Clathrin Is Axonally Transported as Part of Slow Component b: The Microfilament Complex

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ABSTRACT During axonal transport, membranes travel down axons at a rapid rate, whereas the cytoskeletal elements travel in either of two slow components, SCa (with tubulin and neurofilament protein) and SCb (with actin). Clathrin, the highly ordered, structural coat protein of coated vesicles, has recently been shown to be able to interact in vitro with cytoskeletal proteins in addition to membranes. The present study examines whether clathrin travels preferentially with the membrane elements or the cytoskeletal elements when it is axonally transported.

Guinea pig visual system was labeled with tritiated amino acids. Radioactive SDS-polyacrylamide gel electrophoresis profiles from the major components of transport were coelectrophoresed with clathrin. Only SCb had a band comigrating with clathrin. In addition, radioactive clathrin was purified from guinea pig brain containing only radioactive SCb polypeptides. Kinetic analysis of the putative clathrin band in SCb revealed that it travels entirely within the SCb wave. Thus we conclude that clathrin travels preferentially with the cytoskeletal proteins making up SCb, rather than with the membranes and membrane-associated proteins in the fast component.

Coated vesicles have been postulated as playing several roles in intracellular events such as intracellular membrane transport (7, 24), endocytosis (14, 19, 20), and secretion (24, 26, 35). In addition, there is strong evidence that these structures are involved in membrane recycling at the nerve terminal (22). The organelle consists of a membrane vesicle surrounded by a highly ordered protein lattice coat. Occasionally, the coat structures or "baskets" are seen without enclosed vesicles. Biochemical enrichment for these organelles was first attempted by Kanaseki and Kadota (25), who subsequently described the details of the structure. Pearse and co-workers (9, 37, 38) succeeded in highly purifying a 180-kdalton polypeptide (named clathrin) that was postulated to be the major protein constituent of the basket coats of coated vesicles. The physical properties of clathrin have recently been studied in vitro (27, 40, 41, 48). The findings reveal that (*a*) clathrin is capable of binding to α-actinin and actin in vitro, and (*b*) clathrin is capable of two ordered states; it can convert from the "baskets" to a filamentous array of protein molecules with a change in the solution conditions. Thus clathrin forms highly ordered structures and interacts with both membrane elements and cytoskeletal elements.

Because coated vesicles are found in great quantity in axon terminals (22), and the source of nearly all neuronal polypeptides is the neuron cell body (31), clathrin must be axonally transported to the terminal areas. The literature has revealed that the fast component of axonal transport (FC) is the primary source of membrane elements, glycoproteins, glycolipids, and transmitter-related proteins for the axons and terminal regions (6, 8, 10–13, 42).

The two slow components (SCa and SCb), on the other hand, appear to be the source of cytoskeletal elements for the axon and terminal regions; for example, neurofilament triplet proteins, tubulin, and actin are all characteristic of these components (1, 2, 23, 32). Because clathrin is a major structural protein that has been shown to be able to associate with both membranes and cytoskeletal proteins, it was of interest to determine whether clathrin would preferentially travel down

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the axon with the fast or slow components of transport or be present in both components.

In these studies, we have used the spatial and temporal aspects of axonal transport within the neuron to document and characterize the movement of a specific protein, clathrin, within a cell. The results of the studies reported here demonstrate that clathrin does not travel with the membrane elements when it is axonally transported. Instead, clathrin appears to accompany the cytoskeletal proteins of the subcomponent of slow transport we call SCb as they travel from the neuron cell body to the terminal regions.

MATERIALS AND METHODS

Injection and Radiolabeling Polypeptides within the Axon

The visual system of adult male Hartley strain (500–700 g) guinea pigs was used for all experiments. The technique for injection of amino acids into the posterior chamber of the eyes, and thus radiolabeling axonally transported retinal ganglion cell proteins, was a slight modification of that used by Black and Lasek (1). In all experiments, 10 μl of a concentrated (45–48 mCi/ml), one-to-one mixture of tritiated amino acids (4,5-[^{3}H](*N*)L-lysine, 40–80 Ci/mmol, and 2,3-[^{3}H](*N*)L-proline, 20–30 Ci/mmol; New England Nuclear, Boston, Mass.) was injected into both eyes of each animal. After its removal from the animal, the visual system was measured and the initial portion of the optic chiasm was used as a reference point. To obtain preparations that were enriched for radiolabeled polypeptides characteristic of one of the three major components of axonal transport, the postinjection harvest time was varied appropriately. Tytell and Lasek (44) have shown that at 5.0 h postintravitreal injection of tritiated protein precursors the predominant radioactive species in the optic nerve, chiasm, and tract are those characteristic of the fast component. Similarly, Black and Lasek (2) and Garner (15) have shown that the SCb polypeptides are the only radiolabeled polypeptides in the nerve, chiasm, and proximal optic tract at 6 d, and in the terminal and preterminal regions (lateral geniculate and superior colliculus) at 25 d. SCa polypeptides are the predominant radiolabeled species in the nerve and chiasm at 25 d postinjection (2).

SDS–Polyacrylamide Gel Electrophoresis

Samples to be electrophoresed were solubilized in BUST (1% SDS, 5% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 mM Tris, in 8 M urea, at pH 6.8) at 98°C for 1–2 min. Sample electrophoresis was performed in slab (7.5%) or slab gradient (4–17.5%) polyacrylamide gels in the buffer system described by Laemmli (28). An exception is the 5% polyacrylamide disk (11-mm) gel presented in Fig. 6, which was run in the buffer system of Neville (34). Gels were stained with 0.1% Coomassie Brilliant Blue R, and when radioactive samples were electrophoresed, gels were fluorographed according to the method of Bonner and Laskey (4) and Laskey and Mills (33).

Preparation of Clathrin

The procedure used to prepare clathrin from guinea pig brains was a modification of that of Pearse (37) and is graphically summarized in Fig. 1. Whole guinea pig brains were weighed, minced, and rinsed in buffer (0.1 M 2,(*N*-morpholino)ethanesulfonic acid (MES), 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% Na azide, 0.5 mM GTP at pH 6.5; kept at 4°C at all times). Sample was mixed 1:1 (wt/vol) with buffer and homogenized with a motor-driven, ground-glass homogenizer. The sample was then centrifuged as diagramed in Fig. 1. The fraction between 15–45% sucrose (identified on the basis of its cloudiness) was the gradient fraction (designated MID) enriched for clathrin. Aliquots of all fractions were run on SDS gels to monitor the enrichment of the clathrin band (which ran at 170 kdaltons in our gel system).

Electron Microscopy

Samples from the MID fraction were routinely monitored for the presence of coated vesicle "basket" structures by negative staining and electron microscopy. One volume of the fraction was combined with an equal volume of 1 M hexanediol in 0.01 M sodium phosphate, pH 6.2, after the method of Pearse (37). A drop of the mixture was placed on a carbon-coated Formvar (large slot, 3.0 mm) grid (Ladd Research Industries, Inc., Burlington, Vt.) and allowed to stand for 30 s. The drop was shaken off, and one drop of freshly made aqueous 1% uranyl acetate was put on the grid for 30 s. Electron micrographs were taken on the Siemens Elminskop II with magnifications of × 40–120,000.

Comigration Experiments

Experiments testing whether "cold" clathrin standard comigrates in SDS-polyacrylamide gels with radioactive bands in any of the major components of axonal transport were performed as follows. Whole guinea pig brain clathrin, prepared as described previously, was used as the standard. Segments of optic nerve, chiasm, or tract appropriately labeled with one of the three components (FC, SCb, or SCa) were solubilized for electrophoresis as described previously if they were to be run alone, or mixed with clathrin standard before solubilization if they were to be co-run. Approximately 3 μg of clathrin were added to each radioactive sample. After the samples were electrophoresed, the position of the stained clathrin band was marked with India ink before fluorography to facilitate subsequent localization of the band. The stained pattern was then compared to the radioactive profile.

Preparation of Clathrin from Experimental Animals

25-d after intravitreal injection of ^{3}H-labeled amino acids (when the only radioactive proteins present in the retinal ganglion cell terminals are those characteristic of SCb), four animals were sacrificed and their lateral geniculate bodies (LGNs) and superior colliculi (SC) were dissected out and pooled. Clathrin was prepared by the procedure outlined previously. Aliquots from all fractions were run on SDS-polyacrylamide gels, which were then fluorographed. To directly quantitate the radioactivity in the band at the clathrin position, the MID fraction was TCA-precipitated, washed with ethanol and ether, dried, and resolubilized in BUST at room temperature for several hours and at 98°C for 2 min. The sample was electrophoresed as described. The entire gel was sliced in 1-mm segments, which were dissolved in 0.5 ml of 30% H_2O_2 at 60°C overnight. New England Nuclear formula aqueous scintillation cocktail (5 ml) was added to each sample, and the samples were counted. Efficiency of counting was 30–31% by internal standardization.

Axonal Transport Kinetics of the 170-kdalton SCb Polypeptide

To determine the axonal transport kinetics of the 170-kdalton polypeptide at 4, 6, and 9 d postinjection, sequential 1-mm segments of optic nerve, chiasm, and tract were solubilized in 200 μl of BUST, electrophoresed on slab gradient gels, and fluorographed. Fluorographs were used to locate the radioactive 170-kdalton

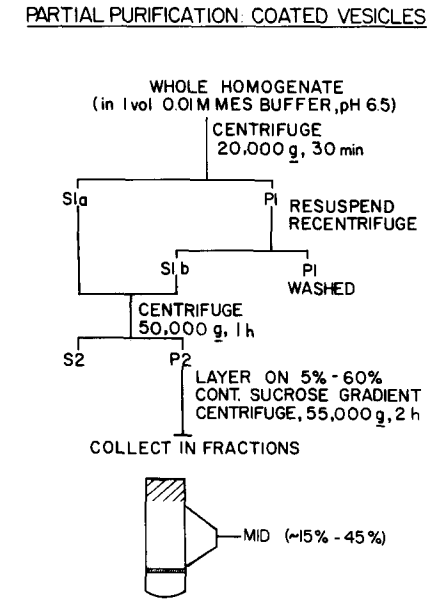

FIGURE 1 A flow diagram of the technique used to enrich for clathrin, the coated vesicle protein. The sample obtained in the *MID* fraction (15–45% sucrose) yielded one major band at 170 kdaltons after SDS-PAGE. *S*, supernate; *P*, pellet.

polypeptide in each gel profile. The appropriate band region in the gel was excised and incubated in 0.5 ml of 30% H_2O_2 at 60°C for 2 d to dissolve the gel. New England Nuclear formula 973 (5 ml) scintillation cocktail was added to each sample and the sample was counted. Counting efficiency ranged from 25–35%; counts were corrected for quenching and converted to disintegrations per minute.

Because the amount of radioactivity incorporated into the retinal ganglion cells in each eye varies considerably, the data for "total SCb radioactivity distribution" and "170-kdalton radioactivity distribution" were standardized. For "total SCb radioactivity," the radioactivity in each segment of visual system was determined, then the total radioactivity in the entire system was summed. Each segment's radioactivity was converted to a percentage of the total sum. The percentage radioactivity in a particular segment was then averaged with the percentage radioactivity in the corresponding segments (located a precise distance from the eye) in other experimental visual systems, and the standard error was computed. The distribution of the radioactivity in the 170-kdalton band was handled similarly: the total radioactivity in the 170-kdalton band throughout the visual system was determined by summing the amount of radioactivity in the band in each segment, then the individual segment radioactivity was expressed as a percentage of the total. Again the percentage for corresponding segments were averaged and the standard error computed. (The number of nerves and tracts analyzed was 8 at 4 d, 8 at 6 d, and 5 at 9 d). The chiasm region was treated differently, as each segment of chiasm received radioactive proteins from both eyes. Therefore, the proportion of radioactivity in a chiasm segment attributable to one eye or the other was estimated based on the proportion of total radioactivity in one nerve and tract relative to the total radioactivity in the opposite nerve and tract.

RESULTS

Preparation of Clathrin from Guinea Pig Brain

The following experiments were performed to establish that our method of sample preparation using whole guinea pig brain and LGN and SC preparations yielded clathrin according to accepted criteria in the literature. First, we obtained increased enrichment of a stained polypeptide found at 170 kdaltons throughout the steps of the clathrin preparation as seen by electrophoresis on SDS-polyacrylamide gels (Fig. 2). The other major bands in the most highly purified sample profile are at ~100 kdaltons and ~55 kdaltons. These polypeptides have been described previously by Blitz et al. (3), who suggested that they may have ATPase activity and a calcium-sequestering function, respectively. We noticed in this part of the study that the electrophoretic migration of the clathrin band changed with the amount of sample added. Therefore, the molecular weight for our enriched polypeptide was only an average molecular weight relative to molecular weight standards. This apparent reduction in molecular weight with increase in protein load was also noted by Woods et al. (47).

The second criterion for identity of clathrin is that the purified fraction (MID) must be highly enriched for "basket" structures, both with and without enclosed membranes. Fig. 3*A* and *B* are negatively stained electron micrographs of routine MID fractions from guinea pig brain. The most common structure seen was a highly ordered basket that was composed of hexagonal and pentagonal faces. Baskets with and without enclosed vesicles were observed. Both observations are typical of the basketlike structures reported by Pearse and co-workers (37) in the negatively stained preparations of their clathrin. This result confirmed that our method of sample preparation enriched for the putative coat protein of coated vesicles, clathrin.

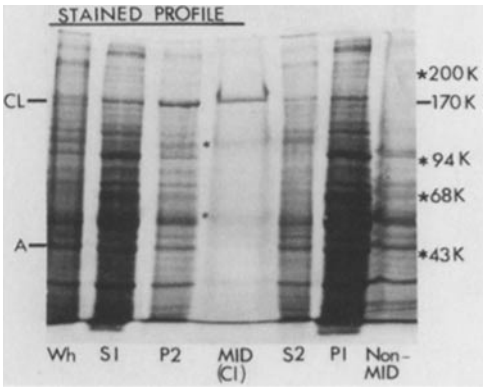

FIGURE 2 Coomassie Blue-stained SDS-PAGE (7.5%) of the major fractions obtained during the enrichment for clathrin. The first four fractions on the left are the subsequent purification steps, whereas the three fractions of the right are fractions that are discarded. The initials below the gel lanes correspond to the initials of the fractions as indicated in Fig. 1. Molecular weight marker positions are indicated on the right of the gel by asterisks. The asterisks next to the *MID* well indicate the positions of the major proteins (at 100 and 50 kdaltons) that copurify with clathrin.

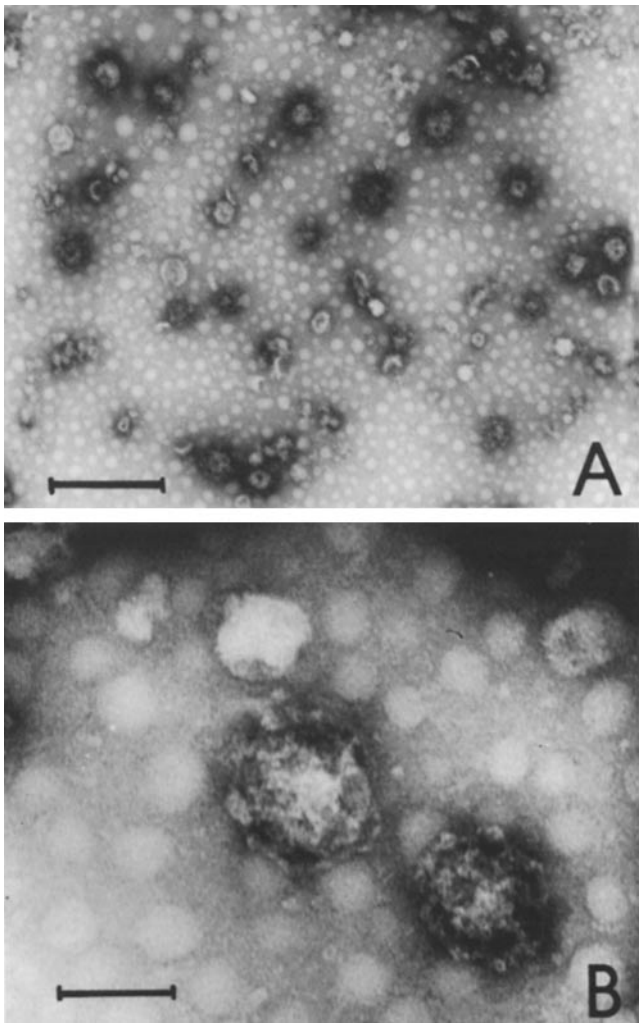

FIGURE 3 Negatively stained electron micrographs of samples taken from the MID fraction (most highly purified clathrin fraction). (*A*) Low magnification of the sample showing that the majority of the structures present look like the "baskets" described by Pearse (37). Bar, 5,000 Å. × 30,000. (*B*) Higher magnification showing the structure of two baskets in greater detail. Bar, 1,667 Å. × 90,000.

Comigration of Clathrin with SCa, SCb, and FC Radioactive Polypeptide Profiles

To determine which of the three major axonal transport components contains a polypeptide with a molecular weight approximating that of clathrin, clathrin was coelectrophoresed with radioactive samples from all three components in one dimension on SDS-polyacrylamide gels. When clathrin prepared from guinea pig brain is coelectrophoresed with either radioactive FC or SCa samples, no radioactive band of significance is seen to comigrate with the stained clathrin band. We obtained the same result when the fluorographs of SCa and FC were overexposed for maximum resolution. This is demonstrated in Fig. 4. When clathrin is electrophoresed adjacent to the radioactive SCb profile, however, the stained clathrin band comigrates with the SCb band at 170 kdaltons. When the two samples (clathrin and SCb) are mixed and electrophoresed, the sharpness of the radioactive band at 170 kdaltons decreases, and the whole, more diffuse, band (stained and radioactive) migrates at ~160 kdaltons. Experiments in which we added increasing amounts of "cold" clathrin to the SCb sample continued to increasingly distort both the stained and radioactive band at 170 kdaltons. Every other band in the profile, however, ran identically regardless of the total amount of added protein. Furthermore, fluorographs of two-dimensional gels (not shown) performed as previously described (36) have confirmed that the stained clathrin band and the radioactivity attributable to the 170-kdalton SCb and behave identically. Both electrophorese in the second dimension as a long streak in the acidic (pH 4.5–6.0) portion of the gel.

Preparation of Labeled, Axonally Transported Clathrin

To test the hypothesis that clathrin is transported in SCb, we purified clathrin from the SCs and LGNs of guinea pigs 25 d after intravitreal injection. The SC and LGN at 25 d were chosen because the only radioactive species in these regions at that time postinjection are the SCb polypeptides (1, 2). Fig. 5 is a fluorograph of a gel with samples from sequential steps in the preparation of clathrin from the injected guinea pigs. The enrichment of the radioactive band at 170 kdaltons can clearly be seen in the first four radioactive profiles. In addition, its loss from S-2 (second supernate) is noteworthy in that it indicates that clathrin is less soluble than many other SCb polypeptides. An experiment in which the MID, or most highly purified fraction, was acid precipitated and electrophoresed on a disk gel and the radioactivity in the gel slices quantified is presented in Fig. 6. It can be seen that there is only one peak of radioactivity in the gel (except for the unspecified radioactivity in the tracking dye region), and this peak was coincident with the stained clathrin band.

Control experiments were performed in which clathrin was purified from areas of the experimental animals' brains that were not directly projected to by the retinal ganglion cells (such as the cortical areas and cerebellum). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography of gels of this preparation revealed no purification of a radioactive band. (In fact, there was no discernible radioactive pattern seen at all, despite the fact the stained profile was normal.) Control experiments in which only one eye per guinea pig was injected were also performed. SDS-PAGE and fluorography of identical samples revealed no radioactive bands in the optic nerve on the control side, and normal SCb radioactive profiles on the injected side. The evidence presented above (*a*) that clathrin and the 170-kdalton SCb polypeptide behave identically during one- and two-dimensional gel electrophoresis, and (*b*) the identical enrichment of the 170-kdalton SCb polypeptide with the stained clathrin band when radioactive sample is subjected to the routine procedure for clathrin preparation, strongly suggests that the 170-kdalton SCb polypeptide is clathrin.

Axonal Transport Kinetics of the 170-dalton SCb Polypeptide

To establish coordinate transport of the 170-kdalton SCb polypeptide (which appears to be clathrin) with SCb, it is required for the transport kinetics of the individual protein to be compared with that of the whole component. This coordinate transport is demonstrated in Fig. 7. The distribution of

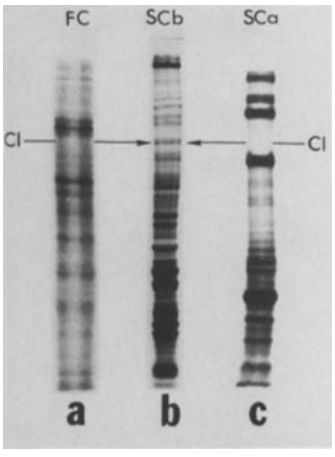

FIGURE 4 (*a*) Radioactive FC (5 h postinjection), (*b*) SCb (6 d), and (*c*) SCa (25 d) samples from guinea pig optic nerve and chiasm were coelectrophoresed with "cold" clathrin. The position of clathrin was marked on the gel with India ink, and the gels were fluorographed. The radioactive and stained profiles were compared. The position occupied by the stained clathrin band is indicated on the figure.

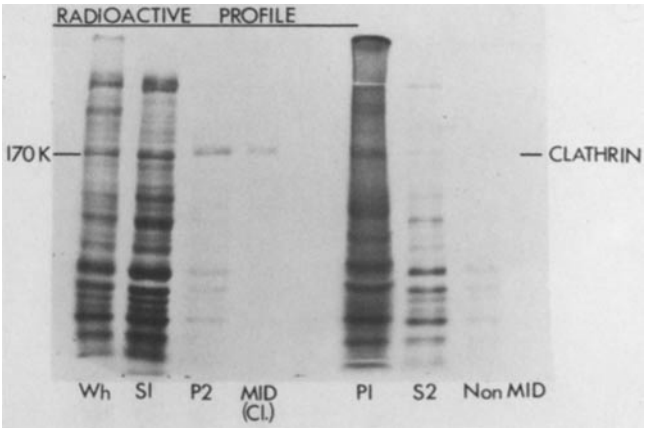

FIGURE 5 LGNs and SCs of animals that had been injected 25 d previously were used to prepare clathrin as in Fig. 1. Samples of each fraction in the preparation were run on a 4–17.5% gradient slab gel and fluorographed as described in Materials and Methods. The first four lanes are the radioactive profiles of the subsequent purification steps as in Fig. 2, whereas the three lanes to the right are the discarded fractions. The labels below the wells refer to the fraction indicated in Fig. 1.

radioactivity attributable to the SCb 170-kdalton band (filled circles) in the entire guinea pig optic nerve, chiasm, and tract is shown at three different times after intravitreal injection of protein precursors. The distribution of radioactivity attributable to the entire SCb wave (open circles) is also shown for comparison. At 4 d, the radioactivity in the 170-kdalton band is concentrated in the portion of the optic nerve nearest the eye. The radioactivity drops sharply with distance away from the eye. At 6 d, a well-defined peak of radioactivity at the optic chiasm can be discerned, with the levels of radioactivity sharply falling to background levels before and after the peak. By 9 d, the 170-kdalton polypeptide has started to enter the preterminal and terminal regions of the axons. Again, the radioactivity behind the peak that is attributable to the 170-kdalton polypeptide has dropped to low levels, implying that relatively little of the polypeptide has been left behind the moving peak. In this short system, it is difficult to determine the extent of the broadening of the peak with time and distance traveled. The distribution of the 170-kdalton radioactivity is coincident to total SCb radioactivity distribution in the region of the approaching "front." Thus, we may conclude that the 170-kdalton polypeptide is transported in SCb. However, the trailing edge of the 170-kdalton peak drops off more sharply than the majority of the SCb radioactivity (seen at 6 and 9 d). This SCb polypeptide is somewhat unique in this property, as the radioactivity distribution of other individual SCb polypeptides tends to more closely match the distribution of the entire SCb wave (15, 16).

To estimate the percentage of total SCb radioactivity present in the 170-kdalton polypeptide, the average radioactivity in the 170-kdalton band was divided by the total radioactivity loaded in the individual gel well for each animal. Only gel profiles in the peak regions at 6 d postinjection were used for this estimate. The average percentage (n = 10) is 2 ± 0.1% of the total SCb radioactivity.

DISCUSSION

These studies demonstrate that clathrin is axonally transported in the group of polypeptides designated SCb, and not in either of the other two major components, FC or SCa. First, it was found that purified clathrin comigrates with the 170-kdalton SCb polypeptide in one-dimensional SDS gels. In addition, only the 170-kdalton SCb band is preferentially enriched (along with the 170-kdalton stained clathrin band) when tissue containing radioactive SCb is subjected to the same rigorous fractionation procedure that has been shown to yield highly purified preparations of clathrin (37). Consistent with these observations, (although neither clathrin nor the 170-kdalton SCb band focuses well in our two-dimensional SDS gel system) both the stained clathrin band and the radioactive 170-kdalton SCb band migrate identically in the acidic portions of two dimensional gels. On the basis of these criteria, there is little doubt that the 170-kdalton SCb polypeptide and clathrin are the same protein. Our conclusion that clathrin does not travel in either of the other two major components is based on the observation that no radioactive band in SCa or FC comigrated with the stained clathrin band, even when fluorographed profiles were overexposed for maximum resolution. The possibility

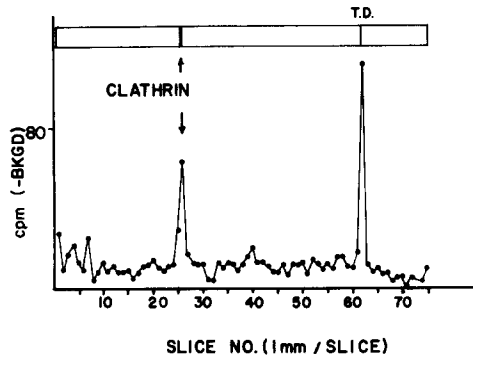

FIGURE 6 The most highly purified fraction (MID) from the radioactive preparation was acid-precipitated and run on a 5% disk gel. The gel was sliced in 1-mm segments and counted. A diagram of the stained profile of the gel is drawn above the graph; the position of the stained clathrin band is indicated. *T.D.*, tracking dye band.

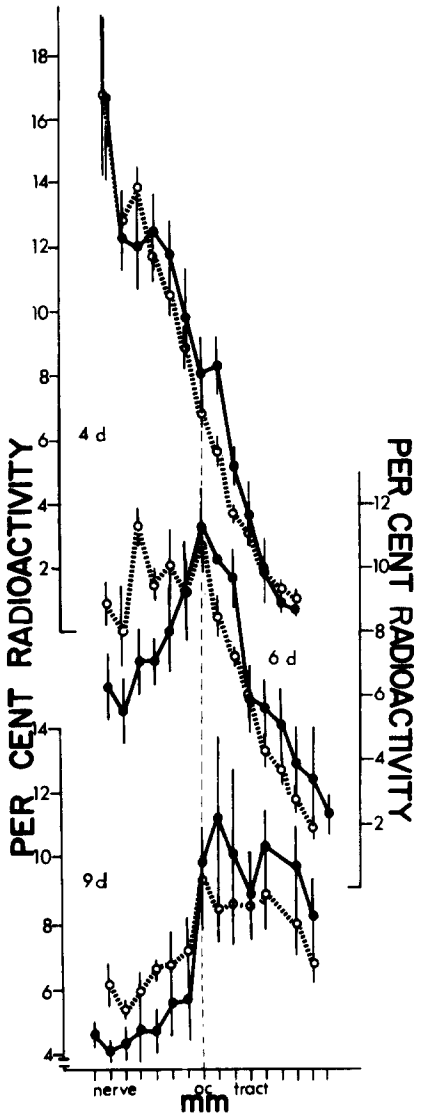

FIGURE 7 The distribution of radioactivity present in the 170-kdalton SCb polypeptide (●) is compared with the distribution of the total radioactivity in the SCb wave (○) at three times (4, 6, and 9 d) after intraocular injection of ^{3}H-labeled amino acids. Disintegrations per minute were standardized by conversion to percentage total dpms in either SCb or the 170-kdalton band (see text). The vertical bars represent the standard error. The number of nerves analyzed at 4 d is 8, at 6 d is 8, and at 9 d is 5. (Maximum total disintegrations per minute in SCb at the peak region at 6 d are 500,000, whereas maximum disintegrations per minute in the 170-kdalton polypeptide at the peak at 6 d are 6,000.) *oc*, optic chiasm.

that clathrin also travels at an intermediate (3–10 mm/d) rate is unlikely; no radioactive band can be detected at the appropriate molecular weight in fluorographed profiles of the intermediate components in this system (Garner, unpublished observations; Tytell et al., manuscript in preparation).

In the introduction we pointed out that the two well-characterized major components of axonal transport, FC and SCa, are unique, both in terms of macromolecular composition and of proposed function. SCb also appears to be unique in macromolecular composition, as indicated by the following pieces of evidence from retinal ganglion cell systems. First, it has been demonstrated that actin travels only in SCb (1, 2, 46). Second, a large actin-binding protein, M-2, whose specific binding is sensitive to ATP, travels exclusively in SCb's counterpart, Group IV, in the rabbit visual system (45, 46). In addition, a comparison of one- and two-dimensional SDS-polyacrylamide gels of the radioactive polypeptide profiles from the various components reveals little homology among the three major components (44; Tytell et al., manuscript in preparation). The present study further demonstrates that SCb has a unique composition, by showing that SCb is the only component in which clathrin is transported.

In the present study we have used the spatial geometry of the axon and the temporal characteristics of axonal transport to demonstrate that (*a*) clathrin can and does move long distances within a cell and (*b*) it travels in phase with the SCb complex of polypeptides. The idea of clathrin moving intracellularly is an integral part of most theories concerning its functions in membrane translocation, endocytosis, and secretion (19, 22, 24, 25). The present study is the first demonstration of its unidirectional movement over many normal cell diameters in distance. The most palpable result of a comparison of the axonal transport kinetics of clathrin with that of the total SCb wave (Fig. 7) reveals that clathrin does travel within the confines of the SCb wave of radioactivity. Clathrin's advancing front, in fact, coincides with that of the SCb wave. The rest of the clathrin curve does fall within the SCb wave. However, the clathrin curve is actually more bell-shaped than the SCb wave: the peak is sharper, and the trailing edge is less skewed. Bell-shaped radioactive distributions that do not diminish in size with distance down the axon have previously been interpreted as evidence that the molecule in question is traveling in a polymerized form, the subunits of which are inaccessible for exchange with "cold" subunits in the rest of the axoplasm (23, 32). Clathrin has been shown to exist in three possible physical forms, as a basket, a filamentous array (41), and as a polygonal network beneath the plasma membrane (21). The evidence presented here that clathrin may travel as a polymer within SCb cannot distinguish which physical form it may take, as any one of the forms mentioned above may be a clathrin "polymer." However, the relative paucity of the "coated vesicle" form within the axon (43), may be indirect evidence that clathrin would be more likely to travel in one of the other two forms.

Clathrin has been noted for its ability to enclose membrane vesicles (14, 25, 39) and to be associated with the plasma membrane (21). In addition, it has been strongly implicated in the mechanism of very rapid intracellular translocation of lipid, protein, and glycoprotein from rough endoplasmic reticulum to Golgi complex and from Golgi complex to plasma membrane (39). It is interesting that during its axonal transport, clathrin has a greater affinity for the SCb polypeptides than it does for the membranes and membrane-associated elements transported in FC. This finding is supported by a recent study using a blockade of FC by cold application (43). Contrary to what might be expected if clathrin-coated vesicles were involved in the translocation of FC membranes, no accumulation of coated vesicles was noted immediately proximal to the block despite a tremendous increase in membranous (vesiculotubular) structures. Although it is possible that clathrin is engaged in the translocation of rapidly transported membranes while in a filamentous nonbasket form (41), this also seems unlikely because a certain amount of clathrin would still be expected to travel somewhat faster than the SCb polypeptides because of its association with membranes. We suggest that it is more probable that the majority of the clathrin remains with the SCb polypeptide complex during transport. However, individual baskets, or local accumulations of clathrin, may be able to dissociate from the SCb complex to act transiently during local axonal membrane translocation, or endocytosis, or membrane recycling.

In vitro studies have previously demonstrated that clathrin binds to the following sequence of proteins increasingly well: tropomyosin, bovine serum albumin, α-actinin, G-actin, clathrin, F-actin, and F-actin + α-actinin (41). In addition, it appears that the presence of membrane is not necessary for clathrin to be able to form baskets. Basket formation in vitro is dependent on pH (40, 41, 48), salts (48), or protein factors (27), rather than on the presence or absence of membrane. It has also been shown that treatment of clathrin baskets with detergents known to disrupt intrinsic membrane proteins has no effect on basket morphology (48). Therfore, it is reasonable to suggest that clathrin is associated primarily with cytoskeletal elements as it is transported in axons, and perhaps, as it exists in the cytoplasm of other cells.

As the nature of the companions of clathrin during its axonal transport may have given us some insight into its physiological association, the nature of clathrin, a morphologically identifiable structural protein, has given us insight into the function of the SCb group of polypeptides. On the basis of previous experiments, in addition to those in the present study, we suggest that SCb has a cytoskeletal role: the SCb polypeptides represent the biochemical counterpart of actin microfilaments and their associated proteins, which we would like to call the microfilament complex. First, SCb contains actin and an ATP-dependent actin-binding protein, as do cellular microfilaments, and is the only source of these proteins to the axon and terminal. Second, microfilaments are structural: they are composed of many proteins that move and operate as a macromolecular complex. Similarly, the diverse array of SCb polypeptides moves as a unit through axoplasm containing many other molecules. They compose a distinct wave in which each individual polypeptide moves in phase with the other SCb polypeptides (15). Our interpretation of this phenomenon is that the many SCb polypeptides form a macromolecular complex, each polypeptide having a greater affinity for the members of the complex than for other elements (i.e., neurofilaments, microtubules, or membranes) in the axoplasm. In addition, the finding that during cell spreading and movement the microfilament bundles can operate independently from the closely linked microtubule/10-nm filament systems (17, 18) is consistent with our evidence that the two systems travel at independent rates in the axon. Also consistent with the hypothesis that SCb represents the microfilament complex is the observation (15) that many of the other characteristic SCb polypeptides have molecular weights approximating those of

proteins known to be present in microfilaments (notably filamin, myosin, α-actinin, and tropomyosin). However, this evidence, based on only one-dimensional gel analysis, must necessarily be considered tenuous. The actual judgement about the identity of these proteins must await more rigorous analysis. The observation in that clathrin travels with the SCb polypeptides adds another dimension to the hypothesis that SCb represents the microfilament complex. It provides the first evidence that SCb not only encompasses known microfilament proteins themselves but includes proteins that can form associations with microfilament proteins as part of a larger complex (29, 30). Recent studies from our laboratory indicating that glycolytic enzymes and calmodulin are also in the SCb group of proteins provide additional insight into the nature of the microfilament complex (5).

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