Re-evaluation of the distributions of dystrophin and utrophin in sciatic nerve

Eric FABBRIZIO, Jacqueline LATOUCHE, François RIVIER, Gerald HUGON and Dominique MORNET*
INSERM U 300, Bâtiment K, Faculté de Pharmacie, Avenue Charles Flahault, 34060, Montpellier, France

Differential expression of proteins belonging to the dystrophin family was analysed in peripheral nerves. In agreement with previous reports, no full-size dystrophin was detectable, only Dp116, one of the short dystrophin products of the Duchenne muscular dystrophy (DMD) gene. We used specific monoclonal antibodies to fully investigate the presence of utrophin, a dystrophin homologue encoded by a gene located on chromosome 6q24. Evidence is presented here of the presence of two potential isoforms of full-length utrophin in different nerve structures, which may differ by alternative splicing of the 3′-terminal part of the utrophin gene according to the specificities of the monoclonal antibodies used. One full-length utrophin was co-localized with Dp116 in the sheath around each separate Schwann cell-axon unit, but the other utrophin isoform was found to be perineurium-specific. We also highlighted a potential 80 kDa utrophin-related protein. The utrophin distribution in peripheral nerves was re-evaluated and utrophin isoforms were detected at the protein level. This preliminary indication will require more concrete molecular evidence to confirm the presence of these two utrophin isoforms as well as the potential 80 kDa utrophin isoform, but the results strongly suggest that each isoform must have a specialized role and function within each specific nervous structure.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive muscular disorder caused by defects in the dystrophin gene. This gene encodes tissue-specific isoforms of dystrophin (a 427 kDa protein), which are generated by alternative splicing or differential promoter use [1–4]. Three short dystrophin products (Dps) of the DMD gene have also been identified and named Dp45 [5], Dp71 [6] and Dp116 [7]. Their transcripts were derived from the 3′ part of the DMD gene using two different internal promoters [6,7]. These short dystrophin products have lost the N-terminal actin-binding domain and most of the long spectrin-like rod region of dystrophin, but they retain the cysteine-rich and highly conserved C-terminal domains of dystrophin which mediate binding to the membrane glycoprotein complex [8]. Moreover, it has been shown that Dp71 is associated with the plasma membrane [9]. Dystrophin C-terminal domains are also homologous to those of utrophin, a dystrophin-related protein mainly localized at the neuromuscular junctions in skeletal muscle [10] and whose gene maps to locus 6q24 [11].

Previous studies have described Dp116 as a rare dystrophin product expressed at post-synaptic densities in cerebral cortex and cerebellum [12] but also present in peripheral nerves [7], while Dp71 is predominantly expressed in liver and brain [13]. The expression of both proteins and their corresponding mRNAs were recently compared [14]. Dp71 and Dp116 were expressed in newborn brains and different cell lines. Dp116 is located on a thin rim around the external myelinated sheath of peripheral nerves and immunological studies have revealed the co-localization of Dp116 and utrophin [15], including a specific perineurium localization for this latter molecule [16,17].

The function of the different Dps is unknown. They may be linked to the dystrophin-associated protein (DAP) complex or related DAP complexes in the membrane. Their expression may be developmentally regulated, with possible involvement in restricted functions of the full-length dystrophin molecule in certain tissues. This property was particularly well demonstrated by the overexpression of Dp71, which led to the recovery of normal membrane glycoprotein levels in dystrophin-deficient muscles but did not provide membrane resistance against degeneration [18,19].

In this study, we have gone further than previous published works in co-localizing Dp116 and utrophin within the peripheral rabbit sciatic nerve using a large panel of specific monoclonal antibodies (more than 15) classified according to six different specificities. The specificity of each of them was demonstrated by labelling the neighbouring muscle (when images were observed by immunofluorescence techniques), complemented by Western blot analysis of nerve-containing or nerveless crude muscle protein extracts. The possible roles of each detected protein are discussed with respect to their specific localizations.

MATERIALS AND METHODS

Preparation of crude extracts

Total extracts of rabbit skeletal muscles or dissected sciatic nerve were prepared from fresh tissues (about 0.1 g) as previously described [20]. Homogenates were centrifuged for 10 min at 8000 *g*. Supernatants were mixed with an equivalent volume of SDS buffer (5% SDS in Tris/HCl, pH 8, containing 50% glycerol, 0.1 mM 2-mercaptoethanol and 0.1% Bromophenol Blue) and the mixture was denatured for 5 min at 100 °C. Samples were separated on 3–9% SDS/polyacrylamide gradient gels.

Monoclonal antibody production

Monoclonal antibody 5F3 was raised against the last 31 residues of Dp71. Its production has been described previously [21].

Monoclonal antibodies targeted against dystrophin or utrophin were produced from two sets of selective fusion proteins expressing either dystrophin (H and C proteins containing residues 3357–3685 and residues 1173–1728 respectively) or

Abbreviations used: DMD, Duchenne muscular dystrophy; Dp, dystrophin product; DAP, dystrophin-associated protein.
* To whom correspondence should be addressed.

utrophin (K protein containing residues 3161–3388) parts expressed in *Escherichia coli*. Their characterizations have been reported previously [22–24].

Western blotting

After SDS/PAGE migration, the proteins were electrotransferred on to nitrocellulose sheets in the presence of 0.1% SDS in transfer buffer [25 mM Tris, 192 mM glycine and 20% (v/v) methanol]. Nitrocellulose sheets were processed normally using specific monoclonal antibodies and purified goat anti-(mouse IgG) coupled to alkaline phosphatase (1/5000, Jackson Immunoresearch Laboratories). During eletrophoretic migration, prestained SDS/PAGE protein markers (high-molecular-mass set from Bio-Rad) were always used as molecular mass standards.

Indirect cytochemical fluorescence labelling

Cryostat sections (10 μm) of unfixed rabbit muscles containing sciatic nerve or isolated nerve included in embedding medium (OCT; Miles) were treated for epifluorescence as described previously [20]. Sections were first labelled with either of the specific monoclonal antibodies and subsequently detected with fluorescein-conjugated goat anti-(mouse IgG) (1/50 from Jackson Immunoresearch Laboratories).

RESULTS

The specificities of the different monoclonal antibodies used in this study are summarized in Figure 1. Because of the strong sequence similarity of sequences of utrophin and both of the short dystrophin products (Dp71 and Dp116) with the C-terminal part of dystrophin, some monoclonal antibodies remained specific to dystrophin or utrophin but others revealed each of these four proteins. However, their use on serial cross-sections allowed clear identification of either one or the other of these

Table 1 Nerve distributions of the antibodies used

The specificity of each antibody in the nerve is underlined.

Antibodies	Molecule recognised	Schwann cell-axon unit	Perineurium
C5G5	Dystrophin	−	−
5F3	Dp71	−	−
H12G9/H2A12	Dystrophin, Dp71, Dp116	+	−
H4C10	Dystrophin, utrophin	+	−
H5A3	Dystrophin, utrophin, Dp71, Dp116	+	+
C4G10	Dystrophin, utrophin	+	+
K5B1	Utrophin	−	+

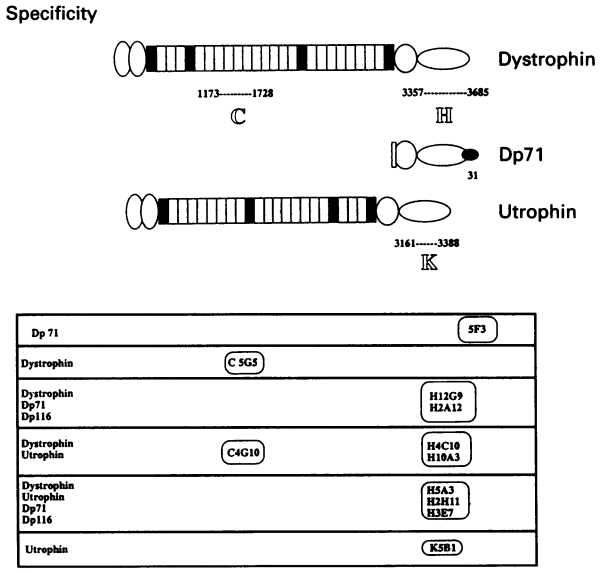

Figure 1 Schematic structures of dystrophin, utrophin and Dp71

Location of binding sites of the different monoclonal antibodies used in this study are shown, as previously described [20–24]. Their specificities for each protein belonging to the dystrophin family are given and analysed in this study within nerve structures. Their locations (C, H or K) and amino acid sequences in the diagram refer to antigenic recombinant proteins against which they were produced. For details see the Materials and methods section.

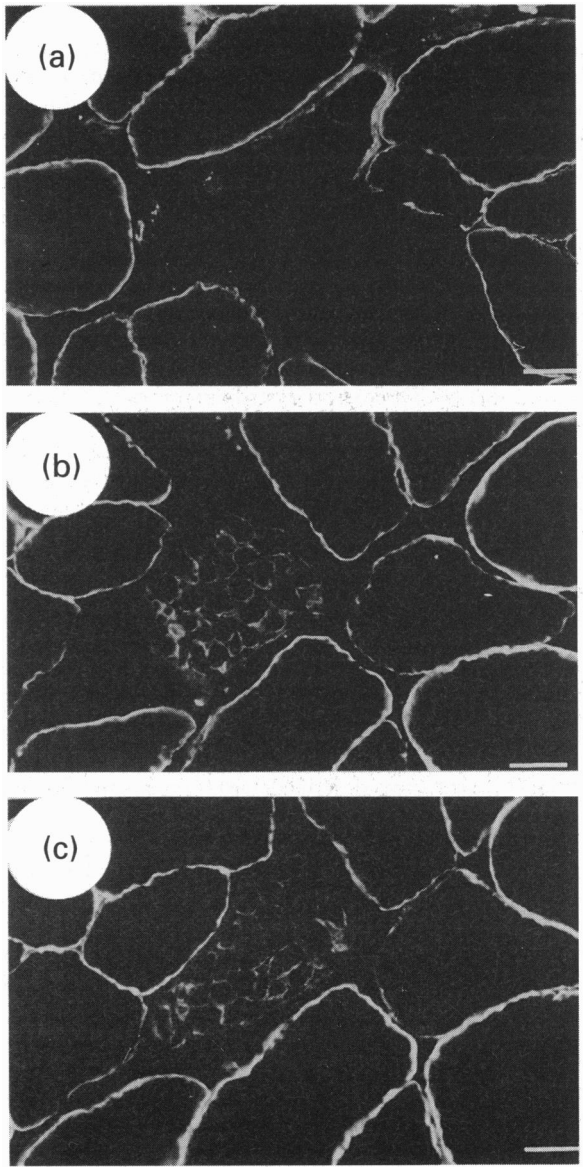

Figure 2 Immunodetection of proteins belonging to the dystrophin family

Rabbit skeletal muscle transverse cryostat sections were stained either with monoclonal dystrophin-specific antibodies C5G5 (**a**), H2A12 (or H12G9) in (**b**), or with monoclonal utrophin and dystrophin antibody H4C10 in (**c**). A fluorescein-labelled second antibody enabled detection of each protein at the periphery of the muscle cell membrane for all three antibodies, and also the sheath around each Schwann cell-axon unit in nerve with H2A12 or H4C10. Bar = 20 μm.

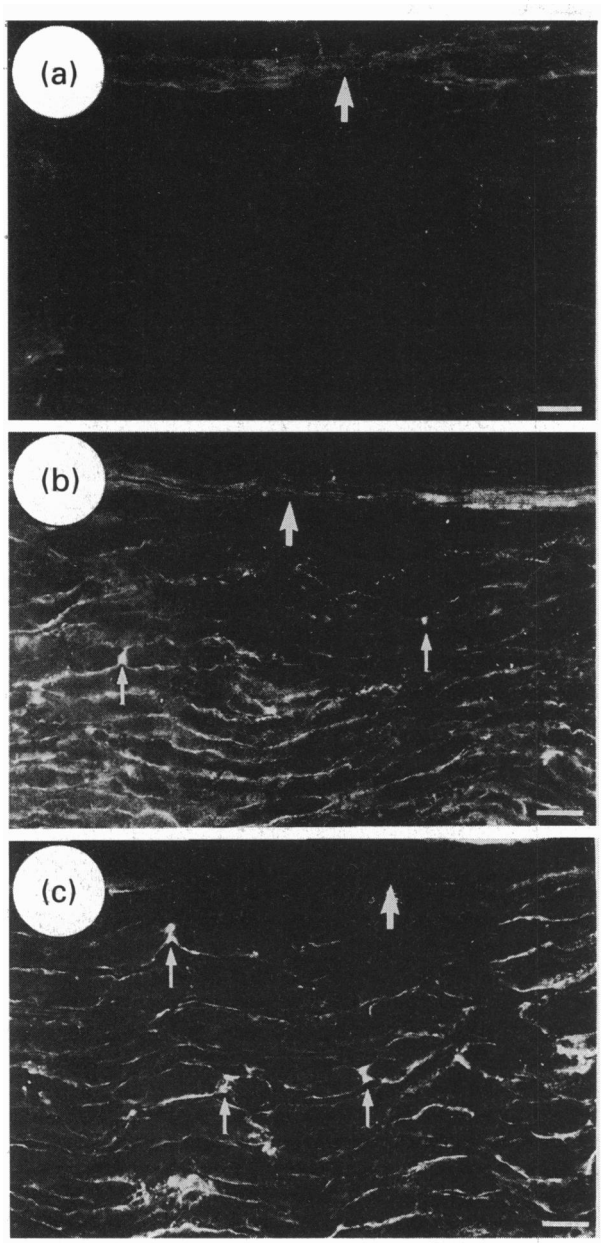

Figure 3 Immunofluorescent detection on longitudinal sections of isolated sciatic nerve

A panel of antibodies similar to that described in Figure 2 was used. (**a**) C5G5 gave no image and corresponded to the control, (**b**) H2A12 (or H12G9) and H4C10 antibodies stained the sheath around Schwann cells and Ranvier's nodes (thin arrows). Thick arrows indicate the absence of labelling in perineurium (**b** and **c**). Bar = 20 μm.

molecules within specific structures, as demonstrated in the following experiments.

Preliminary results in agreement with previous studies

Various antibodies were used on serial transverse cryostat cross-sections of skeletal muscles containing nerves. The specificity of each antibody is given in Table 1 and Figure 1. Positive and negative immunodetections are indicated by + or − respectively, and conclusions concerning the molecules detected in the nerve have been underlined (Table 1), with the following explanations.

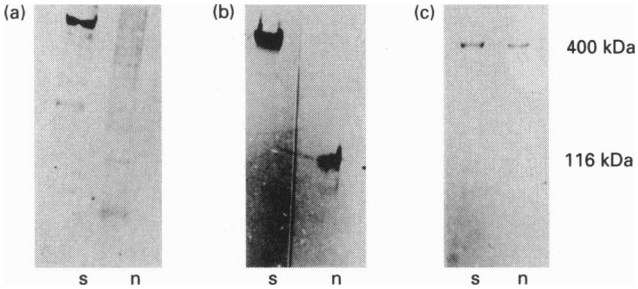

Figure 4 Comparative identification of proteins of the dystrophin family by Western blot

C5G5 antibody detected dystrophin in skeletal muscle (s) but not in nerve (n) extracts as shown in (**a**). H2A12 (or H12G9) antibodies are Dp116-specific in nerves and dystrophin-specific in skeletal muscle extracts (**b**). Only 400 kDa (no 116 kDa proteins) were revealed with H4C10 in (**c**), corresponding to utrophin in nerve extracts.

The C5G5 monoclonal antibody, raised against the dystrophin central rod domain, informed us that no full-length dystrophin was present in peripheral nerves (Figure 2a and Table 1), fully confirming previous reports in this field [7,15]. Use of the 5F3 monoclonal antibody also demonstrated the absence of Dp71 (Table 1 and results not shown). Monoclonal antibodies against the C-terminal domain of dystrophin and specific to this protein (e.g. H12G9 or H2A12), or also staining utrophin (e.g. H4C10), labelled the sheath around each separate Schwann cell-axon unit, as previously reported [7,15]. The same antibodies stained dystrophin on the sarcolemma of skeletal muscle fibres (Figures 2b and 2c).

Longitudinal sections of isolated sciatic nerve were observed to identify proteins stained with the different antibodies further. As shown in Figure 3, the same three monoclonal antibodies were applied. C5G5 antibody gave a clear negative image (Figure 3a), as was also obtained for the blank control (i.e. incubation with only the second fluorescent antibody). The H12G9 (or H2A12) and H4C10 antibodies clearly stained the outer contour of the nerve fibres, with intense immunoreactivity around Ranvier's nodes, and with no myelin lamellae labelling (Figures 3b and 3c).

At this point, we confirmed the types of proteins involved by immunoblot detection, illustrating the deduced monoclonal specificities underlined in Table 1 by Western blot analyses.

Comparative analysis using a similar panel of monoclonal antibodies was undertaken on nerve-containing and nerveless crude muscle extracts. Nerveless crude muscle extracts were analysed using C5G5 antibody (a dystrophin-specific monoclonal antibody as shown in Figure 4a). A 400 kDa protein band was detected, while no band was present in nerve extracts; this corroborates the immunofluorescence results. The dystrophin-specific monoclonal antibody H2A12 (or H12G9) stained a band with a molecular mass of 400 kDa in nerveless crude muscle extracts and only a protein of 116 kDa in nerve extracts (Figure 4b). These monoclonal antibodies therefore appeared to be Dp116-specific in peripheral nerves. The dystrophin/utrophin monoclonal antibody H4C10 reacted with a 400 kDa protein in muscle extracts (corresponding to full-length dystrophin and utrophin), as in nerve extracts (Figure 4c). In this latter tissue, the protein detected by H4C10 could only be utrophin, since dystrophin is absent in peripheral nerves, i.e. in accordance with our results using C5G5 and also with published results [7,15].

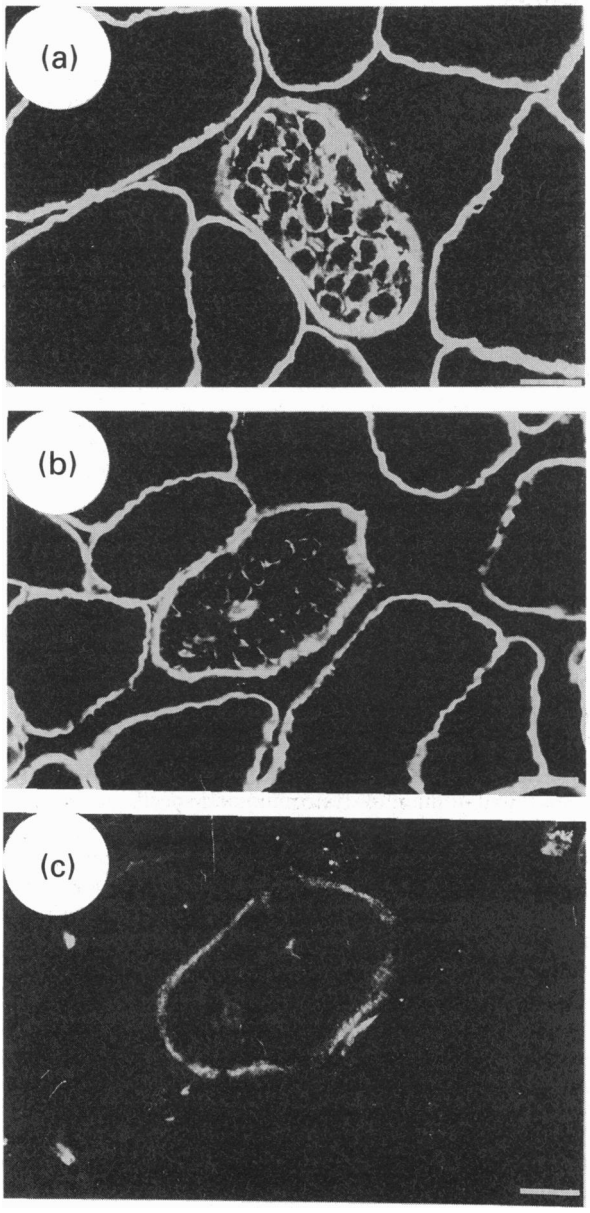

Figure 5 Immunodetection of proteins belonging to the dystrophin family

As in Figure 2, serial transverse cryostat muscle sections were analysed using another set of monoclonal antibodies. The universal H5A3 monoclonal antibody raised against a common epitope of all forms of the dystrophin family clearly labelled the sheath around each Schwann cell and the perineurium in (**a**). The dystrophin/utrophin-specific monoclonal antibody C4G10, unable to detect short-transcript product Dp116, stained the same structures but with less intensity around each Schwann cell. K5B1, a utrophin-specific monoclonal antibody, stained the perineurium, as shown in (**c**).

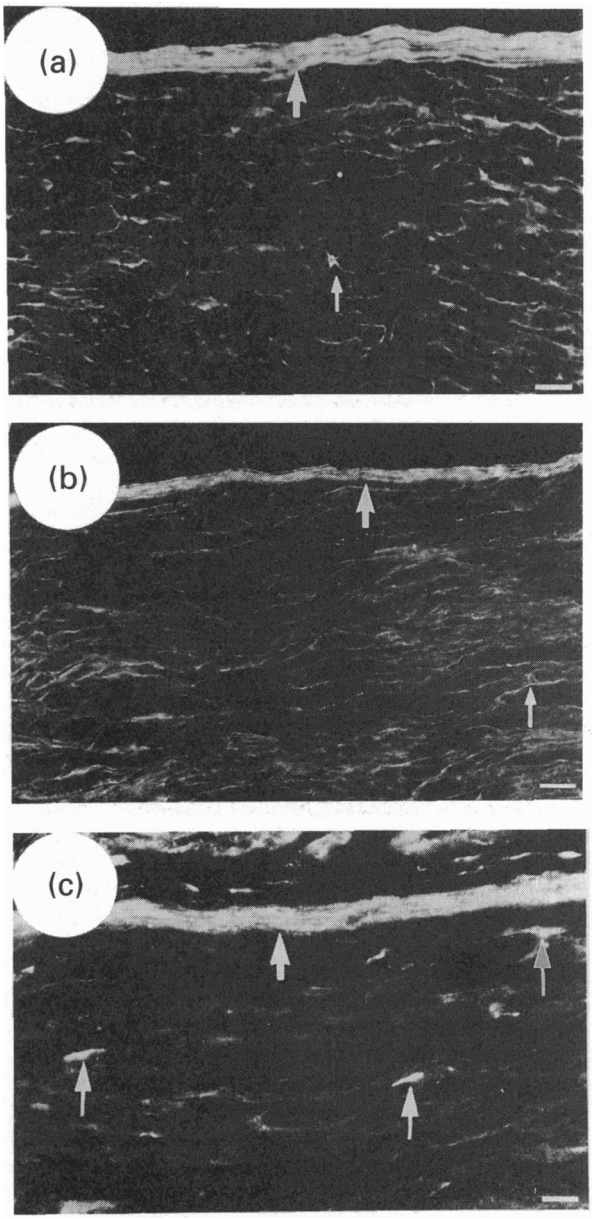

Figure 6 Immunofluorescent detection on longitudinal sections of isolated sciatic nerve

A panel of antibodies similar to that described in Figure 5 was used. (**a**) H5A3, (**b**) C4G10 and (**c**) K5B1. Thick arrows indicated perineurium labelling and thin arrows Ranvier's nodes in (**a**) and (**b**) or Schwann cells in (**c**).

New findings on utrophin distribution in peripheral nerves

New information was obtained through analyses performed using a larger panel of monoclonal antibodies with different specificities to those described above and elsewhere in the literature, i.e. on transverse cryostat cross-sections of skeletal muscles containing nerves (Figure 5) and longitudinal sections of isolated sciatic nerves (Figure 6).

With monoclonal antibody H5A3, raised against the C-terminal domain of dystrophin and utrophin, we revealed labelling of the sheath around each separate Schwann cell-axon unit, as previously observed, but also around the perineurium (Figures 5a and 6a). On the corresponding Western blots, H5A3 stained two bands: a 116 kDa protein, known to correspond to Dp116, and also a 400 kDa protein corresponding to utrophin in the nerve extracts (Figure 7a).

To determine which protein (Dp116 or utrophin) was localized in the perineurium, we used a monoclonal antibody (C4G10) raised against the central part of dystrophin (and utrophin), thus unable to detect short dystrophin forms such as Dp116. C4G10 antibody stained the same structure in rabbit nerve as H5A3

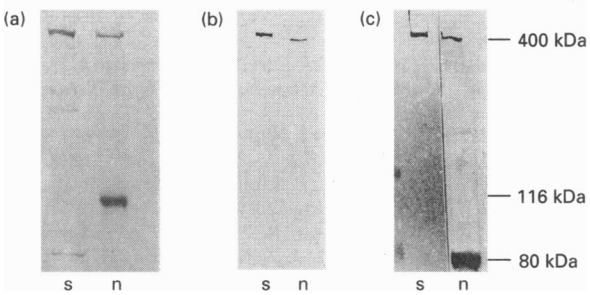

Figure 7 Comparative Western blot analysis of skeletal muscle and isolated nerve extracts

Skeletal (s) and nerve (n) extracts were incubated with H5A3 (**a**), C4G10 (**c**) and K5B1 (**b**) antibodies. Only with the latter was a new 80 kDa protein band detected in isolated nerve extracts, while the 400 kDa protein band was detected in all extracts and specifically corresponded to utrophin only in isolated nerve extracts. H5A3 detected Dp116 in isolated nerve extracts, corresponding to a dystrophin short-transcript specific to peripheral nerves.

(Figures 5b and 6b). In Western blots, C4G10 detected only a 400 kDa protein in nerve extracts (Figure 7b). The results clearly showed that two full-length utrophins must exist, one in the perineurium and one in the Schwann cell membrane, unlike Dp116, which was only found to be present in the Schwann cell membrane. The full-length utrophin present in Schwann cells, which was stained by H4C10, H5A3 and C4G10, must differ from the full-length utrophin detected in perineurium with H5A3 and C4G10 since H4C10 was negative for this structure. The above deduction was strengthened by the following result.

The perineurium and particular structures within the nerves were detected on transverse cryostat cross-sections of skeletal muscle containing nerves using a utrophin-specific monoclonal antibody K5B1 (Figure 5c). In longitudinal sciatic nerve sections, perineurium staining was intense and we noted other clearly labelled structures (Figure 6c). These could correspond to thicker regions in Schwann cells. In Western blots, K5B1 antibody (Figure 7c) showed a 400 kDa utrophin band and also a new 80 kDa protein band specific to the nerve extracts, which could be a potential utrophin short transcript product according to the antibody characteristics (Table 1 and [20]). K5B1 thus revealed a full-length utrophin localized in the perineurium, in agreement with results obtained with H5A3 and C4G10, but also a postulated short product of utrophin, which was localized within Schwann cells.

DISCUSSION

The functional significance of the short dystrophin forms Dp116 and Dp71 is not yet understood. Both proteins are expressed in most cases of DMD and they lack the actin-binding site and most of the long central spectrin-like domain of dystrophin. The functions of Dps may therefore be different and restricted to dystrophin C-terminal properties. The precise localizations of these proteins should be determined to obtain further information on the roles of Dps. Previous studies [7,15–17] and our present results confirmed the absence of dystrophin and Dp71 in adult sciatic nerves, whereas Dp116 was present. In this study, we showed that only the short dystrophin product Dp116 was present in the sheath of each Schwann cell, with staining in Ranvier's nodes but not in myelinated lamellae (in accordance with previous work [7]), and provided preliminary evidence of the presence of two full-length utrophin isoforms and a potential Up80 in peripheral nerves. One of the full-length isoforms was confined to the sheath around each Schwann cell in accordance with previous work [17,15], and the other only to the perineurium. According to the antibody specificities, these utrophin isoforms may differ in their C-terminal domains due to alternative splicing (as previously noted concerning the dystrophin gene [1]), although it was also suggested that such events might not occur in the 3′ part of the utrophin gene [25]. We also observed, at the protein level, a protein of molecular mass 80 kDa located within Schwann cells in peripheral nerves. This product may be a potential short utrophin isoform (which could be called Up80) and could correspond to a short utrophin transcript for which an mRNA was detected at 6.5 kb using a utrophin b3 probe in glioma cell lines [26]. In fact new utrophin short-transcript forms have recently been described, i.e. a 62 kDa tissue-specific isoform referred to a potential 'apo-utrophin' product in C6 glioma cells [17], or an autosomal homologue of dystrophin Dp116 (Up116) noted in developing sensory ganglia [27], and the potential Up80 described here could belong to this utrophin short-transcript family. Nevertheless this 80 kDa protein could be a potential short utrophin product according to this antibody specificity; however, since only one monoclonal antibody (K5B1, whose epitope belongs to a very specific region of the utrophin molecule) detected this protein, we cannot totally overrule the possibility of a spurious cross-reaction with some unrelated protein of this size. All of these results will have to be confirmed by PCR detection to obtain evidence on the existence of new utrophin mRNAs, thus further establishing more accurately these detections and identifications using antibodies.

Nevertheless, antibodies specific to dystrophin and utrophin, but not to Dp116, were then applied to sciatic nerve sections. Both monoclonal antibodies H4C10 and C4G10 only detected utrophin protein bands in nerve extracts (Figures 4c and 7b). However, H4C10 (directed against the C-terminal part of dystrophin) stained the sheath around each Schwann cell (Figures 2c and 3c), unlike C4G10 (directed against the central part of dystrophin) which provided clear labelling in Schwann cells and the perineurium (Figures 5b and 6b). This indicates the existence of a full-length utrophin isoform specific to the perineurium whose mRNA has exon(s) missing which encode the H4C10 epitope (absent also in Dp116), or that this particular sequence was methylated or glycosylated in all of these molecules, i.e. masking this particular epitope.

All of these proteins belonging to the dystrophin family must have specific roles since they are well distributed alone or co-localized in different regions of nerve structures. Dp116 was previously linked via α-dystroglycan with merosin in muscle or co-localized with the brain form of α-dystroglycan, a 120 kDa protein, in peripheral nerves and brain [28]. This indicates that Dp116 may be linked to membranes via a DAP or DAP-like complex. Interestingly, the 50 kDa adhalin is absent in peripheral nerves [15], and adhalin-deficient SCARMD (severe childhood autosomal recessive muscular dystrophy) patients do not suffer from neuropathic disease. In contrast, laminin M is missing in endoneurial basal lamina of intramuscular nerves of some FCMD (Fukuyama congenital muscular dystrophy) patients [29]. The usual mental retardation rate in DMD populations is 30% [30].

However, point mutations were recently described between exons 60 and 74 of the DMD gene in five out of seven DMD patients suffering from mental retardation [31]. In this region, both Dp116 and Dp71 transcripts could be affected and may implicate both of the corresponding proteins in mental retardation. The absence or alteration of these proteins could lead to disruption of local specific structures, lowering nerve influx, which cannot progress from one node to another as easily as in normal nerves and neurons at the synaptic level. As Ranvier's

nodes are known to accelerate propagation of nerve influxes along the whole length of nerves, Dp116 and its related protein complex (mainly α-dystroglycan linked to merosin) could help stabilize structures, thus improving important ionic exchanges in this region. Moreover, elevated intracellular calcium concentrations have been described in skeletal muscles of *mdx* mice and DMD patients [32]. In nerves or neurons of the central nervous system, these high calcium levels could also perturb normal ionic exchange of cells, possibly potentiating mental retardation in the absence of Dp116.

The overall results indicated that localization of all dystrophin family products in peripheral nerves is difficult to evaluate without a large panel of monoclonal antibodies with various specificities. In addition, with the complete absence of dystrophin and Dp71 in this particular structure, it is still difficult to clearly determine which glycoproteins are associated with Dp116 or any utrophin products without careful biochemical analysis of the proteins co-purified from each specific isolated nerve substructure.

Considering all of the different members of the extending dystrophin family present in peripheral nerves and their specific roles, it would be interesting to determine whether specific forms of laminin are co-localized with specific members of the dystrophin family. Merosin is co-localized with Dp116 [28,33] and laminin A specific for epineurium [28]. According to the present study, Dp116 and one full-length utrophin isoform were present in the sheath around each individual Schwann cell, while the other full-length utrophin was found in the perineurium. Moreover, a potential Up80 was found in Schwann cells. Hence, merosin may indirectly link Dp116 and one form of utrophin, and laminins A or S may link the other utrophin form and potential Up80 in thicker regions of Schwann cells, according to the laminin isoform distribution present in the nerve [34]. However, these possibilities need to be fully investigated with respect to utrophin products. As merosin is absent in *dy* mice which suffer from neuropathical muscular diseases [35], it would be of great interest to determine which members of the dystrophin family indirectly bind merosin and to define the influence of their C-terminal parts on the binding of dystroglycan to merosin.

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