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Tubulin and Neurofilament Proteins Are Transported Differently in Axons of Chicken Motoneurons

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Summary

1. We previously showed that actin is transported in an unassembled form with its associated proteins actin depolymerizing factor, cofilin, and profilin. Here we examine the specific activities of radioactively labeled tubulin and neurofilament proteins in subcellular fractions of the chicken sciatic nerve following injection of L-[^{35}S]methionine into the lumbar spinal cord.

2. At intervals of 12 and 20 days after injection, nerves were cut into 1-cm segments and separated into Triton X-100-soluble and particulate fractions. Analysis of the fractions by high-resolution two-dimensional gel electrophoresis, immunoblotting, fluorography, and computer densitometry showed that tubulin was transported as a unimodal wave at a slower average rate (2–2.5 mm/day) than actin (4–5 mm/day). Moreover, the specific activity of soluble tubulin was five times that of its particulate form, indicating that tubulin is transported in a dimeric or small oligomeric form and is assembled into stationary microtubules.

3. Neurofilament triplet proteins were detected only in the particulate fractions and transported at a slower average rate (1 mm/day) than either actin or tubulin.

4. Our results indicate that the tubulin was transported in an unpolymerized form and that the neurofilament proteins were transported in an insoluble, presumably polymerized form.

Keywords: tubulin; neurofilament; actin; cytoskeletal protein; slow axonal transport.

INTRODUCTION

Cytoskeletal proteins have long been known to comprise the majority of proteins carried by slow axonal transport in neurons (Lasek *et al.*, 1984). Nevertheless, there is still controversy as to whether these proteins are transported as subunits or polymers (Bray, 1997). Although pulse-label studies have established that tubulin is carried by slow axonal transport in SCb as well as in SCa and that neurofilament proteins are transported in SCa (Lasek *et al.*, 1984; Filliatreau *et al.*,

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1988; Tashiro and Komiya, 1989), it is unclear in what form they are transported down the axon or where they are assembled into microtubules or neurofilaments (Baas and Brown, 1997; Bray, 1997; Hirokawa *et al.*, 1997).

On the basis of the pattern of movement of pulse-labeled cytoskeletal proteins, it has been suggested that SCa corresponds to the microtubule–neurofilament network and SCb represents a network of proteins complexed with microfilaments (Black and Lasek, 1980; Lasek *et al.*, 1984). Because the faster moving SCb polymers must pass the slowermoving SCa polymers it has been proposed that "polymer sliding" is the mechanism of slow transport in axons (Lasek, 1986). Although this "polymer sliding" model is attractive and explains several features of slow axonal transport, it has been challenged by evidence from *in vitro* and *in vivo* experiments (Bamburg *et al.*, 1986; Okabe and Hirokawa, 1990; Sabry *et al.*, 1995; Takeda *et al.*, 1995; Funakoshi *et al.*, 1996; Miller and Joshi, 1996; for reviews see Hirokawa *et al.*, 1997; Nixon, 1998). The alternative model is that these proteins are transported as subunits (Bray, 1997; Hirokawa *et al.*, 1997).

We previously showed that actin is transported in an unassembled form with its associated proteins actin depolymerizing factor, cofilin, and profilin (Mills *et al.*, 1996a). Here we examine the transport of tubulin and neurofilament proteins in the chicken sciatic nerve following injection of L-[^{35}S] methionine into the lumbar spinal cord. Our evidence suggests that tubulin, like actin, is transported along axons in a subunit form at a slightly slower average rate than actin and that the neurofilament proteins are transported in an insoluble, presumably polymerized form at a slower average rate than either actin or tubulin. A preliminary report of this work was presented at the 1996 Annual Meeting of The Physiological Society of New Zealand (Mills *et al.*, 1996b).

METHODS

Labeling of Chicken Motor Neurons

Adult crossbred hens weighing 1.3–3 kg were anaesthetized with Equithesin (2.5 ml/kg i.m., supplemented as required; Gandal, 1969) and the lumbar spinal cord exposed by laminectomy (Bray *et al.*, 1992). The experimental protocols were approved by the Otago University Institutional Animal Ethics Committee under the guidelines of the National Animal Ethics Advisory Committee of New Zealand. A total of 500 μCi of L-[^{35}S]methionine (>1000 Ci/mmol stabilized with 0.1% 2mercaptoethanol; Amersham International, UK) was injected into the ventral horn as described previously (Yuan *et al.*, 1999).

To study fast transport, the sciatic nerve was ligated with a thread 7 cm from the spinal cord immediately after the injection, and the animal was killed by overdosing with Equithesin 9 hr later. To examine slow transport, the animals were killed at 12 and 20 days after injection of radioactivity. The right sciatic nerve, about 10 cm in length, was removed and cut into 1-cm segments. The segments were eighed, frozen in liquid nitrogen, and stored at –76°C. A segment of the right medianoulnar nerve of equivalent weight was also removed to estimate the amount of blood-borne labeled methionine incorporated into nerves. The radioactivity in the right medianoulnar nerve segments could not be detected by fluorography. Chickens killed at 12 and 20 days were used to determine rates of protein transport as peaks of radioactivity were clearly evident along the nerve at these times.

Sample Preparation for Analysis of Tubulin and Neurofilament Proteins

Nerve segments were homogenized with a tapered glass pestle and tube in 0.6 ml of cold cytoskeletal-stabilizing buffer (0.5% Triton X-100, 0.5 m*M* dithiothreitol, 5m*M* EGTA, 0.5 m*M* phenylmethylsulfonyl fluoride, 1 m*M* benzamidine, 5 m*M* $MgCl_2$,20% glycerol, 5% dimethyl sulfoxide, 0.1 *M* PIPES, pH 6.9). This and subsequent procedures were carried out at 4°C. The homogenates were centrifuged in a microfuge at 15,000*g* for 30 min and the supernatants removed. Aliquots of 5 μl were taken from Triton X-100-soluble supernatants for protein estimation and scintillation counting. Proteins in the Triton-soluble fractions were precipitated with chloroform/methanol (Wessel and Flügge, 1984). The chloroform/methanol precipitates and the pellets from the homogenates were solubilized by stirring for 1 hr with a magnetic stirring bars in 40 μl of lysis buffer (9.5 *M* urea, 2% Nonidet P-40, 2% ampholytes, and 5% 2-mercaptoethanol) for two-dimensional gel electrophoresis (Bamburg *et al.*, 1991).

Gel Electrophoresis, Immunoblotting, and Fluorography

One-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and two dimensional nonequilibrium pH gradient electrophoresis (NEpHGE) were carried out as described by Bamburg *et al.* (1991). For SDS–PAGE, gels were prepared with 10% polyacrylamide, and a mixture of protein standards of high molecular mass, 45–200 kDa (Bio-Rad Laboratories, USA), was run concurrently. Gels were stained with Coomassie brilliant blue R in 50% methanol/10% acetic acid. The protein content of Coomassie-stained spots was analyzed by reflectance densitometry with a Bio-Rad Model GS-700 imaging densitometer, using ovalbumin as a standard.

Proteins in the gels were transferred to polyvinylidene difluoride membranes by electroblotting. After blocking with 5% nonfat milk powder in phosphate-buffered saline, blots were incubated with mouse monoclonal antibodies (Amersham, UK) specific for α-tubulin or β-tubulin (1:100), 68-kDa neurofilament polypeptide (1:20), actin (1:1000), or Hsp 70 (Sigma Chemical Co., USA; 1:5000 diluted in Tween buffer [0.1% Tween-20, 150 m*M* NaCl, 10 m*M* Tris, pH 8.0]), and then with an alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma; 1:12,500) as described before (Yuan *et al.*, 1999). Radioactivity in two-dimensional gels or blots was detected by fluorography as described by Yuan *et al.* (1999).

RESULTS

Transport of Labeled Tubulin in Sciatic Nerve

Tubulin was among the most abundant proteins in nerve extracts and was one of the few proteins, including neurofilament triplets, actin, and Hsc70 (heat shock cognate 70), found in the Triton-insoluble fraction analyzed by two-dimensional gel electrophoresis (IEF/SDS–PAGE) (Fig. 1). The positions of α-tubulin (50 kDa/ pI 5.1–5.2), β-tubulin (50 kDa/pI 5.3–5.4), 68-kDa neurofilament polypeptide (NFL), actin, and Hsp 70 were identified by immunostaining. Middle and high molecular mass neurofilament proteins NF-M and NF-H found also in the particulate fraction were identified by their molecular mass and isoelectric point.

As shown in Fig. 2, tubulin was among the few proteins, including neurofilament triplets, actin, and Hsc70 (heat shock cognate 70), found in the pellet fraction that were labeled in slow axonal transport. Since β-tubulin (pI 5.3–5.4) is less acidic than α-tubulin (pI 5.1–5.2), it seems transported β-tubulin was more strongly labeled than the α-tubulin (Fig. 2). Because Hsc70 and NF-L have similar molecular mass, they could be confused with each other on one-dimensional gels. However, since Hsc70 is more acidic (pI 5.0) than NF-L (pI 5.5), these two proteins were separated on two-dimensional gels (Fig. 1). Fluorographic analysis showed that Hsc70 was transported faster than NF-L, for NF-L was labeled only in the first three nerve segments, whereas Hsc70 was labeled in the first six nerve segments (Fig. 2).

Radiolabeled tubulin in the Triton-soluble fraction of nerve segments appeared to move as a peak (Fig. 3). After 12 days, the specific activity (dpm per pmol) of

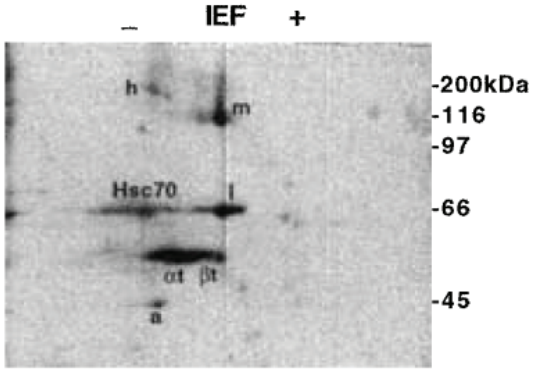

Figure 1. Coomassie blue-stained two-dimensional gel (IEF/SDS– PAGE) of Triton-insoluble nerve extract. αt and βt indicate α-tubulin and β-tubulin, respectively, and l, m, and h correspond to NF-L, NF-M, and NF-H, respectively. Actin is indicated by a and heat shock cognate 70 is labeled Hsc70. Positions of the molecular weight markers are indicated on the right side.

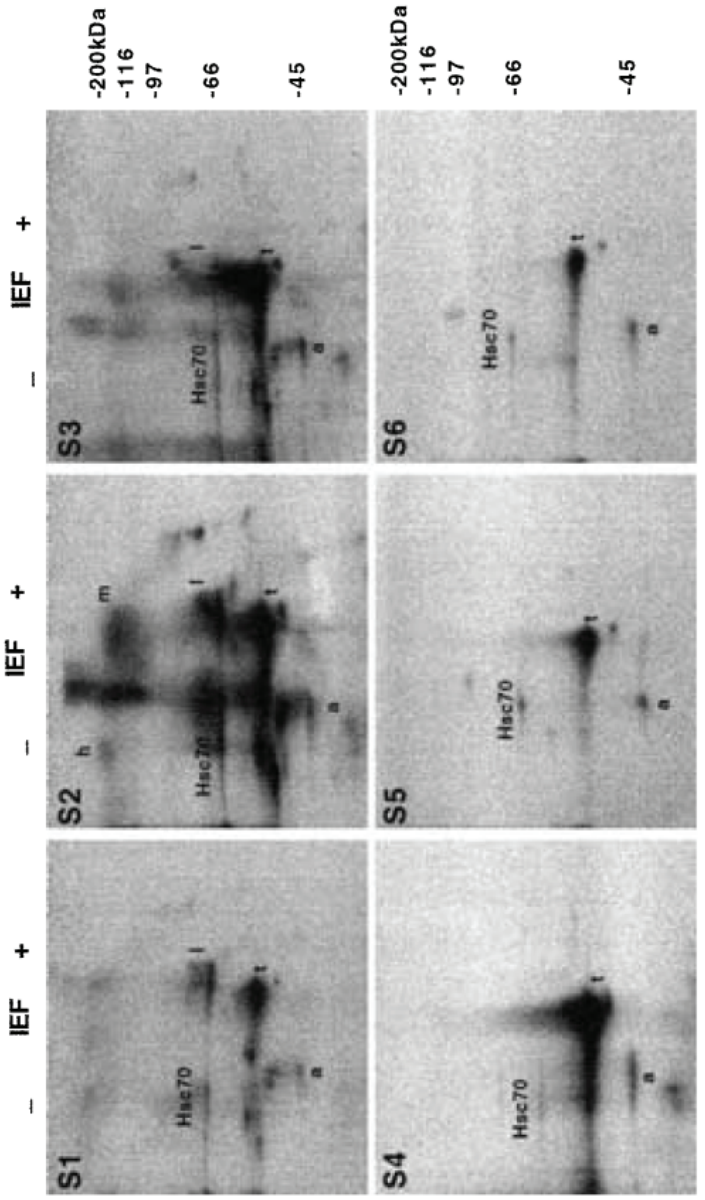

Figure 2. Fluorographs of two-dimensional gels (IEF/SDS–PAGE) of Triton-insoluble fractions of nerve extracts. Twelve days after injection of [^{35}S]methionine into the chicken lumbar spinal cord, fractions were prepared from segments of sciatic nerve 0–6 cm from the spinal cord, each1 cm in length (S1–S6). The positions of the molecular weight markers that were electrophoresed on the SDS gel are shown on the right. t indicates α- and β-tubulin; other labels are the same as in Fig. 1.

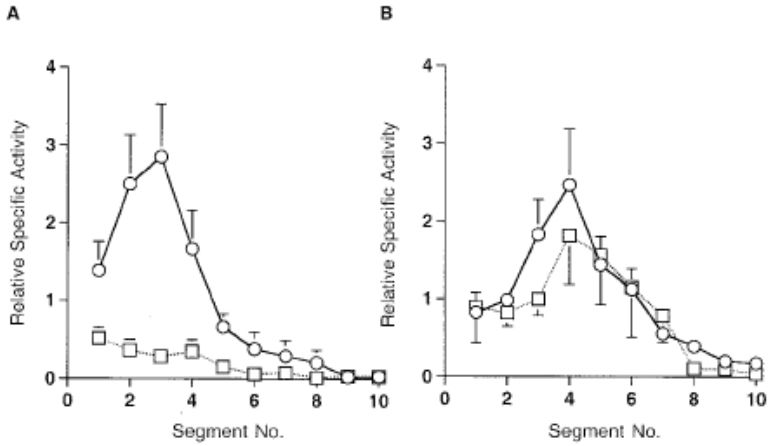

Figure 3. Relative specific activities of tubulin in Triton-soluble (circles) and particulate (squares) fractions of nerve segments 12 (A) and 20 days (B) after injection of [35S]methionine into the lumbar spinal cord. Both Triton-soluble and Triton-insoluble fractions were electrophoresed on two-dimensional gels (IEF/SDS–PAGE) and analyzed by fluorography. Data are the means ± SEM (bars) (n = 4–5). The relative specific activities of tubulin spots were calculated by dividing the specific activities, in dpm per pmol, by the mean for soluble tubulin in the 10 nerve segments. The nerve segments, each 1 cm in length, are numbered from the spinal cord on the horizontal axis.

soluble tubulin was about five times higher than that of its particulate form (Fig. 3A). After 20 days, the rate of migration of soluble tubulin as determined from the distance between the peaks of soluble tubulin at 12 and 20 days was 1.3 mm/ day (Fig. 3B). Alternatively, the rate calculated from the distance traveled at 12 and 20 days from the site of injection, allowing 5-mm nerve in the spinal cord, averaged 2–2.5 mm/day. Although the average rate of tubulin movement calculated by both methods was slower than that of actin and other SCb proteins, the rate at which its front moved was the same as that of actin (Fig. 2, S6).

Distribution of Labeled Neurofilament Along the Nerve

Low molecular mass neurofilament protein NF-L was found only in Triton-insoluble particulate fractions as identified by immunostaining (Fig. 1). Twelve days after injection, the peak of labeled NF-L was still in the spinal cord (Fig. 4A), although NF-L was labeled in the first three nerve segments (Fig. 2). Labeled tubulin, however, was clearly located more than 6 cm from the spinal cord (Fig. 2). After 20 days, NF-L was labeled in the first five nerve segments (Fig. 4B). At this time tubulin was transported even further away from the spinal cord (Fig. 3B). A migration rate of 1 mm/day of labeled NF-L was determined at 20 days based on the distance traveled from the site of injection and allowing 5-mm nerve in the spinal cord. This is likely an underestimation of the true rate, as some time elapses before proteins are synthesized and leave

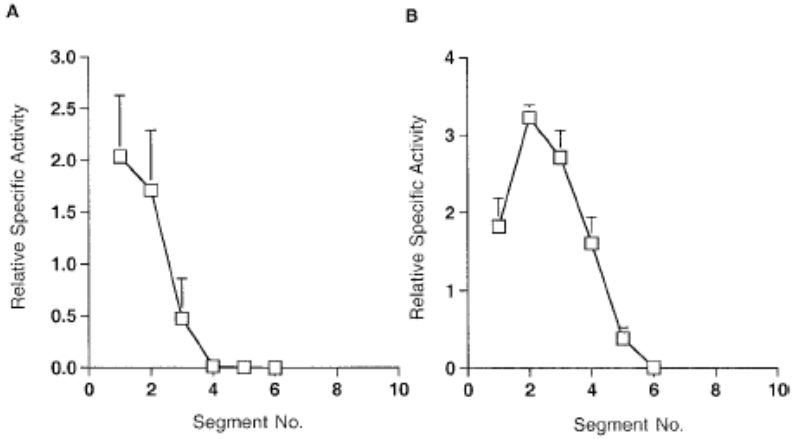

Figure 4. Relative specific activities of NF-L in Triton-insoluble fractions of nerve segments 12 (A) and 20 days (B) after injection of [35S]methionine into the lumbar spinal cord. The pellet fractions were electrophoresed on two-dimensional gels (IEF/SDS–PAGE) and analyzed by fluorography. Data are the means ± SEM (bars) (n = 4–5). The relative specific activities of NF-L spots were calculated by dividing the specific activities, in dpm per pmol, by the mean for NF-L in the 10 nerve segments. The nerve segments, each 1 cm in length, are numbered from the spinal cord on the horizontal axis.

the cell body (Baitinger and Willard, 1987). Nevertheless, NF-L clearly moved more slowly than tubulin if their respective peaks after 20 days are compared (Fig. 3B and Fig. 4B). Both tubulin and neurofilament proteins were not present in fluorographs of rapidly transported proteins that accumulated proximal to a ligation 7 cm from the spinal cord (not shown).

DISCUSSION

The main aim of the present work was to compare the specific activity of the soluble and polymerized tubulin and neurofilament proteins in slow axonal transport. For this purpose chicken sciatic nerve segments were homogenized in a cytoskeletal-stabilizing buffer designed to preserve most of the insoluble polymers in their organized state (Filliatreau et al., 1988). Most of the earlier work on tubulin and neurofilament transport was done by one-dimensional SDS–PAGE (Lasek et al., 1984; Filliatreau et al., 1988; Tashiro and Komiya, 1989), which separates proteins by size only. Other proteins in the tubulin and neurofilament bands may mask their identity, especially in the complex profiles of transported proteins. For example, there is a significant amount of Hsc70 in the NF-L band (Figs. 1, 2). For this reason we compared the specific activity of the soluble and polymerized form of tubulin and neurofilament proteins in slow axonal transport by the higher resolution technique of two-dimensional IEF/SDS–PAGE, which separates proteins first by net charge and then by size.

In the present study tubulin in the chicken sciatic nerve axons was found to exist in two forms: Triton-soluble monomers or small oligomers and Triton-insoluble polymers, presumably microtubules. Consistent with the finding of Tashiro and Komiya (1983), transported tubulin showed a stronger labeling of the β-subunit (Figs. 1 and 2). A likely explanation is that there are 19 methionine residues in β-tubulin and only 10 in α-tubulin (Jacob and McQuarrie, 1996). Previous studies found one third of tubulin in the soluble fraction and two thirds in the particular fraction (Morris and Lasek, 1984). However, we found that the relative specific activity of tubulin in the soluble fraction at 12 days was five times higher than that in the pellet fraction, although the ratio was not as great (1.2 times) at 20 days. These results suggest that the mobile form of tubulin in slow transport was soluble and there was active exchange between soluble and insoluble tubulin as it was transported down the axon. The distribution of soluble tubulin exhibited a unimodal wave (Fig. 3) along the sciatic nerve that moved at an average rate of 2–2.5 mm/ day. It is clear that the moving peak and thus the bulk of radioactive tubulin was in SCb (2–8 mm/day), with only a little trailing in SCa (1 mm/ day) (Figs. 2 and 3).

Also, consistent with earlier reports (Lasek et al., 1984; Filliatreau et al., 1988; Tashiro and Komiya, 1989), we found all the neurofilament proteins NF-L, NF-M, and NF-H recovered in the pellet fraction, presumably transported in the polymerized state. Although it seems unlikely, neurofilament proteins could be moving as oligomers or as complexes with other proteins that are insoluble. Consistent with our findings, Nixon and colleagues have shown that about 75% of neurofilament subunits in the fractions of the retina were Triton-insoluble by 2 hr after injecting mice intravitreally with [35S]methionine, and this percentage increased to 98% by 6 hr (Nixon et al., 1989). Two possible explanations for the failure to recover soluble neurofilament proteins are (1) more than 95% of the neurofilament protein is stably polymerized in axons (Morris and Lasek, 1982) and (2) the critical concentration of NF-L (the concentration at which NF-L remains unassembled at equilibrium with fully formed filaments) is only 38 μg/ml or 0.6 μM (Angelides et al., 1989), which is too low to be detected by the present approach. Consistent with the finding of Hoffman and Lasek (1975), we found that radioactively labeled NF-L, NF-M, and NF-H in the pellet fraction advanced along the chicken sciatic nerve at the slowest rate (1 mm/day) of axonal transport, i.e., in slow component a. This transport rate of neurofilament proteins in the chicken sciatic nerve is slower than the reported rate in cultured neuronal axons (Koehnle and Brown, 1999). It has been suggested that transported neurofilaments can interact with a stationary neurofilament lattice in axons (Nixon et al., 1994).

In a similar study in rat, but using one-dimensional gel analyses, Filliatreau et al. (1988) reported that in addition to the major peak of NF-L in SCa, there is a minor peak in SCb. They interpreted their results as evidence for the existence of two distinct classes of motor axons in the rat sciatic nerve in which the neurofilament transport rates are 1.1 and 2.3 mm/day, respectively. However, their data are based on one-dimensional gels and, as mentioned, NF-L could be confused with Hsc70.

Our findings are consistent with the following three recent reports: (1) Direct visualization of individual axonal microtubeles in Xenopus neurons indicates that they are primarily stationary relative to the substrate (Chang et al., 1999); (2) unpolymerized tubulin and polymerized neurofilament are transported in the squid giant axon (Galbraith et al., 1999); (3) fluorescent neurofilaments assembled from GFP (green fluorescent protein)-tagged NF-M have been observed moving through axon gaps of cultured rat sympathetic neurons (Wang et al., 2000), although virally encoded NF-M can be transported in an unpolymerized form under nonphysiological conditions (Terada et al., 1996). Together with our previous finding on the axonal transport of unpolymerized actin, we propose a differential transport model in which tubulin and actin subunits of polar microtubules and microfilaments are transported in the unpolymerized form, whereas nonpolar neurofilaments were transported in the polymerized form.

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