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Cytoplasmic dynein is associated with slow axonal transport

(microtubules/motor proteins)

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ABSTRACT **Neuronal function is dependent on the transport of materials from the cell body to the synapse via anterograde axonal transport. Anterograde axonal transport consists of several components that differ in both rate and protein composition. In fast transport, membranous organelles are moved along microtubules by the motor protein kinesin. The cytoskeleton and the cytomatrix proteins move in the two components of slow transport. While the mechanisms underlying slow transport are unknown, it has been hypothesized that the movement of microtubules in slow transport is generated by sliding. To determine whether dynein, a motor protein that causes microtubule sliding in flagella, may play a role in slow axonal transport, we identified the transport rate components with which cytoplasmic dynein is associated in rat optic nerve. Nearly 80% of the anterogradely moving dynein was associated with slow transport, whereas only ≈15% of the dynein was associated with the membranous organelles of anterograde fast axonal transport. A segmental analysis of the transport of dynein through contiguous regions of the optic nerve and tract showed that dynein is associated with the microfilaments and other proteins of slow component b. Dynein from this transport component has the capacity to bind microtubules *in vitro*. These results are consistent with the hypothesis that cytoplasmic dynein generates the movement of microtubules in slow axonal transport. A model is presented to illustrate how dynein attached to the slow component b complex of proteins is appropriately positioned to generate force of the correct polarity to slide microtubules down the axon.**

Neurons move proteins and other materials from their site of synthesis in the cell body down axons to synapses and growth cones using several mechanisms that together are termed axonal transport (1, 2). One component, fast axonal transport, is the movement of all membranous organelles and membrane proteins along microtubules (MTs) (2). The accepted paradigm for the mechanism of fast axonal transport is that the motor protein kinesin moves membranous organelles in the anterograde direction, toward the plus ends of MTs, whereas cytoplasmic dynein moves membranous organelles in the retrograde direction, toward the minus ends of MTs (3–5). There are two components of slow axonal transport. In slow component a (SCa) MTs and neurofilaments move at 0.2–1 mm/day. The slow component b complex (SCb) consists of microfilaments and the remaining proteins of the axon, including metabolic enzymes, which are collectively referred to as the cytomatrix (2, 6, 7). SCb proteins move at 2–8 mm/day. Compared to fast transport, very little is known about the mechanisms of slow transport, although it has been hypothesized that motor proteins slide the different cytoskeletal polymers toward the synapse (3, 8, 9). It is well established that dynein generates sliding between the MTs of ciliary and flagellar axonemes (10–12). The polarity of dynein force generation (10, 11) and the orientation of MTs in the axon (13) are consistent with the hypothesis that dynein generates SCa movement by sliding the MTs toward the synapse (4). We therefore initiated a study to determine whether cytoplasmic dynein is associated with slow axonal transport.

MATERIALS AND METHODS

Radiolabeling and Isolation of Axonally Transported Proteins. Axonal proteins were radiolabeled as described (14–16); one mCi (MBq) of Tran^{35}S-label (ICN) was injected into the vitreous of the left eye of adult Sprague–Dawley rats. Four rats were used for each time point. To analyze proteins moved in fast anterograde axonal transport, the optic nerves were isolated 4 hr and 24 hr after injection, respectively. To examine the proteins of SCb as well as the leading edge of the wave of MTs and neurofilaments in SCa, the optic nerves were isolated 4 days and 21 days after injection, respectively. For the segmental analysis of SCb transport, rat optic nerves and tracts were isolated 2, 4, and 6 days after injection. The optic nerves and tracts were divided into three ≈5-mm segments. Segment 1 corresponds to the proximal half of the optic nerve, segment 2 corresponds to the distal half of the optic nerve, and segment 3 corresponds to the optic chiasm and the proximal portion of the optic tract [see Fig. 1 in Elluru *et al.* (ref. 16)].

Immunoprecipitation and Electrophoretic Procedures. The isolated optic nerves or segments were pooled and homogenized in Triton X-100 lysis buffer; cytoplasmic dynein was immunoprecipitated using monoclonal antibody 74.1 as described (14). Kinesin was immunoprecipitated by using monoclonal antibody H2 (17). Our immunoprecipitation procedure was found to be >99% efficient, based on quantitation of sequential immunoprecipitations. SDS/PAGE (8% acrylamide or 4% acrylamide/8 M urea) and visualization of radiolabeled proteins using a PhosphorImager (Molecular Dynamics) were done as described (14).

Binding of SCb Dynein to MTs. Five rats were injected intravitreally with 1 mCi (MBq)/eye (both eyes) of Tran^{35}S-label. Radiolabeled rat optic nerves were isolated 4 days after injection. The nerves were pooled and homogenized; and a high speed supernatant was prepared as described by others (18, 19), except that apyrase and inhibitors of proteases, kinases, and phosphatases (14) were included in the homogenization buffer. Purified tubulin (20) was polymerized with taxol (Calbiochem) to form MTs that were added to the high-speed supernatant. After incubation, MTs were pelleted through a 10% sucrose cushion, yielding a MT-depleted supernatant and a MT pellet.

Quantitative Analyses. Quantitation of radioactivity associated with individual polypeptides was done by using autoradiography with storage phosphor screens and ImageQuant image analysis software (Molecular Dynamics), as described (14). The amount of radiolabeled protein associated with each

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Abbreviations: MT, microtubule; SCb, slow component b; SCa, slow component a.

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time point of the SCb study was expressed as a percentage of the total radiolabeled protein from all time points of the SCb study. Quantitation of total radiolabeled protein was performed by trichloroacetic acid precipitation of radiolabeled rat optic nerve homogenates and scintillation counting (21).

RESULTS

Rat optic nerve was used to examine the anterograde axonal transport of cytoplasmic dynein (for method, see ref. 15). Brain cytoplasmic dynein is composed of two ≈530-kDa heavy chains, two or three ≈74-kDa intermediate chains, and four 50- to 60-kDa polypeptides (22). Fig. 1*A* shows an autoradiograph of radiolabeled 530-kDa heavy chain of the dynein complex immunoprecipitated from optic nerves at four time intervals after injection of the vitreous of the eye with Tran^{35}S-label (14, 16). The greatest amount of dynein appears to be associated with the 4-day time point (SCb), whereas a lesser amount of radiolabeled dynein is found in the fast time intervals, 4 hr and 24 hr. Quantitation of the dynein polypeptides associated with the various rate components (Fig. 1*B*) revealed that ≈80% of the anterogradely transported dynein is associated with SCb. Approximately 15% of the dynein is associated with membranous organelles. The remainder (≤5%) is most likely the trailing edge of the wave of SCb transport, although it may also be associated with SCa. Thus, the bulk of cytoplasmic dynein appears to be traveling down the axon in SCb. To control for the synthesis of radiolabeled cytoplasmic dynein in regions other than the retina, the unlabeled contralateral optic nerve was also removed, and cytoplasmic dynein was immunoprecipitated and quantified. At each time interval, the contralateral nerve had <5% of the radiolabeled cytoplasmic dynein found in the ipsilateral (injected) nerve. As a control for the selected postinjection time points,

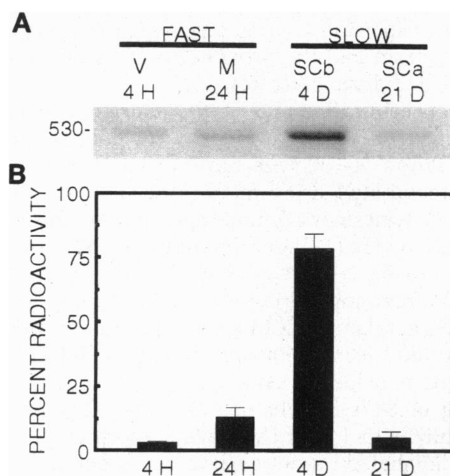

FIG. 1. Association of the dynein heavy chain (530 kDa) with various rate components of axonal transport. (*A*) Autoradiograph of radiolabeled cytoplasmic dynein immunoprecipitated from the optic nerve of rat retinal ganglion cells at various times after injection of Tran^{35}S-label into vitreous as described. The two lanes marked FAST are the two time intervals used to analyze the fast axonal transport of dynein associated with membranes; a 4-hr (4H) interval for the small tubulovesicular organelles (V) and a 24-hr (24H) interval for the mitochondrial proteins (M). The two lanes marked SLOW are the time intervals used to analyze the slow components: 4 days (4D) for the proteins associated with microfilaments and the cytomatrix of SCb and 21 days (21D) for the proteins associated with the leading edge of the MTs and neurofilaments of SCa. (*B*) Quantitation of radiolabeled cytoplasmic dynein heavy chain present in each rate component of anterograde axonal transport was as described (averaged results with SDs, $n = 3$). To ensure constant specific activity, the same lot of Tran^{35}S-label was used for each set of experiments. The quantitation of the radioactivity associated with the other dynein polypeptides yielded results similar to that of the dynein heavy chain.

kinesin transport was also quantified. In agreement with Elluru *et al.* (16), kinesin, a motor for anterograde fast axonal transport, was found primarily in fast component (data not shown).

The transport of cytoplasmic dynein in SCb was confirmed by following the time course of the movement of the dynein polypeptides through segments of the optic nerve and tract, a technique known as segmental analysis (7, 23) (Fig. 2). The amount of radiolabeled dynein in three ≈5-mm contiguous segments of the optic nerve and optic tract was analyzed over several days. The results obtained were those expected for a protein moving through the optic nerve and tract in SCb. Two days after injection of Tran^{35}S-label, most of the dynein is seen in segment 1, the segment most proximal to the retina. At longer time periods after injection, the more distal segments show increased amounts of radiolabeled dynein. At 4 days after injection more dynein is seen in segment 2, and at 6 days after injection the amount of dynein seen in segment 3 has increased (Fig. 2*A*). The same pattern of movement is observed for each of the dynein polypeptides, indicating that all subunits necessary for the enzymatic activity of dynein are transported coherently. When quantified, the movement of dynein (Fig. 2*B*) through these segments closely parallels the bulk movement of proteins associated with SCb (Fig. 2*C*). These results confirm that cytoplasmic dynein is transported with the microfilaments and proteins of the SCb complex.

To further characterize SCb dynein, we determined whether or not it could bind to MTs (Fig. 3). Four days after injection, the optic nerves were homogenized. The membranes were pelleted, yielding a high-speed supernatant containing radiolabeled dynein and other SCb polypeptides. The 530-kDa heavy chain of cytoplasmic dynein is the most heavily labeled high-molecular-weight SCb polypeptide extracted from the optic nerve. Unlabeled taxol-stabilized MTs were incubated with the high-speed supernatant and then pelleted by centrifugation. Cytoplasmic dynein was depleted from the supernatant and enriched in the MT pellet. This result indicates that dynein from the SCb pool can bind MTs *in vitro*. Because microtubule-associated protein 2 is a dendritic protein not found in the optic nerve axons, no radiolabeled microtubule-associated protein 2 is observed in this experiment (3). No radiolabeled kinesin heavy chain (≈120 kDa) is seen in the MT pellet because kinesin is a component of fast axonal transport (16).

DISCUSSION

There is broad evidence that cytoplasmic dynein is the motor for retrograde fast axonal transport of membranous organelles from the synapse to the cell body (3–5). Because all axonal protein synthesis occurs in the cell body, dynein must be transported to the synapse, presumably in an inactive form. This study of the anterograde axonal transport of cytoplasmic dynein in the rat optic nerve demonstrates that most dynein is transported in the slow component. The segmental analysis of the movement of dynein through the optic nerve and tract indicates that the dynein is transported with the microfilaments and other proteins of SCb. The movement of dynein in SCb may represent bulk transport of inactive dynein to the synapse for eventual use as the motor for retrograde transport. However, while inactive dynein transported with the microfilaments and other proteins of SCb would not be expected to bind MTs, we found that dynein from SCb binds to MTs in an ATP-dependent manner *in vitro*. This result suggests that SCb dynein is functionally active, a result consistent with the hypothesis that SCb dynein is the motor for the slow axonal transport of MTs. Although the possibility that the dynein is activated when it is extracted from the SCb complex cannot be ruled out, this appears to be unlikely. To date, only one putative cytoplasmic dynein regulatory protein has been identified, the dynactin complex, and it is reported to activate the motility of membrane-bound dynein (24). Also, inhibitors of

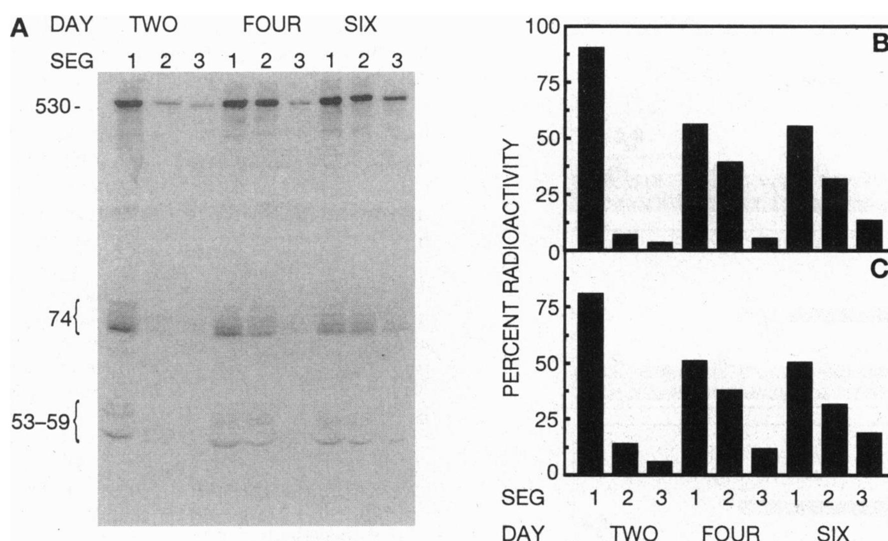

FIG. 2. Association of cytoplasmic dynein with SCb of axonal transport. A segmental analysis was done to confirm the association of cytoplasmic dynein with SCb. Axonally transported proteins were radiolabeled with Tran^{35}S-label via intravitreal injection. (*A*) Autoradiograph of radiolabeled cytoplasmic dynein immunoprecipitated from segments of the optic nerve and tract at the indicated times after injection. The number of days after injection that the optic nerve and tract were isolated (TWO, FOUR, and SIX) is indicated above each set of three lanes. Arabic numerals (1, 2, and 3) indicate the isolated segment of the optic nerve or optic tract analyzed in that lane. The dynein polypeptides, the 530-kDa heavy chain, 74-kDa, and 53- to 59-kDa polypeptides, are identified at left. Increased amounts of each of the cytoplasmic dynein polypeptides are seen in the more distal segments at later times. (*B*) Quantitation of radiolabeled cytoplasmic dynein heavy chain shown in *A*. (*C*) Quantitation of trichloroacetic acid-precipitable radiolabeled proteins in the optic nerve and tract at the indicated times after injection.

phosphatases and kinases were included in the extraction buffer to prevent changes in the phosphorylation state of the dynein that might alter its functional properties (14). It is interesting to note that when brain is homogenized, many proteins in the SCb transport complex are apparently extracted, including metabolic enzymes (2, 25, 26) and dynein (Fig. 3). The association of dynein with the SCb complex thus provides an explanation for the large soluble pool of active dynein in brain.

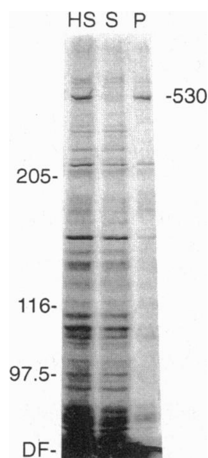

FIG. 3. Cytoplasmic dynein associated with SCb binds to MTs. A supernatant containing radiolabeled SCb dynein extracted from the axon was obtained as described using optic nerves isolated 4 days after injection. Taxol-stabilized MTs were added and, after incubation, the MTs were pelleted through a sucrose cushion to yield a MT protein-depleted supernatant and a MT pellet. The radiolabeled high-molecular-weight polypeptides of the optic nerve axons in these fractions were analyzed by SDS/PAGE using a 4% acrylamide/8 M urea separating gel and autoradiography. Gel lanes are as follows: HS, the high speed supernatant; S, depleted supernatant; P, the MT pellet. The identity of the 530-kDa dynein heavy chain (530) was confirmed by the immunoprecipitation of the dynein after its release from the MTs with ATP. The molecular mass standards are as follows: 205 kDa, rabbit skeletal muscle myosin; 116 kDa, β-galactosidase; 97.5, phosphorylase B. DF, dye front. Tubulin has run off this gel.

Members of the dynein family are known to associate with a variety of cargo: flagellar dyneins with MTs, and cytoplasmic dynein with membranous organelles, centrosomes, and kinetochores (22). Our data suggest that cytoplasmic dynein also binds either to microfilaments or another protein of the SCb complex in the axon. As there is no evidence that dynein binds directly to actin, it is tempting to speculate that the dynactin protein complex mediates the binding of dynein to the microfilaments of the SCb complex. Dynactin copurifies with dynein on sucrose density gradients. The major constituents of this large protein complex are p150^{glued}, p50, actin, and actin-related protein (24). The p150 component of dynactin binds dynein *in vitro* (27, 28), and it has recently been suggested that the actin and actin-related protein subunits of dynactin may interact with microfilaments (24, 29, 30).

Axonal transport has been extensively studied since it was first identified by Weiss and Hiscoe in 1948 (31). Although recent breakthroughs have advanced our understanding of the mechanism of fast axonal transport, less is known about the mechanism(s) of slow transport (3, 4). From their axonal transport studies, Lasek and associates proposed that the MTs and the other cytoskeletal complexes in the slow components are moved as polymer by sliding (2, 3, 8). Additional evidence for the movement of MTs in the axon comes from two recent studies. Using newly cultured rat sympathetic ganglia neurons treated with low concentrations of vinblastine, Baas and Ahmad (32) demonstrated the transport of MTs from the cell body into the axon. Reinsch *et al.* (33) used *Xenopus* neural tube cultures from embryos injected with photoactivatable tubulin to demonstrate that MT polymer does indeed move along axons. As discussed in a recent review, MT movement down the axon does not exclude the possibility that there is exchange of subunits during transport (3).

The models for MT movement in SCa are derived from the precedent of dynein-generated MT sliding in flagella (3, 8, 9). As was observed in the flagellar axoneme, the models proposed that the motor protein for SCa movement is bound to the MT. Surprisingly, our results indicate that very little, if any, cytoplasmic dynein is associated with the MTs moving in SCa. Instead, most of the cytoplasmic dynein is associated with SCb. Nevertheless, as illustrated in a model (Fig. 4), dynein attached to the

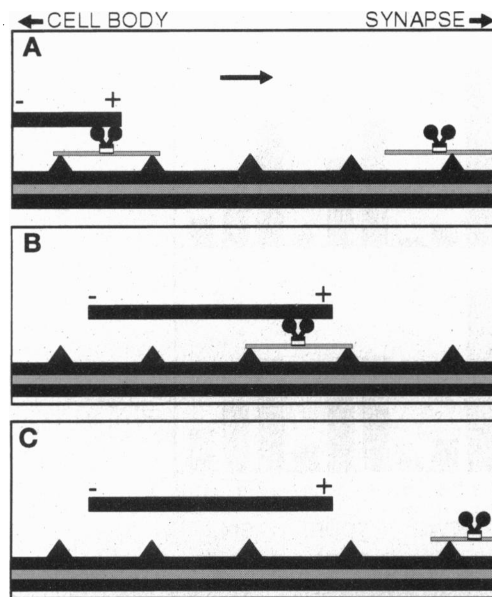

FIG. 4. Model illustrating the anterograde movement of MTs generated by cytoplasmic dynein associated with SCb. Thin gray line, microfilament (and associated proteins of the cytomatrix); triangle, motor for SCb movement (myosin); gray area bounded by black lines, plasma membrane; thick bar, MT with + end on right; [symbol], dynein; [symbol], protein linking dynein to microfilament. (*A*) At left dynein is bound to a moving microfilament through a linking protein attached to its cargo-binding end. The dynein is also bound to an MT through its globular motor domain. The polarity (+/− ends) of the MT is shown. The large arrow points in the direction of anterograde movement. At right, a microfilament and dynein are leaving the field of view. (*B*) The polarity of dynein force generation and the moving microfilament translocate the MT toward the synapse. (*C*) Dynein releases from the MT, and the MT stops moving forward. The microfilament and dynein continue to move toward the right edge of the field.

microfilaments or another protein of the SCb complex is appropriately positioned to generate force of the correct polarity to slide the plus ends of axonal MTs toward the synapse. This is the orientation observed for almost all MTs in the axon.

In Fig. 4*A*, the cargo-binding domain of dynein is attached through a linker protein to a moving microfilament. The microfilaments, dynein and other proteins of the SCb complex, move toward the synapse at 2–8 mm per day. The mechanism of movement of SCb is unknown. Perhaps, as drawn, it is moved by a myosin attached to the plasma membrane that slides the microfilaments (5). The dynein also binds a MT through its globular motor domain. The plus end of the axonal MT is oriented toward the synapse. The polarity of dynein force generation and the moving microfilament translocate the MT toward the synapse, as is seen in Fig. 4*B*. When dynein releases the MT, the MT stops moving toward the synapse. The microfilament and dynein continue to move along the axon (Fig. 4*C*). The net speed of movement of a MT would depend on how frequently or how long it interacted with dynein over time. This could be modulated by the arrangement of the cytoskeletal complexes in the axon or by specific regulatory protein(s). Net slow movement would be produced by infrequent interactions of the MT with dynein. Frequent interactions would generate faster net MT movement. The microfilaments and other proteins of the SCb complex would continue moving during the cycles of dynein binding to and releasing from MTs. While our results are consistent with the hypothesis that SCb dynein has a role in the slow transport of MTs, testing this model will require additional experiments, including the purification and characterization of SCb dynein.

This report demonstrates that ≈80% of the anterogradely moving dynein is associated with SCb and that this dynein is capable of binding to MTs *in vitro*. Active SCb dynein capable of generating force would move MTs with their plus end leading to the synapse, exactly as seen in the axon. The association of dynein with the microfilaments and cytomatrix (SCb) suggest that, in addition to its role in the retrograde movement of membranous organelles, dynein may be the motor for the anterograde axonal transport of MTs in SCa.

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