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The blood–nerve barrier is rich in glucose transporter

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Summary

The glucose transporter of the facilitated diffusion type has been localized in sections of innervated rat diaphragm muscle and sciatic nerve by immunofluorescence, using affinity-purified antibodies against both the entire transporter and the carboxy-terminal peptide. In both tissues the transporter was very abundant in the perineurial sheath of cells surrounding the nerve fibres. The transporter also appeared to be abundant in the endoneurial blood vessels of the sciatic nerve. The identity of the antigen as the glucose transporter was established by extracting sciatic nerve with sodium dodecylsulphate and immunoblotting the extract. A single reactive polypeptide with the expected molecular weight of 55 000 was found. The high concentration of glucose transporter in the cells of the blood–nerve barrier presumably ensures an adequate supply of glucose to the nerve fibres.

Introduction

Peripheral nerves are ensheathed by one or more layers of flattened cells with tight junctions at their contact surfaces, which make up the structure known as the perineurium (Thomas & Olsson, 1984). In addition, blood vessels within the nerve fibre are surrounded by endothelial cells sealed with tight junctions (Thomas & Olsson, 1984). Together these structures constitute the blood–nerve barrier, through which small, hydrophilic compounds, such as L-glucose and sucrose, pass very slowly (Rechthand *et al.*, 1987). On the other hand, D-glucose is a major fuel for peripheral nerves (Greene & Winegrad, 1979). Consistent with this fact, a kinetic study of the uptake of D- and L-glucose by rat tibial nerve has shown that the blood–nerve barrier contains a transport system specific for D-glucose (Rechthand *et al.*, 1985).

In this report we describe the localization of the glucose transporter (GT) of the facilitated diffusion type in innervated diaphragm, muscle and in sciatic nerve by immunofluorescence. In both tissues the GT is remarkably abundant in the cells of the blood–nerve barrier.

Materials and methods

Antibodies

Two previously described preparations of affinity-purified rabbit antibodies against the GT were used. One consisted of antibodies raised against the purified GT from human erythrocytes (Schroer *et al.*, 1986). The other consisted of antibodies against the carboxy-terminal peptide of the GT (residues 477 to 492) (Davies *et al.*, 1987). The cDNAs encoding the GT in a human hepatoma line (Mueckler *et al.*, 1985) and in rat brain (Birnbaum *et al.*, 1986) have been sequenced, and this peptide is identical in both. Other antibodies were from the following sources: mouse monoclonal antibody against the 68 000 Da neurofilament protein, Amersham; fluorescein-goat anti-rabbit immunoglobulin (IgG) and rhodamine-goat anti-mouse IgG, Cappel.

Immunofluorescence

Strips of rat diaphragm muscle taken from regions containing nerve fibres were rolled up like a scroll and frozen in isopentane slush cooled in liquid nitrogen. Sections (8 μm thick) were cut with a cryostat at − 20° C, mounted on glass slides and allowed to air dry. Rat sciatic nerve was placed in a slot cut in a small piece of rat liver prior to freezing, and 8 μm sections were cut as described for the diaphragm muscle. Sections were incubated with 150 mM NaCl/10 mM sodium phosphate (pH 7.4) containing 5% goat serum (antibody dilution buffer) for 30 min at room temperature, and then a mixture of rabbit anti-transporter IgG (3.7 μg ml^{-1}) and mouse anti-neurofilament (1/5 dilution) was applied. The latter is a mouse monoclonal antibody directed against the 68 000 neurofilament protein. In some cases a 10-fold molar excess of purified human erythrocyte GT (Baldwin *et al.*, 1982) was incubated with the anti-transporter antibody for 15 h at 4° C prior to

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application to the sections. After incubation with the first antibody, the sections were washed in the phosphate-buffered saline and incubated with a mixture of fluorescein-goat anti-rabbit IgG (4 μg ml^{-1}) and rhodamine-goat anti-mouse IgG (10 μg ml^{-1}). After 30 min the sections were again washed in phosphate-buffered saline and mounted for viewing in a fluorescence microscope equipped with epifluorescence and filters that permitted independent visualization of the two fluorochromes. Sections were photographed with Ilford HP5 film.

Extraction of sciatic nerve and immunoblotting of the glucose transporter

Freshly dissected rat sciatic nerve (about 0.5 cm) was stored frozen at − 70° in a 1.5 ml microfuge tube. Initially it was vortexed intermittently at room temperature for 30 min with 450 μl 4% sodium dodecylsulphate (SDS)m pH 7.0, containing a mixture of protease inhibitors, di-isopropyl fluorophosphate (1 mM), pepstatin A (1 μM), ethylene-diaminetetra-acetic acid (5 mM), and L-*trans*-epoxysuccinyl-leucylamido-(3-methyl)butane (0.1 mM). The nerve fibre, which remained visibly intact, was pelleted in the microfuge, and the supernatant was assayed for protein by a modification of the Lowry method (Peterson, 1977); the concentration was 700 μg ml^{-1}. In an effort to solubilize more of the nerve, the supernatant was made to 25 mM in dithiothreitol from a 1 M stock. It was then added back to the pellet and the mixture was ground with a small Teflon pestle. The insoluble material was sedimented and the supernatant was again assayed for protein. The concentration this time was 680 μg ml^{-1}, and thus this subsequent treatment did not solubilize additional protein.

SDS samples were electrophoresed on 10% polyacrylamide gels, the polypeptides were transferred electrophoretically to nitrocellulose, and the nitrocellulose was probed first with various rabbit antibodies and then with ^{125}I-labelled goat antibodies against rabbit IgG and was then subjected to autoradiography. The details of this method are described elsewhere (Biber & Lienhard, 1986).

Results

Localization of the glucose transporter in muscle and nerve by immunofluorescence

Immunofluorescence studies of rat diaphragm muscle showed little, if any, staining of muscle fibres by antibodies against the GT, probably because the site density of the GT was below the level needed for detection by this method. However, intense staining of structures that appeared to surround bundles of nerve fibres within the muscle was seen. The association of anti-GT staining with nerve was most easily demonstrated by double-label immunofluorescence, in which transverse sections of muscle were first incubated with a mixture of rabbit antibodies against the GT and a mouse monoclonal antibody directed against the 68 000 molecular weight polypeptide of neurofilaments, followed by a second incubation with fluorescein-anti-rabbit IgG and rhodamine-anti-mouse IgG. Bundles of nerve fibres were brightly stained by anti-neurofilament and were completely encircled by anti-GT staining (Fig. 1A–H). This result was obtained with two different preparations of anti-GT antibodies, one raised against purified GT from human erythrocytes (Fig. 1A–D) and the other raised against a synthetic peptide corresponding to the carboxy-terminal sequence of the transporter (Fig. 1E–H). Staining by anti-GT was completely abolished by incubation of either antibody with purified GT prior to application to the section (Fig. 1I–L). Furthermore, no staining of this structure was seen with several control rabbit IgG preparations (data not shown).

A similar pattern of staining was observed in transverse sections of rat sciatic nerve. As shown in Fig. 2A and B, anti-GT (raised against the C-terminal peptide) stained structures that extended around the periphery of bundles of nerve fibres. Structures that appeared to encircle blood vessels that lie within the nerve bundles were also stained (Fig. 2A). There was little GT staining of the nerve fibres themselves, of the region containing the nerve fibres (the endoneurium) and of the region between the fascicles of fibres (the epineurium) (Fig. 2A). As in the muscle sections, this staining was eliminated by prior incubation of the antibody with purified transporter (Fig. 2C, D). Similar results were observed with the other preparation of anti-GT antibodies.

Identification of the glucose transporter polypeptide in sciatic nerve

Isolated sciatic nerve was extracted with SDS, and the resulting sample of proteins was subjected to immunoblotting (Fig. 3). The nerve sample showed a single band of M_r 55 000 when probed either with antibodies against the entire GT (lane 1) or with ones against the carboxy-terminal peptide (lane 5). Binding of the latter antibodies was blocked by an excess of the peptide itself (lane 7). It can be confidently concluded that this M_r 55 000 polypeptide is the glucose transporter, both because it is the only immunoreactive protein and because the transporter in a number of other tissues from rat and other species has been shown to be a polypeptide of this molecular weight (Mueckler *et al.*, 1985; Birnbaum *et al.*, 1986, and references therein).

Lanes 2 and 6 of the immunoblot show the human erythrocyte GT. The transporter from this cell is more heterogeneously glycosylated than that from other tissues, and consequently runs as a broader band on SDS gel electrophoresis (Lienhard *et al.*, 1984). The carboxy-terminal peptide of the human erythrocyte and rat brain GTs are identical (Mueckler *et al.*, 1985; Birnbaum *et al.*, 1986), and since the rat brain and sciatic nerve transporters are likely to be the same polypeptide, the antibodies against the carboxy-

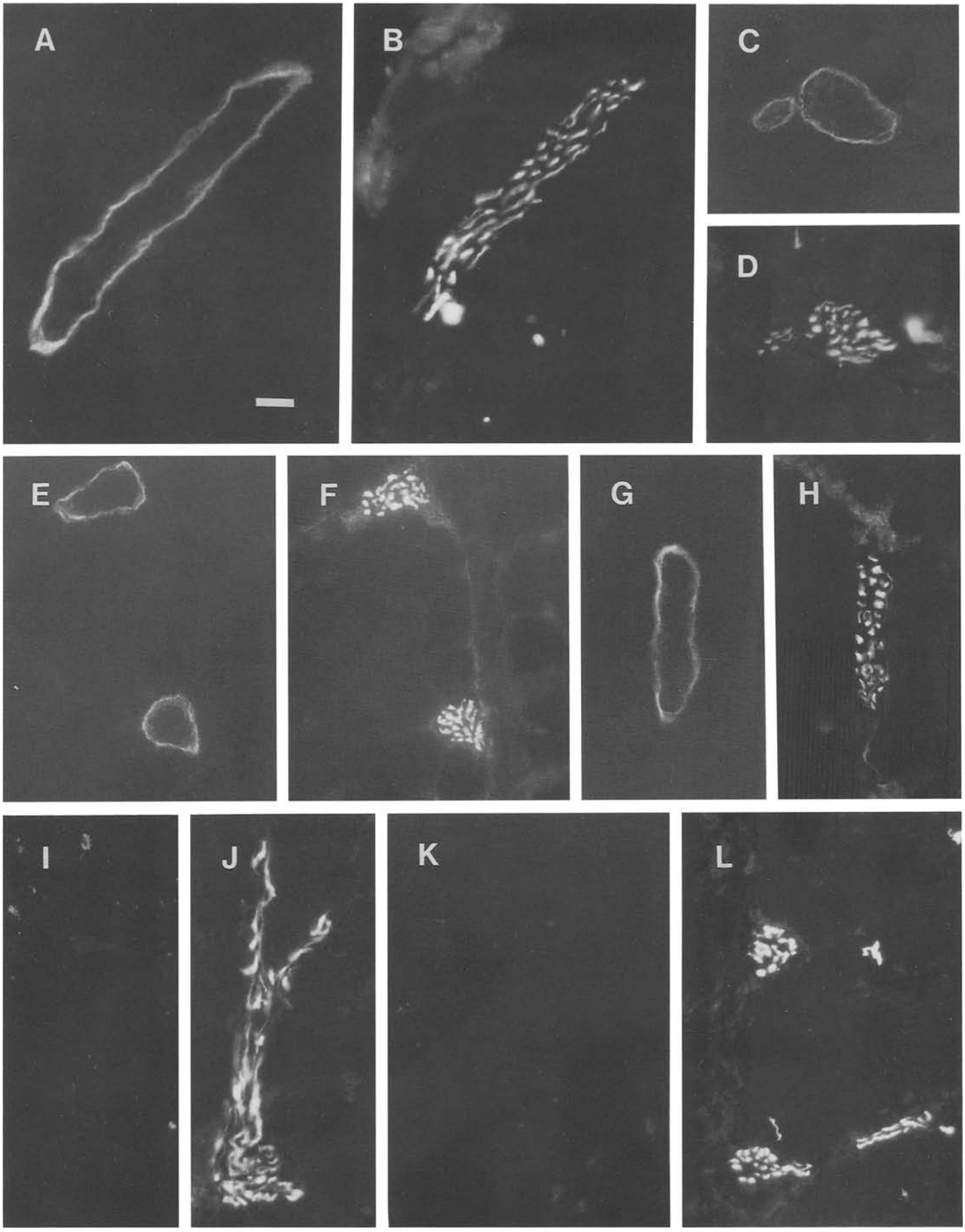

Fig. 1. Immunofluorescence staining of nerve bundles in rat diaphragm muscle. Sections of muscle were incubated with a mixture of anti-neurofilament and either anti-GT raised against purified human erythrocyte transporter (A, C, I) or anti-GT raised against the C-terminal peptide (E, G, K). In each pair of photographs, fluorescein fluorescence (A, C, E, G, I, K) shows the anti-GT staining, and rhodamine fluorescence (B, D, F, H, J, L) shows the anti-neurofilament staining. In two photographs (I, K), anti-GT was incubated with purified GT prior to application to the section. Scale bar: 50 μm.

terminal peptide should bind with equal affinity to the erythrocyte and sciatic nerve GTs. Consequently, on the assumption that the radioactivity in the GT band is roughly proportional to the amount of GT, an estimate of the amount of GT in the sciatic nerve sample can be made by comparison of the band in lane 5 with that in lane 6. On this basis, lane 5 contained 10 ng GT, and thus the GT constituted 0.07% of the protein in the sample.

Discussion

The cells at the periphery of the nerve fibres that were labelled with the anti-GT antibodies must be the perineurial cells. The only other possible assignment would be the epineurium, which is a loose condensation of connective tissue containing fibroblasts, adipocytes, mast cells and collagen bundles that surrounds the perineurium (Thomas & Olsson, 1984).

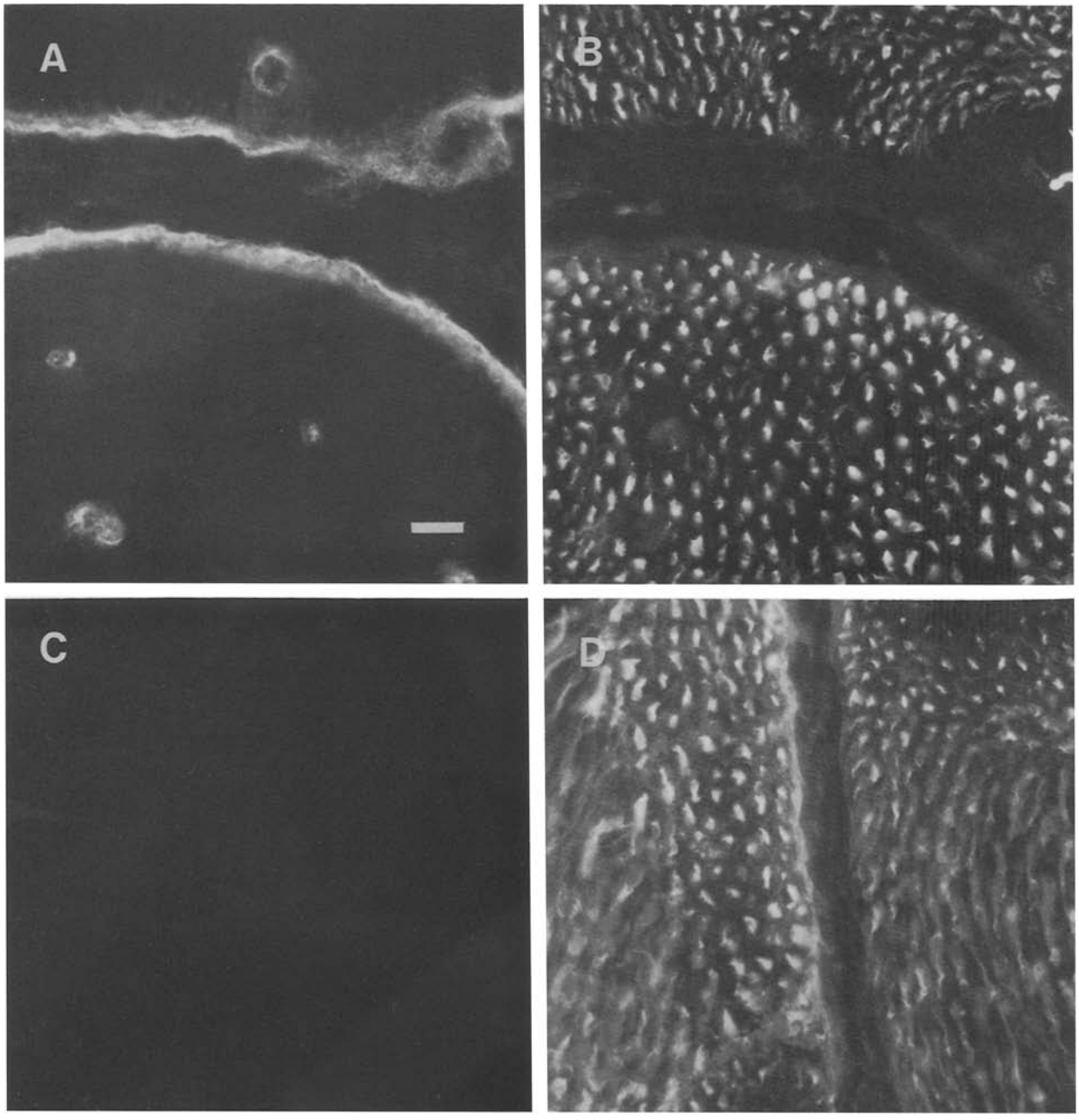

Fig. 2. Immunofluorescence staining of rat sciatic nerve. Sections of nerve were incubated with a mixture of anti-neurofilament and anti-GT against the C-terminal peptide. Fluorescein fluorescence (A, C) shows anti-GT staining, and rhodamine fluorescence (B, D) shows anti-neurofilament staining. In (C) anti-GT was incubated with purified GT prior to application to the section. Scale bar: 50 μm.

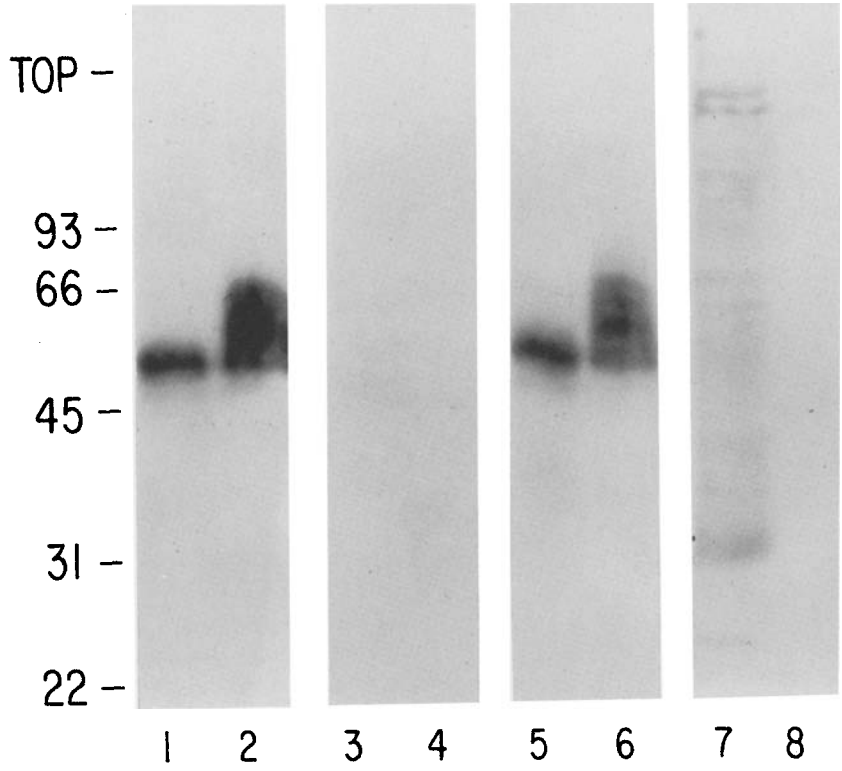

Fig. 3. Immunoblot of the GT in rat sciatic nerve. SDS samples of rat sciatic nerve (14 μg protein) and of purified human erythrocyte GT (20 ng protein) were run on alternate lanes of a single slab gel, along with molecular weight marker proteins. The polypeptides were transferred to nitrocellulose, and strips containing adjacent lanes were probed with the following antibodies, all at 0.3 μg ml^{-1}: lanes 1 and 2, anti-GT raised against purified GT; lanes 3 and 4, irrelevant purified rabbit IgG; lanes 5 and 6, anti-GT raised against the carboxy-terminal peptide; lanes 7 and 8, the latter antibodies mixed with 1 μg ml^{-1} GT carboxy-terminal peptide. In each case the sciatic nerve sample is on the left and the purified GT is on the right. The molecular weight standards were detected by staining the appropriate strips with India ink (Hancock & Tsang, 1983), and are given in thousands. After correction for the blank (about 500 cpm), the values for the radioactivity in the GT band were 1100, 4012, 1434 and 2180 for lanes 1, 2, 5 and 6, respectively.

However, this possibility is ruled out by the observation that there was no labelling of the epineurial region between fascicles of the sciatic nerve. In addition to the intense labelling of the perineurium, the sciatic nerve shows strong labelling of small circular structures within the fascicles. These are almost certainly blood vessels within the endoneurium, although at the level of resolution in this study we cannot decide whether the GT is located in the endothelial cells that actually line the vessels, in any pericytes that may surround the vessels, or both. Thus, the blood–nerve barrier is highly enriched with GT. The biochemical analysis supports this conclusion. The estimated amount of GT (0.07% of the total extracted protein) is relatively large. By comparison, human erythrocytes, in which the GT is very abundant and constitutes 5% of the plasma membrane protein, contain about 0.13% GT, expressed as a percentage of the total cellular protein (Allard & Lienhard, 1985).

Previous studies have shown that the cell membranes from cerebral microvessels have a high content of GT, as measured by the binding of the specific ligand, cytochalasin B (Dick *et al.*, 1984; Kasanicki *et al.*, 1986) Moreover, the molecular weight of the GT in these cells is 53 000 to 55 000. Thus, the blood–brain barrier and blood–nerve barrier are very similar with regard to the GT system.

Neither the muscle cells nor the axons exhibited significant immunofluorescence. Consequently, the density of GT in these cells is probably substantially lower. Studies have appeared describing: (i) the insulin-induced translocation of the GT in rat diaphragm muscle from an intracellular location to the plasma membrane, as measured by cytochalasin B binding to subcellular fractions (Wardzala & Jeanrenaud, 1981); (ii) the identification of the GT in skeletal muscle membranes as a polypeptide of $M_r \sim$ 50 000 by immunoblotting with the anti-GT anti-

bodies used here (Klip *et al.*, 1983); and (iii) the distribution of the GT between muscle plasma and transverse tubular membranes, as measured by cytochalasin B binding (Burdett *et al.*, 1987). Since bundles of nerve fibres are found throughout skeletal muscle, the possibility that a significant portion of the GT seen in these studies was derived from the cells of the blood–nerve barrier should be considered. Thus some of the conclusions of these studies about the properties of muscle cell GT may not be valid.

Finally, we note that antibodies against the GT may prove valuable as specific staining agents for the blood–nerve barrier.

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