detected, a point of interest as these species probably correspond to other variants within the 70-kDa *hsp/hsc* family (Welch and Feramisco, 1985; Chappell et al., 1986; Ungewickell, 1985). We conclude that the 70-kDa band that coimmunoprecipitates with SCb clathrin is indeed the UP.

Clathrin-uncoating protein complexes in cultured neurons

We have determined whether clathrin-UP complexes similar to those observed in SCb of rat retinal ganglion cell axons also exist in cultured sympathetic neurons. In addition to determining whether such complexes exist in other types of neurons, the culture system affords a much greater opportunity than the axonal transport system for detailed biochemical analyses of the nature of the clathrin-UP complex.

We first determined whether clathrin-UP complexes could be immunoprecipitated from extracts of cultured neurons. As shown in Figure 5, lane 1, clathrin heavy and light chains and a 70-kDa polypeptide were major components present in immunoprecipitates prepared in buffer C, along with many other less prominent bands in the M_r range of 70–150 K. Identical results were also obtained when cultures were extracted with RIPA buffer (data not shown). The profile of bands present in the immunoprecipitates from extracts of cultured neurons was strikingly similar to that obtained in immunoprecipitates from SCb (see Fig. 2A, lane 4 or Fig. 3, lane 6), suggesting that clathrin-containing complexes like those present in SCb of retinal ganglion cells are also present in cultured neurons. In this regard, various controls such as preadsorbing the antibody with purified clathrin trimers or using nonimmune serum blocked the appearance of labeled clathrin heavy and light chains, the 70-kDa band and most of the lighter bands between 70 and 150 kDa (data not shown). These observations confirm that these nonclathrin polypeptides are specifically coprecipitated with clathrin. The most prominent of these coprecipitating proteins has an apparent M_r of 70,000 and corresponds to the UP.

To evaluate properly the biological significance of the coprecipitation of clathrin-UP complexes, it is necessary to determine whether the complex existed *in situ* or formed only artifactually following lysis of the neurons. If the complex formed postlysis, then unlabeled UP included in the lysis buffer should compete with the labeled UP and thereby reduce the levels of labeled UP in the resulting immunoprecipitates. We tested this prediction by including 1.2 or 6.0 μ g of purified brain UP in the lysis buffer. A typical culture yields ≈ 200 μ g of total protein. Assuming that UP represents $\approx 0.1\%$ of total protein (Schlossman et al., 1984), then the levels of exogenous UP included in the lysis buffer are expected to exceed the endogenous UP by 6- or 30-fold. As shown in Figure 5, including unlabeled UP in the lysis buffer had no detectable effect on the levels of labeled UP present in immunoprecipitates (compare lanes 2 and 3 with lane 1). Quantitative studies confirm the impression based on visual inspection; the ratio of DPM in clathrin to DPM in UP was 8.9 in control precipitates and 8.7 in precipitates prepared with a 30-fold excess of exogenous unlabeled UP in the lysis buffer.

In a final series of experiments, we asked whether the clathrin-UP complex recovered by immunoprecipitation possessed a functional UP, as characterized by its ability to dissociate from clathrin in the presence of ATP (Flynn et al., 1989; Heuser and Steer, 1989). Immune complexes bound to protein A-Sepharose beads were prepared from soluble extracts of cultured neurons as described in Materials and Methods, and were then rinsed three times with buffer C. One aliquot of the clathrin-UP complexes bound to the beads was then incubated with ATP in the presence of an ATP-regenerating system for 30 min at 26°C, while the other sample was incubated in parallel but without ATP. The beads were then sedimented through a cushion of buffer C supplemented with 0.5 M sucrose and 2 M urea, and

Figure 4. Clathrin uncoating ATPase is immunoprecipitated by anti-LC β antiserum. Immunoprecipitates from optic axons containing labeled SC β proteins in RIPA buffer were analyzed by 2-D isoelectric focusing $\times$ SDS gel electrophoresis; purified UP was included in the sample as an internal marker. *Panel A* shows a Coomassie blue-stained profile of the portion of the gel containing the UP. The basic end of the gel is to the left. The *arrow* in panels A and B identifies the position of the major Coomassie blue-stained spot. *Panel B* shows a fluorograph of the same gel used to prepare panel A. Note that a labeled protein in the immunoprecipitates comigrates specifically with the most acidic variant of the UP preparation.

then further rinsed using the standard immunoprecipitation protocol. ATP treatment caused the selective release of the 70-kDa UP (Fig. 5, compare lanes 4 and 5), demonstrating that the UP is present in a functionally active form in the immunoprecipitates, and by extension, in the intact cell. We also note that none of the other coprecipitating proteins in the immunoprecipitates were detectably affected by ATP treatment. Thus, we can also conclude that their presence in the immunoprecipitates is not due to their binding to the UP.

Discussion

The present studies provide the first demonstration that clathrin and UP form stable complexes in intact cells. The essential finding is that a polyclonal antibody specific for clathrin LC β coprecipitates clathrin in complex with UP under nondenaturing conditions. The specificity of the antibody was established by immunoblotting against purified clathrin and whole brain extracts as well as by immunoprecipitation out of heterogeneous nerve extracts prepared under denaturing conditions (Figs. 1 and 2 and data not shown). All of these assays showed that the antibody recognized a single band on SDS gels that corresponded to clathrin LC β . The results from a variety of control immunoprecipitations indicate that the coprecipitation of clathrin and UP from labeled neuronal extracts reflects specific interactions (see Results). A specific and functional association is further supported by the ATP-dependent release of UP from the immunoprecipitated complexes (Fig. 5). These properties of the labeled rat neuronal proteins are entirely consistent with previous studies of clathrin-UP interactions using purified bo-

Figure 5. Immunoprecipitation of clathrin-UP complexes from extracts of cultured neurons. *Lanes 1–3*, ^{35}S -L-Met labeled cultures of sympathetic neurons were lysed in buffer C and immunoprecipitated with anti-clathrin antibodies (lane 1). Parallel cultures were lysed in the presence of 1.2 μg or 6.0 μg of UP/hsc70 (lanes 2 and 3, respectively) and processed similarly (see Results for details). *Lanes 4 and 5*, Immune complexes bound to protein A-Sepharose, prepared from radiolabeled cells processed as in lane 1, were incubated at room temperature for 30 min in the presence of an ATP-regeneration system without ATP (lane 4) or with ATP (lane 5) and then washed and processed as described in Results. *HC* and *LC* refer to heavy and light chains of clathrin, respectively, while *70 K* marks the UP/hsc70 band.

vine brain preparations (Braell et al., 1984; Schlossman et al., 1984; Schmid et al., 1985; Ungewickell, 1985; Chappell et al., 1986; Heuser and Steer, 1989).

Do clathrin-UP complexes normally exist in intact neurons? To answer this question, neurons labeled with ^{35}S -methionine were lysed in the presence or absence of up to a 30-fold excess of unlabeled UP and then assayed for clathrin-UP complexes by nondenaturing immunoprecipitation. These experiments showed unambiguously that inclusion of exogenous UP in the lysis buffer had no detectable effect on the levels of endogenous (i.e., metabolically labeled) UP in the immunoprecipitates. The absence of measurable competition is strong evidence that complexes of clathrin and UP existed in the intact neurons prior to lysis. The complex as isolated *in vitro* is also evidently quite stable under the prevailing solution conditions, as the UP remains bound to clathrin throughout the immunoprecipitation procedure, which lasts several hours. Furthermore, addition of exogenous UP during the immunoprecipitation reaction does not appreciably displace cellular (i.e., labeled) UP from clathrin.

Collectively, these observations provide the first demonstration of a relatively stable interaction between clathrin and UP in intact cells and lend strong support to the concept that UP plays an intimate role in clathrin dynamics in cells.

There is an apparent paradox in the premise that a clathrin–UP complex exists as a stable entity within the cell, yet the UP can be readily released from the clathrin immunoprecipitate by physiological (or even lower) levels of ATP. One possible explanation for this apparent discrepancy is that the comparatively rapid ATP-dependent dissociation, which takes place on a time scale of minutes, has been shown to occur at very low protein concentrations (Flynn et al., 1989; Heuser and Steer, 1989), conditions that apply in the immunoprecipitation experiment reported here. In contrast, there is evidence suggesting that at higher protein concentrations (which presumably prevail in the intact cell) ATP-dependent release can be dramatically slower (Greene and Eisenberg, 1990), with an apparent half-time of many hours. Of course, other cellular factors may well affect the ATP-associated release of clathrin, which appears to be a complicated step in the overall uncoating reaction (Schmid and Rothman, 1985a,b; Schmid et al., 1985; Rothman and Schmid, 1986; Greene and Eisenberg, 1990). While resolution of this riddle will require further knowledge of the clathrin–UP interaction and the cellular environment, the data reported here clearly indicate that the clathrin–UP complex exists in the intact cell.

In addition to the UP, numerous other metabolically labeled polypeptides with apparent subunit M_r in the range of 70–150 K also coimmunoprecipitated with clathrin, though with lower apparent stoichiometries. Although we have not studied these proteins as extensively as the UP, the available data suggest that their presence in the immunoprecipitates is also due to specific interactions. This interpretation is supported by the absence of these bands in control immunoprecipitates (see Results), by the similarity in profiles obtained in immunoprecipitates from cultured neurons and SCb from optic nerves, and by the fact that most of the coimmunoprecipitating proteins are minor components of their respective starting materials. In view of the ability of the UP–*hsc70* to bind many unrelated peptides and presumably diverse proteins as well (Flynn et al., 1989; Rothman, 1989; Beckmann et al., 1990), it was anticipated that the presence of the heterogeneous group of coimmunoprecipitating proteins reflected interactions with UP. However, this expectation is not supported by the data, as treatment of the immune complexes with ATP resulted in the selective and nearly quantitative removal of UP without any discernible effect on the presence of the other immunoprecipitated proteins. We thus conclude that the presence of the coimmunoprecipitating proteins is not due to interactions with the UP but rather to binding directly or indirectly to clathrin. For simplicity, we refer to the material immunoprecipitated by anti-clathrin antibodies under nondenaturing conditions as “clathrin complexes,” recognizing that the precise nature of these “complexes” remains to be defined.

It has been hypothesized that SCb corresponds to the transport of one or more multicomponent complexes (Garner and Lasek, 1981; Tytell et al., 1981). The evidence for this is indirect and is based in part on the observations that all proteins of SCb are transported together in the axon at a uniform rate and that the movement of the SCb proteins is absolutely distinct from the movement of the proteins transported in other rate components such as SCa and FC (for a complete discussion of this

issue, see Garner and Lasek, 1982; Lasek et al., 1984). The present studies provide the first direct evidence in support of this hypothesis by showing that relatively stable complexes consisting of clathrin, UP, plus a discrete subset of other SCb proteins exist and can be isolated by immunoprecipitation from axonal extracts. The associations revealed by these immunoprecipitation experiments may represent only a subset of those occurring among the SCb proteins *in situ*. For example, the association of some SCb proteins with clathrin or other of the coprecipitating proteins may not have survived the dilution and/or solution conditions associated with the immunoprecipitation protocol; this may explain why AP, which is in SCb (Chestnut et al., 1986), is not evident in our immunoprecipitates. Thus, the complexes identified by the present experiments may be part of one or more larger entities that comprise SCb. Nondenaturing immunoprecipitation experiments similar to those performed here, but using antibodies to other SCb proteins and different solution conditions, should be useful to define better the organizational form of the SCb proteins.

What are the forms of axonally transported clathrin and of the complexed clathrin immunoprecipitated from neurons in culture? In considering these issues, it is relevant that clathrin is transported in both FC and SCb. Because the materials transported by these two rate classes are fundamentally different in their biochemical properties (Grafstein and Forman, 1980; Lasek et al., 1984), it is likely that the clathrin of SCb and FC are distinct in their intracellular organization. It is well documented that most and possibly all integral membrane components are delivered to the axon by FC, and that these membrane components are transported in the form of vesicles (Grafstein and Forman, 1980; Brady, 1984). We have shown that a portion of the axonally transported clathrin enters the axon with FC (Fig. 3). Because membrane components of coated vesicles are also transported in FC (Stone et al., 1984), and coated membrane profiles have been observed ultrastructurally in axons (Baas and Heidemann, 1986; Morre and Walter, 1982), it is likely that the clathrin of FC is transported as coated vesicles.

In contrast, the available evidence indicates that SCb clathrin is not transported in the form of coated membranes (see Garner and Lasek, 1981). First, integral membrane components have not been detected in SCb (Grafstein and Forman, 1980). Second, as shown here, a substantial fraction of SCb clathrin exists in a discrete complex with the ATP-dependent uncoating protein. Under *in vitro* conditions, clathrin is stabilized in a noncoat form when complexed with the uncoating protein (Schlossman et al., 1984; Rothman and Schmid, 1986). Finally, immunoelectron microscopic localization of clathrin in neurons reveals that while some is membrane associated, a substantial amount of clathrin is associated with nonmembranous components of the cytoplasm (Cheng et al., 1980; Lin et al., 1982).

What are the functions of the nonmembranous clathrin complexes transported in SCb? In considering this question, we note that coprecipitation with clathrin of major 70-kDa as well as heterogeneous 70–150-kDa polypeptides under nondenaturing conditions is not unique to experiments with neurons or to analyses with our polyclonal antibody. Strikingly similar patterns to those reported here were obtained by Brodsky (1985) in immunoprecipitation analyses of human lymphocyte cell lines using two different monoclonal antibodies against clathrin heavy chain. At the time it was not possible either to identify these bands or to be certain that the associations had actually pre-existed in the intact cell, but the reasonable inference was made

that they might represent clathrin-binding proteins (Brodsky, 1985). Recently, similar or identical complexes have also been observed in nondenaturing immunoprecipitates of labeled HeLa cells using monoclonal anti-*hsp72.73* antibodies (Beckmann et al., 1990). These results then are consistent with the view that relatively stable complexes of clathrin, UP, and heterogeneous 70–150-kDa polypeptides exist in cells generally. If this is correct, then these complexes, rather than reflecting specializations unique to neurons, are likely to contribute to the structure and function of cells generally.

These clathrin-containing complexes might be expected to comprise the soluble pool of clathrin that may represent both storage forms and intermediates in the cycle of disassembly and reassembly of the clathrin coated pit at the plasma membrane (Goud et al., 1985). However, in preliminary extraction experiments using the conditions described by Goud et al. (1985) to release the soluble form of clathrin, we found that while the percentage of clathrin released from cultured neurons was comparable to that reported for brain (Goud et al., 1985), the clathrin complex(es) remained insoluble (I. Pleasure, M. Black, and J. Keen, unpublished observations). If these clathrin-containing complexes participate in the cycle of coated membrane assembly and disassembly, then they represent novel intermediates in this cycle. However, most of the nonclathrin proteins of this complex do not correspond to identified components of the clathrin coat structure. These considerations suggest that the clathrin complexes may not represent intermediates in the coated membrane cycle, but rather may possess functions not commonly ascribed to clathrin. To begin defining these functions, it will be necessary to identify these other proteins and to learn more about the nature of the interactions that give rise to the complexes.

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