Neurochemical Research, Vol. 12, No. 10, 1987, pp. 967–976

Examination of a Nerve Injury-Induced, 37 kDa Protein: Purification and Characterization*

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(Accepted November 17, 1986)

Following traumatic injury to the adult rat sciatic nerve the synthesis and accumulation of soluble, extra-cellular, 37 kDa protein is increased. This protein, which accumulates in the extracellular space of the injured nerve, accounts for nearly 5% of the total soluble pool of protein in an injured nerve 3 weeks after injury. 8 weeks after injury, when regeneration is nearly complete, this accumulated pool returns to control levels, yet if regeneration is blocked synthesis of the 37 kDa protein remains high. Recently this 37 kDa protein has been shown to be nearly identical to apolipoprotein E, the protein component of various lipoprotein particles. This finding suggests a role for the 37 kDa protein in cholesterol and lipid transport and metabolism during nerve repair within the nervous system, functions that have been ascribed to apo E in serum. Results are presented here describing the purification of the nerve injury induced 37 kDa protein and the subsequent production of specific rabbit antisera directed against it. By centrifugation analysis in a sucrose gradient, a native mass of 37 kDa was determined, revealing the 37 kDa protein's monomeric, native structure. Additionally injections of [^{35}S]methionine directly into the injured nerve allowed 1) a comparison of 37 kDa synthesis in vivo versus in vitro and 2) an examination of the presence or absence of retrogradely transported 37 kDa protein. The in vitro and in vivo collected material were found to share identical 2-dimensional electrophoretic mobilities, and no appreciable amount of transported 37 kDa protein was found in proximal regions of the injured nerve.

KEY WORDS: Regeneration; degeneration; apolipoprotein E; HDL/LDL; nerve growth.

INTRODUCTION

Axons in the peripheral nervous system unlike those axons contained within the brain and spinal cord, are able to successfully regenerate after traumatic injury. The cell bodies of the regenerating fibers can be located either inside the CNS, such as motor neurons, or like sensory or most autonomic neurons they can reside in the periphery. Of importance in determining whether a giving axon will regenerate is not the location of the nerve cell body but the environment encountered by its severed axon (1, 5, 7, 8, 18, 23, 33, 34, 39). For example normally non-regenerating CNS axons will regenerate when presented with a grafted section of peripheral nerve (3, 36, 37, 46). Conversely regenerating peripheral axons when presented with a grafted section of CNS tissue will stop growing.

The influence that peripheral nerves can exert on growth has prompted a search for molecules that might underlie this function. The synthesis and secretion of several proteins by sheath cells surrounding injured nerves have been shown to increase as assayed by protein gels of injured rat sciatic nerves,

* Special Issue dedicated to Dr. E. M. Shooter and Dr. S. Varon.

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0364-3190/87/1000-0967$05.00/0

and of regenerating goldfish optic nerves (14, 30, 32, 34, 40). By tissue culture studies several laboratories have identified diffusible substances in denervated peripheral nerves which promote neurite outgrowth (24, 35). A number of studies as well have implicated the importance of components of the extracellular matrix in peripheral nerves, including laminin and fibronectin, in at least facilitating growth if not initiating it (2, 21, 22, 27, 38, 45).

Attention recently has focused as well on a 37 kDa protein which has been shown by two-dimensional electrophoretic analysis to increase approximately 100-fold in both synthesis and accumulation after injury of an adult rat sciatic nerve, (34, 40). This soluble 37 kDa protein is secreted and accumulates in the extracellular space of peripheral nerves during axonal regeneration. It is synthesized as well during neonatal development of the peripheral nervous system (PNS) and central nervous system (CNS) of the rat (29, 41, 42). The elevated levels of synthesis of this protein correlate with periods of growth, returning to control levels when regeneration is known to be complete. Yet when regeneration is blocked, elevated levels of synthesis persist for months (30). All of these observations suggest that the 37 kDa protein may be involved in some aspect of axon elongation. Synthesis of this protein has also been detected in injured adult CNS tissue (29, 40, 42), but while it accumulates in injured PNS tissue, it does not accumulate in injured CNS fiber tracts (29), suggesting that it might be one of the responsible factors limiting regeneration in the CNS.

In a recent set of papers this 37 kDa protein has been identified as being similar if not identical to apolipoprotein E (17, 43). Apolipoprotein E in serum is involved in the transport and metabolism of lipid and cholesterol (6, 25). These findings and others led to the hypothesis that the 37 kDa protein may help in the metabolization of lipid and cholesterol in the nervous system as well (as detailed in 17, 43). Specifically, during regeneration the 37 kDa protein may aid in the preparation of the distal environment of the nerve for growth, by first aiding in the metabolization of the lipid rich myelin and axonal debris generated after injury and secondly by facilitating the reutilization of this debris in the reassembly of both axonal and myelin membranes.

In this communication the purification and biochemical characterization of the 37 kDa protein is described. This analysis includes a determination of the native molecular weight of the 37 kDa protein, a comparison of its synthesis in vivo versus in vitro, and the production of specific anti-sera to the purified protein. In addition, the presence or absence of retrogradely transported 37 kDa protein is examined by injection of labeled methionine into the injured nerve. These results are discussed in terms of how they lend additional support to a proposed function for the 37 kDa protein in events of nerve regeneration.

EXPERIMENTAL PROCEDURE

Surgery and Collection of Labeled Sheath Cell Protein. All surgeries and procedures for collection of labeled sheath cell proteins are as described in detail in (30). Briefly, adult male Sprague Dawley rats were anesthetized, then the sciatic nerve was exposed in the hip, and crushed for 10 sec with jewelers forceps. One to three weeks later rats were killed with a saturated CO_2 atmosphere, and the sciatic nerve segment distal to the crush was removed, along with removal of the contralateral control nerve. The nerve was then cut into 1 to 2 mm pieces and placed into serum free Dulbecco's modified Eagle's medium. For metabolic labeling methionine free medium was used and [^{35}S]methionine was added. After 4 hours the collected media fractions, containing soluble proteins released into the medium, along with newly synthesized, labeled proteins, were centrifuged and proteins in the supernatants precipitated in 10% trichloroacetic acid.

Retrograde Labeling. Sciatic nerves were crushed as before. One week later, the nerves were re-exposed and injected subepineurally in the region of the lesion via a 31 gauge needle with 5 μl of a solution containing the dye bromphenol blue and 50 μCi of [^{35}S]methionine in distilled H_2O. Possible leakage out of the injection site was monitored by the spread of the dye, which was never seen. In half the animals the sciatic nerve on the unoperated side was also exposed and injected in a comparable region. As an additional control contralateral uninjected, unoperated nerves were analyzed for the labeling of proteins that might have arisen by retrieval of labeled methionine from the lymph or blood that may have leaked out of the original injection site. In all cases these later controls demonstrated no detectable labeling (data not shown).

After injection, animals were resutured and allowed to survive for one or four days. Sciatic nerves, and their associated spinal roots and dorsal root ganglia, from both sides of the animal were then removed, cut into equal segments and frozen at −80°C in a dry ice ethanol bath. To collect the labeled, $NaDodSO_4$ soluble proteins, nerves were homogenized in a solution of 10 mM Tris HCl at pH 7.5 containing 2% $NaDodSO_4$, 5 mM EDTA and 1 mM dithiothreitol with a ground glass homogenizer and supernatants from a 10,000 *g* spin were collected. The presence or absence of transported 37 kDa protein was determined by loading equal portions of nerve on lanes of 10% polyacrylamide gels. The resultant Coomassie stained gels were photographed and left to expose on film for up to 45 days. Two dimensional gels of samples of nerve near the site of injection confirmed the identity of the 37 kDa protein.

Sucrose Gradient Analysis. To determine the native molecular weight of the 37 kDa protein the following experiment was

done. Linear gradients of sucrose, from 5% to 20% (by weight) in 10 mM Tris-saline, pH 7.4 were poured in Ultra clear centrifuge tubes (Beckman, CA) ($\frac{9}{16}$ inches by $3\frac{1}{2}$ inches, 12 ml total volume). Samples, in a total volume of 100–200 μl were overlayed on the sucrose and mineral oil overlayed over the sample. One nerve equivalent of three week crushed conditioned medium, either whole or enriched by DEAE 52 ion-exchange chromatography was used. 2 mg each of carbonic anhydrase (MW 29,000), β-lactalbumin (36,800), bovine serum albumin (66,000), β-galactosidase (116,000) and bovine gamma globulin (150,000) were loaded on identical gradients as molecular weight standards. Tubes were loaded in a SW T1 41.1 rotor (Beckman) and spun at 40,000 rpm for 36 hr (w^2t = 100,000 $rad^2/sec \times 10^7$). Fractions (0.5 ml) were removed with an 18 gauge needle inserted in the bottom of the tube. Absorbance at 280 nM (A280) was used to determine the depths of sedimentation of the protein standards. Fractions from gradients containing conditioned medium were TCA precipitated and run on 10% polyacrylamide gels. The Coomassie blue stained gels were photographed and the negatives were scanned densitometrically to determine the peak of migration of the 37 kDa protein.

37K Purification and Antibody Production. Conditioned media from a total of 150 3-wk post crush adult rat sciatic nerves, in a total volume of 150 ml of DMEM was collected and centrifuged at 12,000 *g* for 5 min. The supernatants were dialyzed against 50 mM sodium phosphate, pH 6.0 at 4°C for 48 hrs with 2 changes. Fractions of this material were loaded onto a DEAE 52 ion exchange column in 50 mM sodium phosphate, pH 6.0. The column was then washed in several column volumes of 100 mM sodium phosphate pH 6.0. To elute the 37 kDa protein a linear gradient of 150–500 mM sodium phosphate pH 6.0 was applied to the column. Samples of each fraction were analyzed on a 10% polyacrylamide gel to determine which fractions contained the 37 kDa protein. Fractions found to contain the 37 kDa protein were pooled, dialyzed against 50 mM ammonium acetate, lyophilized, then resuspended in 2 ml of 0.1% SDS, 2 mM DTT and 10% glycerol.

The 37 kDa protein was purified to homogeneity by a final step of preparative polyacrylamide gel electrophoresis, separating denatured proteins according to their electrophoretic mobilities. Protein samples in solubilization buffer (20) were loaded onto a 10% polyacrylamide tube gel (diameter 1.2 cm, height 8 cm), with a 2 cm, 3% polyacrylamide stacking gel, mounted in a preparative gel electrophoresis apparatus (Bethesda Research Labs., MD). 200 to 500 μl of starting material was loaded, a constant current of 10 mA was applied and a flow rate of 10 to 15 ml an hour was used for sample collection. Again fractions were analyzed on a 10% polyacrylamide gel to determine which fractions contained the 37 kDa protein.

Rabbits were inoculated with 250 μg of purified 37 kDa protein suspended in Freunds complete adjuvant (DIFCO), injected subcutaneously at 8 sites along the back of New Zealand White rabbits. After one month, the rabbits received three subsequent boosts every other week with 50 μg of purified 37 kDa protein in Freunds incomplete adjuvant, yielding antiserum of high titer, directed against both the native and denatured 37 kDa protein.

Immuno-Protein Blots and Immuno-Precipitations. Protein blots or Western analyses were done essentially according to (44). Proteins were separated on 10% acrylamide gels and transferred to Zeta-Probe blotting membranes (BIO-RAD) in $NaDodSO_4$ free Laemmli buffer (20) at 200 mA for 4 hrs at 4°C. After blocking the paper with 10% bovine serum albumin (Sigma) at 50°C for 6 h, primary antibody, at dilutions of up to 100,000×, was added in 3% BSA and allowed to incubate overnight at room temperatures. Bound primary antibody was visualized with peroxidase labeled second antibody (Vector Labs, Burlingame, CA) using 4-chloro-napthol (SIGMA) at 3 mg/ml in 20% methanol/Tris saline, pH 7.4 with H_2O_2 (50 μl of 30% solution per 100 mls). Controls with non-immune sera were done at 100× dilution.

Sample Preparation and Electrophoresis. For one-dimensional polyacrylamide gel electrophoresis (PAGE), protein samples were solubilized by boiling in sample buffer containing 0.5% $NaDodSO_4$ and 2 mM dithiothreitol according to (20).

Two dimensional PAGE was carried out according to O'-Farrell (31). The first dimension of isoelectric focusing was in 1.5 mm diameter tube gels containing 4% pH 3.5 to 10 and 2% pH 4 to 6 ampholytes. Focusing was carried out at 350 volts for 18 h. The second dimension $NaDodSO_4$ gels were again according to Laemmli (20) using 10% polyacrylamide.

Gels were stained for protein with either Coomassie brilliant blue staining (10), or silver staining (28). Destained Coomassie gels were prepared for autoradiography by first impregnating the gel with 2,5-diphenyloxazole (PPO, Sigma) according to the APEX procedure (19) then dried under heat and reduced pressure, and exposed to XAR-5 X-ray film (Kodak) for one to two weeks at −70°C.

RESULTS

In Vitro and In Vivo Synthesis of the 37 kDa Protein After Nerve Crush. In Figure 1, two dimensional gels are shown which depict both the accumulated proteins collected in vitro and the newly synthesized proteins labeled in vivo from an injured rat sciatic nerve. As has already been described, (34, 40) the synthesis and accumulation of a slightly acidic (pH 5.3 to 5.5) protein with an apparent molecular weight of 37,000 is markedly increased after injury. The Coomassie blue stained gel of the soluble component of the nerve three weeks after a crush shows the accumulated 37 kDa protein (Figure 1a). The protein runs as a heterogeneous smear, in a characteristic, comet shape (arrow in each panel). This unique protein profile has aided in the identification of this protein throughout these studies. The fluorograph shown in Figure 1b shows the identical comet shaped electrophoretic profile for metabolically labeled 37 kDa protein when [^{35}S]methionine was injected directly into the nerve.

This set of in vivo labeling experiments provided an opportunity to examine whether the characteristic two-dimensional electrophoretic profile of the protein seen for in vitro collected material was similar to the in vivo labeled material and not an artifact produced by either our culture conditions, or by injury to the nerve when it was cut into 1 to 2 mm pieces. As can be seen in the pair of two

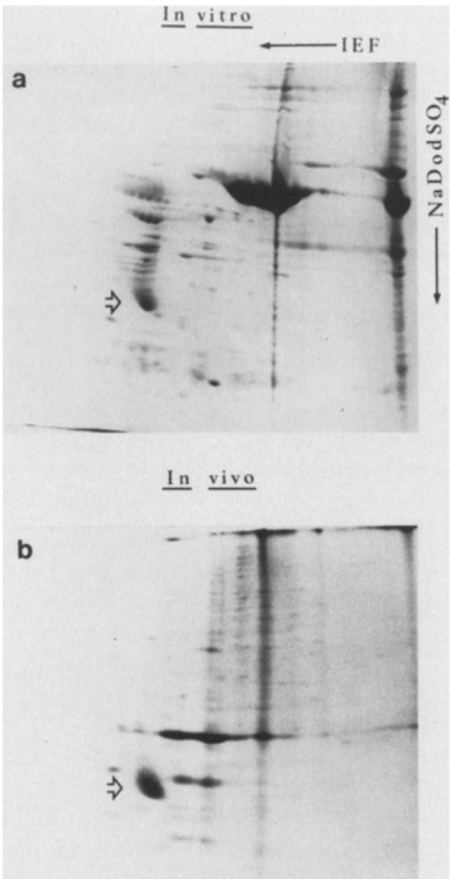

Fig. 1. Two-dimensional electrophoretic analyses of soluble proteins in the distal sheath of crushed rat sciatic nerves collected both in vivo and in vitro. The Coomassie stained gels of the accumulated proteins collected in vitro from crushed nerves is shown in (a). In panel (b) are shown the accumulated newly synthesized, $NaDodSO_4$ soluble proteins collected in vivo after direct injection of [^{35}S]methionine into the sub-epineurial space of a crushed nerve. Crushed nerves were examined in vivo 1 week after injury and in vitro nerves 3 weeks after injury. The arrow defines the position of the 37 kDa protein. In both instances the 37 kDa protein runs as a characteristic comet shaped smear.

dimensional gels, this was not so, since the same comet shaped profile is observed (arrows, Figure 1a and b). This finding indicates that the in vitro collected media would be a suitable source of native 37 kDa protein for purification, described later.

Retrograde Transport. Recent evidence has demonstrated that growing neurons in culture, specifically their growth cones, can bind and internalize lipoprotein particles containing apo E (17). The eventual target of the internalized protein was not determined, although it was suggested that it was locally utilized in the assembly of plasma membrane in the growth cone. Alternatively the protein and its associated lipid could be taken up by the growth cone and transported back to the cell bodies. This later possibility was tested in the following way. [^{35}S]methionine was injected subepineurally 1 to 5 mm distal to a crush site. Injections were done 1 week after the crush when synthesis of the 37 kDa protein is at a maximum. Animals were resutured and sacrificed 1 to 4 days later, allowing enough time for both the localized synthesis and secretion of labeled protein and its subsequent transport through more proximal segments back to their cell bodies. At the time of sacrifice proximal segments of nerve, spinal nerves and dorsal root ganglia were removed and assayed on one and two dimensional gels for the presence of labeled, transported 37 kDa protein.

Figure 2 is a summary of one such set of experiments. This particular experiment screened proximal segments 4 days after injection of label (results were similar one day after injection as well). No radioactive proteins except for a prominent 60 kDa protein seen in every lane, both labeled and unlabeled, were found to be retrogradely transported (labeled) in the proximal segments (Figure 2,

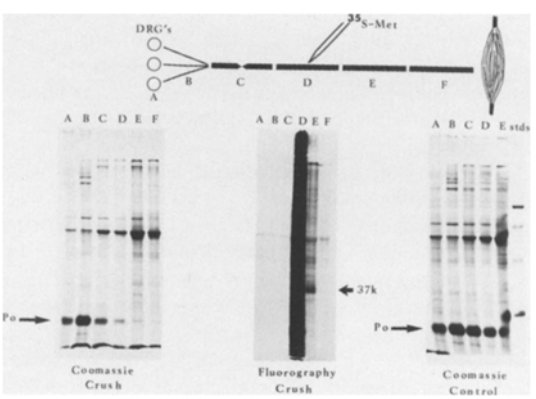

Fig. 2. Analysis of the capacity for regenerating sciatic nerve axons to retrogradely transport the 37 kDa protein. Surgery, injection protocol and sample analysis were as described in the Materials and Methods. In the first panel is a Coomassie blue stained gel of the SDS soluble material collected from various segments of an injured nerve as diagrammed at the top of the figure. In the third panel is a Coomassie blue stained gel profile from an uninjured nerve. The center panel is a fluorograph (45 day exposure) of the injured nerve, 4 days after injection of labeled methionine. As expected the 37 kDa protein is synthesized in the distal segments (arrow) but no evidence of the 37 kDa protein can be seen in more proximal segments. Transport, if it is occurring is below the level of detection of the analysis used. The major glycoprotein of myelin "P_O" can be seen in only those segments where intact fibers are present, as shown in the Coomassie stained gels. Molecular weight standards in the right panel (stds) were 92,500, 66,000, 45,000, 31,000.

lanes a–c in center panel). (The identity of this 60 kDa protein is unknown). The bulk of the labeled 37 kDa protein, seen near the site of injection (Figure 5, lane d, arrow in fluorogram) is not transported but is retained locally. If any 37 kDa protein is being transported, it is below levels detectable in our system. (The identity of this band as the 37 kDa protein was confirmed in two-dimensional gels Figure 1(b), in which a sample of nerve from near the injection site was analysed). In control experiments with contralateral uninjured nerves, either injected with label or left alone, little 37 kDa protein synthesis was detectable in any segment (data not shown).

Several other protein changes are apparent in nerves when the Coomassie blue stained gels of control and injured nerve segments are compared (Figure 2, left hand and right hand panels). The most prominent is the disappearance of the major glycoprotein of peripheral myelin, the 29 kDa P_O protein (13). In a number of similar experiments, the 37 kDa protein was never found in segments where large amounts of the P_O protein remained, indicating presumably intact, uninjured, myelinated fibers.

Sucrose Gradient Analysis. The physical analysis of the 37 kDa protein in previous experiments was confined to studies done under denaturing conditions. It has not been established whether under native conditions 1) the protein exists as a multimer of identical 37,000 molecular weight subunits, or 2) it exists in association with other molecules, an association that is disrupted by the various denaturing agents used such as $NaDodSO_4$, DTT or urea used for gel analysis. To answer these questions a preparation of partially enriched 37 kDa protein from in vitro collected material (pooled samples from a DEAE 52 chromatographic separation, described later in section on purification) was layered on top of linear gradient of sucrose in phosphate buffered saline and spun for 30 hr at 200,000 *g*. By comparing the relative rates of sedimentation of native standards in gradients carried out in parallel, an indication of the native molecular mass for the 37 kDa protein was established. The results of this analysis are shown in Figure 3. The nerve injury induced 37 kDa protein migrated the same distance as the 37,000 kDa molecular mass protein standard (Figure 3b), indicating that the nerve injury induced 37 kDa protein exists as a monomer of 37,000 molecular mass in the nerve.

As noted above, a partially enriched preparation of the 37 kDa protein was used in these experiments. The enrichment step was obtained on a

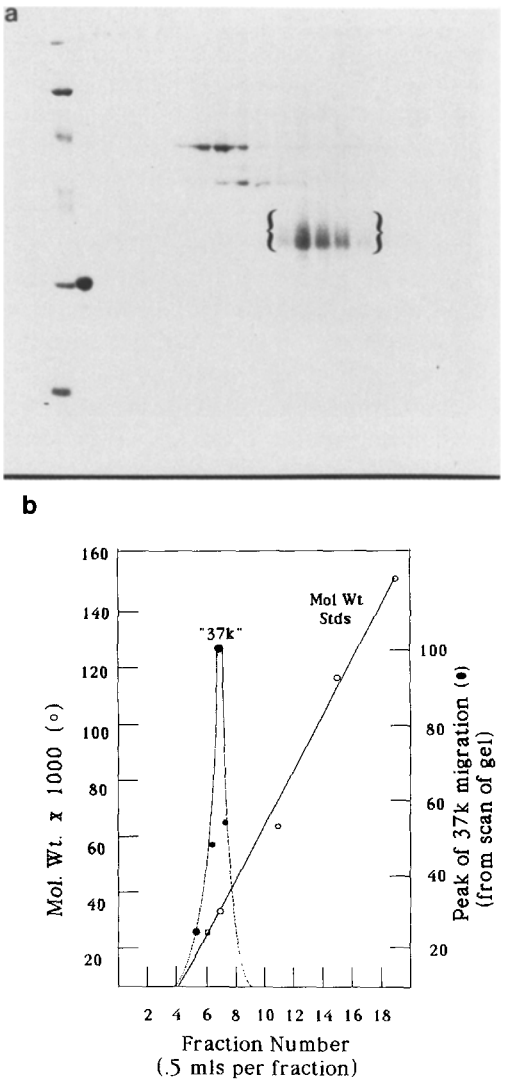

Fig. 3. Sucrose gradient analysis of the native 37 kDa protein. Partially enriched conditioned medium containing the 37 kDa protein was layered on top of a linear gradient of sucrose from 5 to 20% in physiological salt and pH and spun for 30 hours at 200,000 *g*. In (a) is a Coomassie blue stained $NaDodSO_4$ gel profiling fractions collected from one representative run. The top of the gradient or fraction number 1 is represented by the far right hand lane. Molecular weight standards used were 92,500, 66,000, 45,000, 31,000, 21,000. The peak of the 37 kDa protein containing fractions is shown by the brackets. (In addition to revealing its native mass, this analysis also suggested an effective enrichment step to be used in the purification of the native protein).

In order to determine the native molecular weight of the nerve injury induced 37 kDa protein, molecular weight standards were centrifuged and analyzed in parallel. The results of this analysis are in panel (b). Standards used and their masses were carbonic anhydrase (29 kDa), β-lactalbumin (37 kDa), bovine serum albumin (66 kDa), β-galactosidase (116 kDa) and gamma-globulin (150 kDa). The peak of the 37 kDa nerve injury induced protein was determined by scanning the Coomassie blue stained gel shown in (a). Rates of migration of standards were derived by scanning eluted fractions at 280 angstroms to determine their various peaks of absorbance. As can be seen (b) the 37 kDa nerve injury induced protein co-sediments with the β-lactalbumin, 37 kDa standard.

DEAE 52 column run at pH 6.0 in 50 to 200 mM sodium phosphate followed by overnight dialysis against 50 mM ammonium acetate pH 7.0. It is possible that some molecular interactions may have been altered or disrupted by either the pH change during the column analysis or by dilution of the complex during dialysis. When a sample of whole conditioned medium containing the 37 kDa protein that had not been enriched or dialysed was analyzed, the 37 kDa protein sedimented with an apparent weight between 45,000 and 66,000 (data not shown). One possible explanation for this result is that the high concentration of serum albumin, present in conditioned medium, but not in the DEAE-enriched material, altered local densities of the sucrose enough to disrupt the normal sedimentation profile. Alternatively the 37 kDa protein in conditioned medium may, in fact, be complexed with other molecules. Snipes et al. (42) have found that the 37 kDa protein in conditioned medium is excluded from a G-200 sizing column, indicating a molecular weight in excess of 200,000 kDa. We have recently demonstrated that the 37 kDa protein in conditioned medium is associated with lipid in the form of high density lipoproteins (17) which would likely account for these results as well. It is apparent though that the protein moiety is a monomer of 37,000 kDa.

Purification and Antibody Production. By three weeks after a sciatic nerve injury, maximal levels of 37 kDa protein have accumulated in the distal nerve stump, accounting for 2–5% of the total soluble protein which can be recovered by washing the nerve stump in vitro (30, 40). Therefore purification of the 37 kDa protein was undertaken using 3-wk conditioned medium. The slightly acidic net charge of this protein allowed the use of DEAE 52 ion-exchange chromatography as a first step. Three week post crush conditioned medium, dialysed against 50 mM sodium phosphate pH 6.0 was loaded on a DEAE 52 column in 50 mM sodium phosphate. A subsequent 100 mM wash removed the bulk of the serum albumin and a number of other proteins (Figure 4a). After this step a linear gradient of sodium phosphate from 150 mM to 500 mM yielded fractions eluting around 200 mM sodium phosphate in which the 37 kDa protein was enriched (Figure 4a). These fractions were pooled and dialysed against a volatile solvent, 50 mM ammonium acetate, to facilitate concentration of the pool by lyophilization.

To determine whether proteins of identical molecular weight, but different isoelectric points to the 37 kDa protein were co-purifying during the DEAE chromatography step, the pooled 37 kDa enriched samples were analyzed by 2-dimensional gel electrophoresis. Only the nerve injury induced 37 kDa protein with an isoelectric point near 5.3 was found in the region of 37,000 kDa in these enriched samples (data not shown). This result allowed a final step of purification to be used where proteins were separated on the basis of size by preparative polyacrylamide gel electrophoresis. Appropriate fractions from the DEAE chromatography step were solubilized in sample buffer and loaded onto a 10% polyacrylamide tube gel in a preparative gel apparatus. Fractions eluted from the preparative gel were again analyzed by electrophoresis in Na-$DodSO_4$ (Figure 4b). Both a two dimensional, Coomassie blue stained gel (Figure 4c) and a one dimensional silver stained gel (Figure 4b) demonstrate the purity of the preparation. 166.7 mg of starting material (from 100 ml of 1 sciatic nerve/ml conditioned medium) yielded 480 μg of purified proteins. This material was used for amino acid sequence determinations described elsewhere (16) as well as for antibody production described below.

Rabbits were innoculated as described in the Materials and Methods first with 250 μg of the purified denatured protein followed by boosts with 50 μg of the same preparation of protein. The antiserum raised against the denatured protein was tested for its ability to recognize the 37 kDa under both native and non-native conditions in the following ways. Antisera diluted 1:100 was incubated with [^{35}S]methionine labeled crushed nerve conditioned medium. Precipitation of antigen-antibody complexes with protein-A Sepharose revealed the specific binding of the labeled 37 kDa protein and a 20 kDa polypeptide as well (Figure 5a). The 20 kDa protein is likely a fragment of intact 37 kDa protein as determined from A.A. sequence data (16). The ability of antisera to specifically recognize the native molecule established its suitability for immunofluorescence studies, on tissue samples.

In Western or immuno protein blots, the antibody specifically recognized the denatured 37 kDa protein from medium conditioned by injured nerves at serum dilutions of 1:10,000 (Figure 5b). Barely detectable levels of the 37 kDa protein were present in conditioned medium from control nerves (Figure 5b). The immuno-reactive band just below the 37 kDa smear in this immuno blot, is likely one of the isoforms of normal serum derived apolipoprotein E. When purified apolipoprotein E, which cross-reacts

with the antisera to the 37 kDa protein, is run along side nerve crush conditioned medium and probed on nitrocellulose with 37 kDa antisera, this lower immunoreactive band corresponds to the most prominent of three bands of apolipoprotein E (16). It is therefore possible that residual serum, present in the nerve which contains apolipoprotein E, accounts for the source of this additional band, as well as the faint staining seen in control nerves from other blots (not shown).

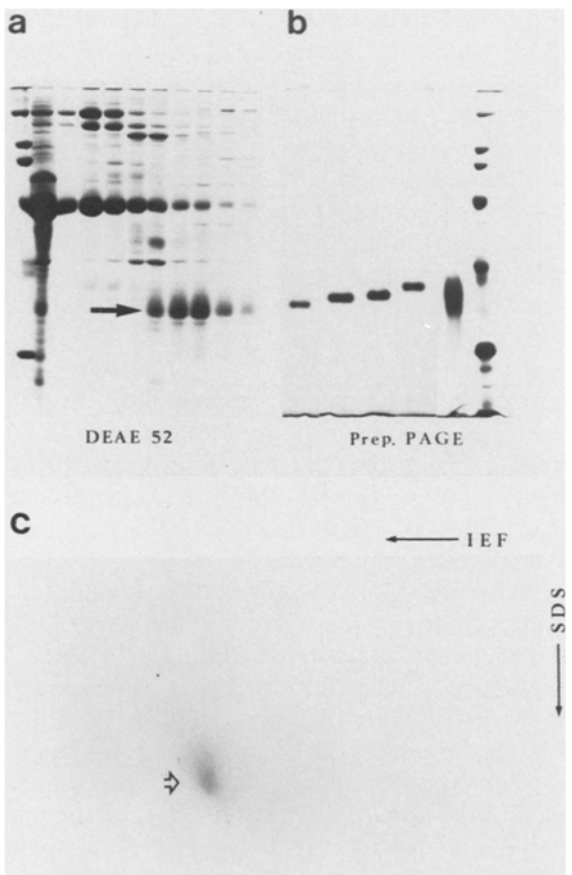

Fig. 4. Purification of the 37 kDa protein from conditioned medium. In panel (a) are lanes representing eluted fractions from one DEAE 52 analysis. Whole, dialyzed conditioned medium (lane 2) was first loaded in 50 mM sodium phosphate pH 6.0. The column was then washed with several column volumes of 100 mM sodium phosphate, pH 6.0 (lanes 3 through 6) which removed the bulk of the serum albumin. Fractions enriched for the 37 kDa protein (arrow) were eluted at the beginning of a gradient of sodium phosphate, pH 6.0, from 150 mM to 500 mM (lanes 7 through 11). Fractions represented by lanes 7–11 were pooled and concentrated, prior to the second step of preparative polyacrylamide gel electrophoresis (Prep PAGE), panel (B).

Panel B (lanes 1–4) represent fractions from one Prep PAGE run after silver staining. In lane 5 is the pooled material, showing the heterogeneity of the protein. A two-dimensional, Coomassie blue stained gel of this pooled material is shown in (C), revealing the purity of the preparation. Yield from 150 crushed nerves was approx. ½ mg of 37 kDa protein.

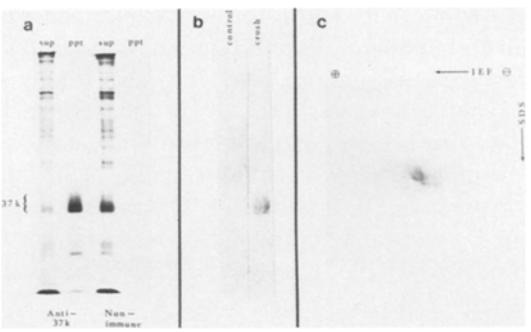

Fig. 5. Rabbit sera antibodies to the 37 kDa protein recognize both the native and denatured 37 kDa protein. In panel (a) are supernatants (sup) and precipitates (ppt) from samples of [^{35}S]-methionine labeled conditioned media of 1 week post-crush nerves, that were incubated first with anti-sera for 4 h at 4°C then with protein—A Sepharose for 1 hr. Anti-sera to the 37 kDa protein at 1:100 dilution precipitated the 37 kDa protein and a 20 kDa protein, that is likely a fragment of the intact 37 kDa protein. Non-immune serum at 1:50 did not precipitate any labeled proteins (panel a). In panel (b) are immunoblots of unlabeled media collected from control rat sciatic nerves ("Cont") and crushed sciatic ("crush"). At 1:10,000 dilution the denatured 37 kDa protein is recognized in both crushed sciatic and crushed optic nerve samples. Conditioned medium from a control nerve had little detectable immunoreactivity in this blot.

In panel (c) conditioned medium from segments of sciatic nerve, removed 3 weeks after injury was first separated by two dimensional polyacrylamide gel electrophoresis then blotted onto nitrocellulose paper and probed with antisera to the 37 kDa protein. Staining was allowed to proceed to exhaustion to rule out binding to any low abundant cross reactive proteins. The antisera recognizes the comet shaped 37 kDa protein.

To further characterize the antisera to the 37 kDa protein, immuno protein blots of two dimensional PAGE gels of conditioned medium from sciatic nerves crushed three weeks earlier were made. The separated proteins were transferred to nitrocellulose and reacted with antisera (Figure 5c). Binding of the antisera to the characteristic comet shaped 37 kDa protein was observed, along with staining of lower molecular weight material. Again this additional staining likely represents serum apolipoprotein E, co-migrating with the slightly more heterogenous, nerve injury induced 37 kDa protein.

DISCUSSION

By several criteria, including immuno-cross reactivity and amino acid sequence homology the 37 kDa protein has been shown to be homologous to apolipoprotein E (16, 43). Apolipoprotein E,

along with other lipoproteins, mediate the recognition and uptake of lipoprotein particles containing cholesterol, triglyceride and phospholipid by specific receptors on various cells in the periphery (6, 25, 26). The binding of apolipoprotein E containing lipoprotein particles such as low and high density lipoproteins (LDL's and HDL's) stimulates receptor mediated uptake of the particles, which serve as a primary source of cholesterol for normal cellular metabolism (11, 12). The recent discovery of apolipoprotein E in the brain (4, 9, 15) along with our finding that a nearly identical protein is synthesized in response to nerve injury has indicated that neural tissue may use similar pathways for acquiring lipid.

The sucrose gradient sedimentation analysis described here further supports the structural homology between the nerve injury induced 37 kDa protein and apolipoprotein E. Apolipoprotein E exists as a monomer of approximately 35 kDa, and the 37 kDa protein from DEAE enriched material although slightly heavier exists as a monomer as well. Also under conditions where an association with lipid is likely not to be disrupted, as with the whole conditioned medium, the apparent mol. mass of the 37 kDa increased, indicating that like apo E, the 37 kDa protein is associated with lipid. Results from potassium bromide centrifugation analysis have confirmed that the bulk of the 37 kDa protein detectable in the nerve is associated with lipid (17).

The capacity for regrowing axons to retrogradely transport the 37 kDa protein was examined by injecting labeled methionine into the injured sheath in the region of regenerating axons. The results of these experiments exclude the possibility that transport of any significant or measurable amount of the labeled 37 kDa protein to the dorsal root ganglia occurred despite the high levels of its synthesis distal to the injured site. This experiment does not rule out the possibility that the 37 kDa protein could be bound and internalized by the growing tip of the axon and not subsequently transported or that amounts below the level of sensitivity of our analysis were transported. The 37 kDa protein or more specifically its associated lipid could be locally incorporated into the axon and utilized thereby clearing it from the extracellular space of the nerve. The coincidence of the time course for the disappearance of the 37 kDa protein after injury and the regrowth of the axon, argues that a relationship exists between the extent of regrowth and the level of the 37 kDa protein present in the soluble pool of the distal sheath. Utilization or degradation of the protein by the regrowing axon (growth cone) or Schwann cells in the region of the axon could yield such a result.

This interpretation is consistent with a portion of a model that has been proposed (16, 17, 43) in which the 37 kDa protein, functioning in a manner similar to that known to occur for apolipoprotein E, delivers cholesteryl ester rich lipid particles to regrowing axons. The regenerating fibers would metabolize this lipid for use in membrane synthesis at the growing tip thereby facilitating their elongation. Apo E and its associated lipid would therefore be utilized locally by the axon growth cone but not transported to more promimal segments. Accumulated extracellular 37 kDa protein and any associated lipid would be used up as regeneration continued. In support of this are the findings that in cases where regeneration in the sciatic nerve is blocked, levels of accumulated 37 kDa protein have been shown to remain high for as long as two months (30) as if utilization were not occuring, while in normal regenerating nerves levels of 37 kDa protein are at control levels by two months post-injury. Recently it has been shown that lipoprotein particles containing apo E can be bound and internalized by PC12 growth cones in vitro (17). Whether this material is in fact metabolized in the assembly of new membrane is under investigation.

A variety of purification schemes exist for enriching apolipoprotein E from whole sera (47) that take advantage of apo E's affinity for heparin. The isolation procedure described here provides a simple method, giving high yields that is suitable for isolating the nerve derived homologous protein from conditioned medium. The antisera generated with this purified preparation recognizes both the native and denatured 37 kDa protein as well as apolipoprotein E, allowing an examination of the tissue distribution of the protein. Such studies to date have indicated that the 37 kDa protein is present in adult astrocytes, non-myelinating Schwann cells, a subpopulation of dorsal root ganglion neurons along with macrophages (J. Boyles and R. W. Mahley, personal communication). The distribution of apo E in the rat brain using antibodies to the serum derived apo E has been well characterized and (4, 15) and is nearly identical to that seen for the 37 kDa protein with the exception that no immunoreactivity was detectable in neurons. The staining in neurons with 37 kDa antisera may be an artifact produced by either preexisting antibodies in the pre-immune sera or cross-reactivity of the immunized sera. How-

ever, using another antisera to apolipoprotein E, others have seen staining in neurons as well (E. Zimmerman, personal communication). It may be that the 37 kDa protein, while bearing nearly identical primary structural homology differs in some post-translational modifications thereby creating a unique epitope. Apo E when first secreted is more heavily sialylated, yet once in serum it is processed to its 35 kDa size, presumably by extra-cellular glycosidases (4, 48). Yet the 37 kDa nerve injury derived proteins retains its more heterogenous, glycosylated form up to 14 weeks post-injury. These differences, and the possible disparities seen in the immuno-localization studies emphasize the importance of utilizing separate purification schemes for the two proteins, so that any differences in the processed proteins, if they exist, can be resolved.

Many gaps still persist in our understanding of how the peripheral nervous system repairs itself after injury, and much less is known about why the central nervous system fails to regenerate. It is intriguing to imagine that one or several molecules might provide the necessary stimulus and/or substrate for growth, such as the growth factors and the various matrix proteins, laminin, collagen IV etc. It is more likely though that many molecules acting in concert will be required, ranging from growth factors and structural proteins to proteases and protease inhibitors, similar to the cascade of events following capillary wall injury or clot formation. The 37 kDa protein may well represent one significant part of such a multi-step process.

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