Presence of long and short dystrophin and/or utrophin products in *Torpedo marmorata* peripheral nerves

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Abstract Peripheral nerves from rabbit and *Torpedo marmorata* were comparatively analyzed for the presence of short dystrophin products. Western blot analyses of *Torpedo marmorata* peripheral nerve extracts revealed the existence of three proteins belonging to the dystrophin family: a M_r 400 kDa protein band detected with dystrophin/utrophin, dystrophin-specific and *Torpedo* utrophin-specific antibodies, a molecule identified as Dp116 and, for the first time at the protein level, a new protein probably corresponding to Up116. All of these products were carefully identified according to the specificities of the monoclonal antibodies used. In immunofluorescence studies, clear staining of the thin rim surrounding each Schwann cell-axon unit was observed in both *Torpedo marmorata* and rabbit peripheral nerves, showing colocalization of all of these molecules. Their potential functions were discussed in comparison to similar products found in rabbit peripheral nerves.

Key words: Duchenne muscular dystrophy; Dystrophin; Dp116; Up116; Peripheral nerve; *Torpedo marmorata*

1. Introduction

Proteins of M_r 400 kDa, corresponding to dystrophin and dystrophin-related protein isoforms, have been detected in acetyl choline receptor-rich membranes of *Torpedo californica* [1] and *Torpedo marmorata* electrocytes [2–4]. This tissue is a syncytium that is embryologically derived from skeletal muscle stem cells [5]. This electric organ thus provides a new and distinct source of dystrophin and dystrophin-related protein which were previously available in skeletal muscles.

The mammalian dystrophin gene, mapped on Xp21 [6], encodes tissue-specific isoforms of dystrophin generated either by alternative promotors or differential splicing [7–10]. In addition to the full-length 400 kDa dystrophin, four short dystrophin products (Dps) called Dp71 [11], Dp116 [12], Dp140 [13,14] and Dp260 [13,15] have been identified. Their transcripts are derived from the 3′ part of the DMD gene using four different internal promoters [11–15]. 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 cystein-rich and highly conserved C-terminal domains of

dystrophin (for review see refs. [16,17]). These two terminal domains interact with a dystrophin associated protein complex (DAPC) to form a link between the cytoskeleton and the extracellular matrix [18–20]. Dystroglycan is at the center of this link with a trans-sarcolemmal glycoprotein, β-dystroglycan, which bind the cysteine-rich domain of dystrophin and a sub-sarcolemmal glycoprotein, α-dystroglycan, binding laminin [21]. The dystrophin-related protein (also named utrophin), which is 80% homologous with dystrophin but encoded by a gene mapped on 6q24 [22], has the same link with this protein complex but its main localization is at the neuromuscular junction in skeletal muscle [23]. According to recent studies [24–27], dystroglycan localized with utrophin in this structure could be an agrin receptor and would thus play a key role in synapse formation via acetyl choline receptor clustering. *Torpedo* electrocytes were the first tissues in which interactions between agrin and dystroglycan were tentatively identified [28]. The electric organ and its innervation with electrocyte nerves provide a giant model for neuromuscular junction formation and exchanges between peripheral motor nerves and their effectors.

We describe for the first time, in reference to previous data from mammal peripheral nerves [12,29,30], the presence and distribution of short dystrophin family products in peripheral nerves from *Torpedo marmorata* electrocytes. A panel of monoclonal antibodies previously characterized in human skeletal muscles [4,31] was used with two complementary techniques: Western blot identification and immunofluorescence detection.

2. Materials and methods

2.1. Preparation of crude extracts

Fresh *Torpedo marmorata* were collected alive and electric organs and electrocyte peripheral nerves were immediately dissected after death and homogenized with extraction buffer (2 times for 30 s) in a Waring blender. Extraction buffer (0.05 M Tris HCl, pH 9.0) was freshly made with extemparaneous addition of a cocktail of protease inhibitors (10 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml of soybean trypsin inhibitor, 5 μl/ml of leupeptin) in the presence of 1% Triton X-100. The different mixtures were centrifuged for 10 min at 10,000 × g and then the supernatant was boiled for 5 min in the presence of an equal amount of loading buffer. All samples were then analyzed by the SDS-PAGE technique followed by Western blot immunodetection. The same protocol was used for fresh dissected rabbit sciatic nerve.

2.2. Monoclonal antibody production

Two sets of monoclonal antibodies were produced from selective recombinant proteins expressing either C-terminal chicken dystrophin (H protein with residues 3357 to 3660) or central chicken dystrophin (C protein with residues 1173 to 1728) fragments in *E. coli*. Their specificities were previously characterized [30,31]. Monoclonal antibodies used in this study were also *Torpedo* utrophin-specific and previously shown to be able to detect neuromuscular junctions and vessels in human skeletic muscles [4].

2.3. Immunoblot analysis

All samples were applied on 0.75 mm SDS-polyacrylamide 8% gels with stacking gel. After SDS-PAGE migration, the proteins were electrotransferred onto nitrocellulose sheets in the presence of 0.1% SDS in the transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol). Nitrocellulose sheets were processed normally using the panel of specific monoclonal antibodies presented in Fig. 1 and purified goat anti-mouse IgG coupled to alkaline phosphatase (1/5000, Jackson ImmunoResearch Laboratory). The following proteins were always used as molecular weight markers: myosin (199 kDa), β-galactosidase (120 kDa), bovine serum albumin (87 kDa) and ovalbumin (48 kDa).

2.4. Immunofluorescence detection

The panel of monoclonal antibodies used, were applied on serial 10 μm transverse and longitudinal cryostat sections of electrocyte nerves from Torpedo marmorata and on serial 10 μm transverse and longitudinal cryostat sections of rabbit sciatic nerves. Specific labelling of each antibody on sections were detected with fluoresceine-conjugated goat anti-mouse IgG (1/100, from Sigma Immuno Chemical).

3. Results

3.1. Comparative Western blot analyses of Torpedo and rabbit nerve extracts

With monoclonal antibodies H12G9, H2A12 and H4B9, which detected only the C-terminal domain of dystrophin (and not utrophin), we revealed, as expected [2–4], a 400 kDa protein band in electrocyte extracts corresponding to Torpedo marmorata dystrophin (Fig. 1A, lane 2). The same panel of antibodies stained a unique protein band corresponding to Dp116 in rabbit sciatic nerves (Fig. 1A, lane 4), as expected [30], and two protein bands in Torpedo electrocyte nerves: a single 116 kDa band and a 400 kDa protein band (Fig. 1A, lane 3).

Monoclonal antibodies H'5A3, H'4A1, H'4C10 and H'2H11 directed against the highly conserved C-terminal part of dystrophin/utrophin revealed a unique 400 kDa protein band in electrocyte extracts (Fig. 1B, lane 2), corresponding to Torpedo marmorata dystrophin and Torpedo marmorata dystrophin-related protein, in agreement with previous studies [2–4]. In rab-

bit sciatic nerve extracts, these antibodies stained a 400 kDa band corresponding to utrophin, and a 116 kDa single band corresponding to Dp116 (Fig. 1B, lane 4), in agreement with previous studies [12,29,30]. The same monoclonal antibodies identified three proteins in Torpedo electrocyte nerve extracts, a 400 kDa protein band and two M_r 116 kDa proteins migrating as a doublet band (Fig. 1B, lane 3).

The presence of a 400 kDa protein band detected in electrocyte nerve extracts with antibodies such as H12G9 (unable to detect utrophin) was confirmed with monoclonal antibody C5G5 (which is raised against the central part of dystrophin) (Fig. 1C, lane 3). This antibody did not detect any proteins in rabbit sciatic nerve extracts (Fig. 1C, lane 4) as expected [30]. In electrocytes, antibody C5G5 only stained a 400 kDa protein band corresponding to Torpedo marmorata dystrophin, as expected [2–4] (Fig. 1C, lane 2).

Monoclonal antibody C4G10, directed against the central part of dystrophin/utrophin and which was inefficient in identifying Dps, only stained a 400 kDa protein band in all extracts: electrocytes (Fig. 1D, lane 2) as well as electrocyte nerves (Fig. 1D, lane 3) and rabbit sciatic nerves (Fig. 1D, lane 4). This 400 kDa rabbit protein corresponded to utrophin, in agreement with the protein content in rabbit sciatic nerve extracts [12,29,30].

Among the six Torpedo monoclonal antibodies which were previously characterized in our laboratory [4], T2C8 (Torpedo utrophin-specific) clearly revealed presence of 400 kDa protein band in all extracts, i.e. electrocytes as well as Torpedo and rabbit nerves, and the new 116 kDa band was only detected in Torpedo nerve extracts (Fig. 2).

3.2. Colocalization of dystrophin family proteins in Torpedo electrocyte nerves

The specific monoclonal antibodies H12G9 (as well as H2A12 and H4B9), was used on transverse cryostat cross-section of Torpedo electrocyte nerves, we observed clear la-
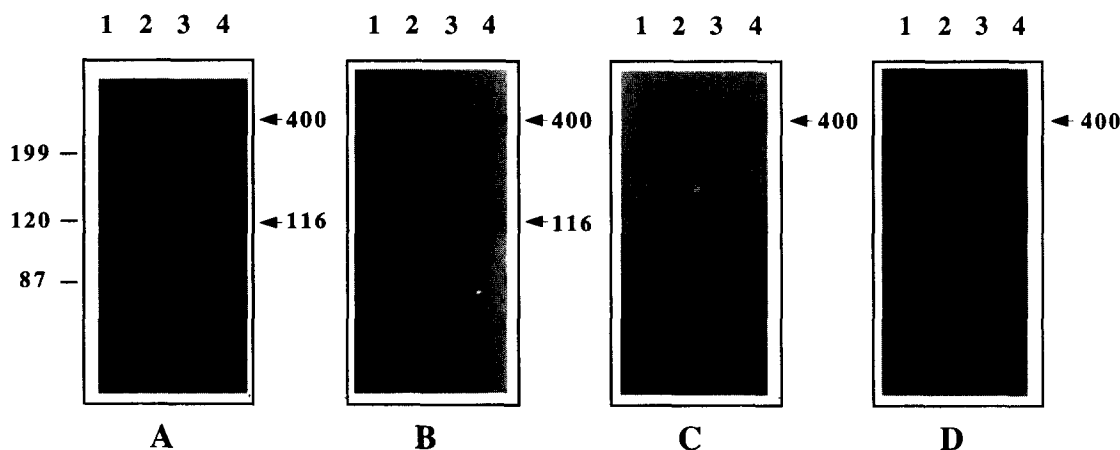

Fig. 1. Comparative Western blot analyses with monoclonal antibodies of various specificities. Lane 1 corresponds to molecular weight markers: myosin (199 kDa), β-galactosidase (120 kDa), bovine serum albumin (87 kDa) and ovalbumin (48 kDa). Different crude protein extracts: Torpedo electrocytes (lane 2), Torpedo electrocyte nerves (lane 3), rabbit sciatic nerves (lane 4) were separated by SDS-PAGE and transferred to nitrocellulose. Nitrocellulose strips were labelled with specific antibodies, H12G9 (A), H'5A3 (B), C5G5 (C), or C4G10 (D) and visualized with alkaline phosphatase-conjugated secondary antibody. Panel A: the dystrophin antibody H12G9 specifically identified the short dystrophin isoform Dp116 in rabbit nerves, unlike in Torpedo nerves where H12G9 stained both a 400 kDa protein band and a single 116 kDa protein band. Panel B: with H'5A3 we identified a 400 kDa protein band in both nerves from Torpedo marmorata and rabbit, but also a single band corresponding to Dp116 in rabbit nerve, while two proteins were detected in Torpedo marmorata nerves corresponding to Dp116 and Up116. Panel C: with the dystrophin specific monoclonal antibody C5G5, we only identified a 400 kDa protein band in electrocytes and nerves from Torpedo marmorata without any staining in rabbit nerve extracts. Panel D: in all extracts, the dystrophin/utrophin monoclonal antibody C4G10 stained a unique M_r 400 kDa protein band.
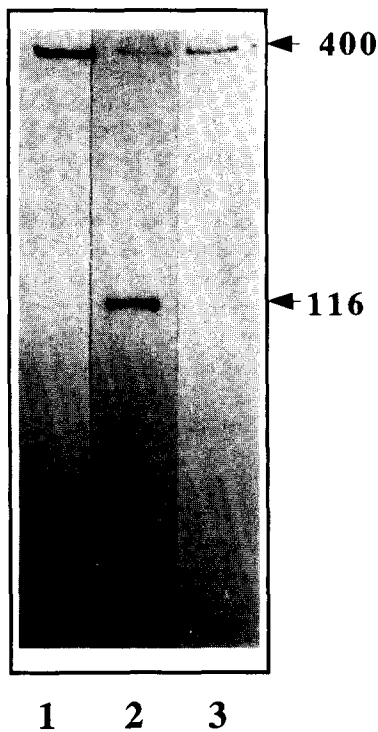
Fig. 2. Western Blot analysis with *Torpedo* utrophin-specific antibody. The same crude extracts tas in Fig. 1 were analyzed with *Torpedo* utrophin-specific antibody. Lane 1 correponds to *Torpedo* electrocytes, lane 2 to *Torpedo* electrocyte nerves and lane 3 to rabbit sciatic nerves. Nitrocellulose strips were treated with monoclonal antibody T2C8 which was previously characterized [4]. The utrophin protein band (400 kDa) was revealed in all lanes and the new 116 kDa band was detected in lane 2.

belling of the sheath surrounding each Schwann cell-axon unit. Images obtained on longitudinal sections of electrocyte nerves with this antibody showed staining of the outer contour of the nerve fibers, constricting at the Ranvier's nodes without myelin lamellae labelling. The same staining was observed in rabbit sciatic nerves with these antibodies, in agreement with previous studies [12,29,30], on transverse and longitudinal sections.

The same nerve structure was labelled with the panel of monoclonal antibodies directed against the C-terminal part of dystrophin/utrophin: H'543, H'4A1, H'4C10 and H'2H11, corresponding to the Schwann sheath with clear staining at the Ranvier's nodes in longitudinal sections when applied to either *Torpedo* electrocyte nerves (Fig. 3A,C), or rabbit sciatic nerves (Fig 3B,D). Similar results were obtained with *Torpedo* utrophin-specific antibodies.

The monoclonal antibodies C5G5, or C4G10, gave us the same results as H12G9 or H'5A3 on transverse and longitudinal sections of *Torpedo* electrocyte nerves. Only C4G10 labelled the same structure in rabbit sciatic nerve sections, whereas C5G5 did not stain anything.

4. Discussion

Torpedo dystrophin shares 60–70% amino acid sequence identity with the central rod domain and 90% homology with the C-terminal regions of human dystrophin [32]. Seven monoclonal antibodies used in this study, which were previously characterized [30,31], were raised against this high interspecies

homologous C-terminal part of dystrophin and their high specificity was certified both in mammals and *Torpedo* tissues.

In electric organ from *Torpedo marmorata*, all of these antibodies labelled the innervated membrane (colocalization with the staining of acetyl-choline receptors by α-bungarotoxine) (data not shown) and always recognized, in crude electrocyte extracts, a unique 400 kDa protein band corresponding to *Torpedo* dystrophin and *Torpedo* dystrophin-related protein, according to antibody specificities and previous studies [1–4]. We identified, as in electric organ with the same panel of monoclonal antibodies, a 400 kDa protein band in *Torpedo marmorata* electrocyte nerves. This 400 kDa protein band was revealed with dystrophin/utrophin and dystrophin specific monoclonal antibodies, it thus corresponded to *Torpedo* dystrophin and perhaps also to *Torpedo* dystrophin-related protein [3,4].

In contrast to electrocytes, we identified two potential short dystrophin family products in *Torpedo* nerves (comparatively to rabbit nerves where only Dp116 is described). To our knowledge, this is the first time that short dystrophin family products have been identified in such an ancestral animal, and this is of particular interest. Indeed, phylogenetic studies often propose a model to understand the development stages in mammals and humans. The 116 kDa bands recognized as doublets with dystrophin/utrophin antibodies but as singletons with dystrophin antibodies unable to detect utrophin certainly corresponded to different proteins. The slowest migration of these two 116 kDa protein bands could correspond to a short *Torpedo* dystrophin isoform that we assume to be named Dp116. In fact, this 116 kDa *Torpedo* protein band comigrates with rabbit Dp116 and H12G9 is specific to this single protein in rabbit nerves [30]. The faster migrating 116 kDa band could correspond to a short C-terminal isoform of *Torpedo* dystrophin-related protein (utrophin) according to its detection obtained with monoclonal antibodies directed against the C-terminal part of dystrophin/utrophin but also with *Torpedo* utrophin-specific antibodies. In a recent paper, Blake et al. [33] identified a homologue of the short dystrophin transcript Dp116 in mouse brain, but proceeding from the utrophin gene. Then we assumed that the faster 116 kDa *Torpedo* protein band could correspond to this new utrophin transcript, which we propose to call Up116. However, in the absence of cDNA identification of this new protein, the following hypothesis cannot be totally excluded to explain this new 116 kDa protein band: it could be a different post-translational modification of the slowest 116 kDa protein, e.g. a loss of glycosylation or phosphorylation which would prevent its detection with any dystrophin monoclonal antibodies.

Full-length *Torpedo* dystrophin and 116 kDa proteins were all colocalized by immunofluorescence studies in a thin rim surrounding each *Torpedo* nerve fiber sheath corresponding to Schwann cells as shown in Fig. 3 and schematically summerized in Fig. 4. Utrophin and Dp116 were previously identified in the same structure of mammalian peripheral nerves [12,29,30]. These 400 kDa products of the dystrophin family and specific short M_r 116 kDa isoforms of the same protein family undoubtedly have a key role in peripheral nerves, probably in myelinogenesis and propagation of nerve impulses, according to their high phylogenetic conservation within particular tissues and their cell expression. As in skeletal muscle, dystroglycan and the extracellular matrix protein merosin are present in mammalian peripheral nerves. Dystroglycan has been localized in the same
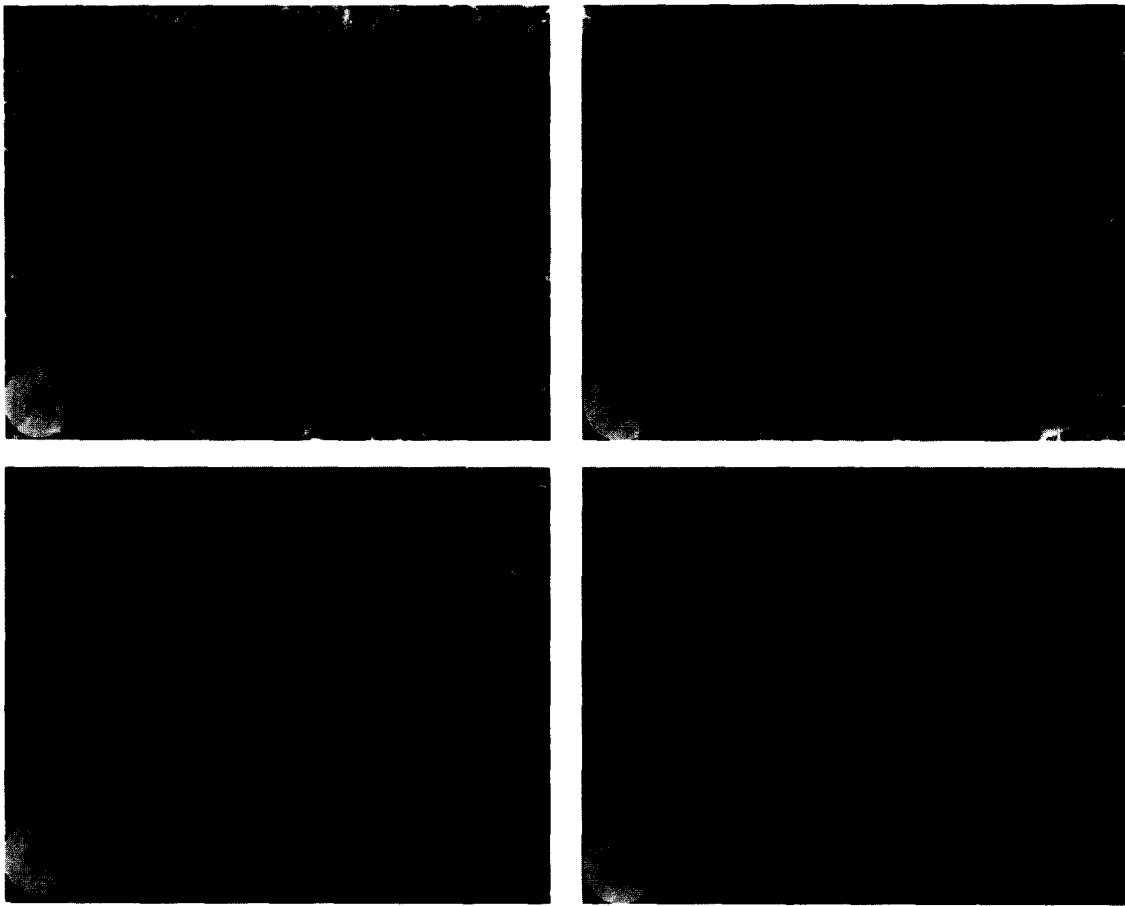
Fig. 3. Comparative immunodetection in *Torpedo marmorata* electrocyte nerves versus rabbit sciatic nerves. A fluoresceine-conjugated anti-mouse IgG enabled us to reveal the H'5A3 dystrophin/utrophin-specific detection. On transverse 10 mm cryostat cross-sections of *Torpedo* (A) and rabbit (B) nerves, H'5A3 labelled a thin rim around each nerve fiber. The same antibody clearly stained this particular structure on longitudinal 10 mm sections of *Torpedo* (C) and rabbit (D) nerves. White arrows point to the intense staining observed in the Ranvier's nodes. Similar images were obtained using H12G9 dystrophin-specific monoclonal antibody. Bar = 25 μm.

structure as utrophin and Dp116, e.g. the Schwann sheath [34], and could permit a link between utrophin/Dp116 and merosin in mammalian peripheral nerves. Peripheral nerve dysmyelination noted in merosin deficient dystrophic dy mouse [35] suggests a putative utrophin and Dp116 role in peripheral nerves,
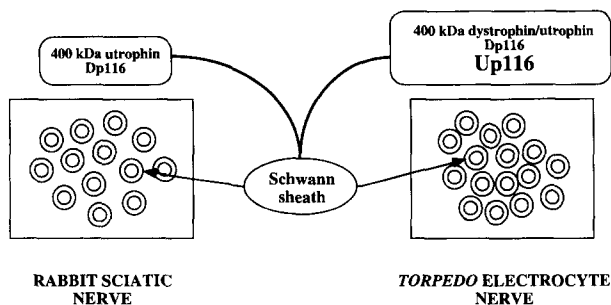

Fig. 4. Schematic representation of the dystrophin/utrophin localization in nerves from rabbit and *Torpedo marmorata*. All proteins detected in this study were colocalized in the sheath surrounding each Schwann cell-axon unit but their identity was not the same in rabbit (presence of only utrophin and Dp116 molecules) and in *Torpedo marmorata* nerves (presence of three proteins belonging to the dystrophin superfamily: a dystrophin/utrophin long transcript of M_r 400 kDa, and two short transcripts of M_r 116 kDa corresponding to a dystrophin product (Dp116) and a new utrophin product (Up 116).

as previously proposed. *Torpedo* electrocyte nerves must synthesize a high quantity of agrin to control AChR clustering on the innervated membrane of the electric organ. We assumed that the new *Torpedo* Up116 could, via dystoglycan, be a special agrin receptor, regulating agrin concentrations in electrocytes. It is also possible that both long and short utrophin products are linked to various agrin isoforms in *Torpedo* nerves. These proteins thus may have a determining role in synapse construction in *Torpedo*. It would be interesting to investigate whether this new utrophin short isoform (Up116) is present during the developmental stages in mammal nerves and to determine its putative role in synaptogenesis.

References

[1] Chang, H.W., Bock, E. and Bonilla, E. (1989) J. Biol. Chem. 264, 20831-20834.
[2] Jasmin, B.J., Cartaud, A., Ludosky, M.A., Changeux, J.-P. and Cartaud, J. (1990) Proc. Natl. Acad. Sci. USA 87, 3938-3941.

[3] Cartaud, A., Ludosky, M.A., Tomé, F.M.S., Collin, H., Stetzkow-ski-Marden, F., Khurana, T.S., Kunkel, L.M., Fardeau, M., Changeux, J.-P. and Cartaud, J. (1992) Neuroscience 48, 995–1003.

[4] Fabbrizio, E., Leger, J., Leger, J.J. and Mornet, D. (1993) Neurosci. Lett. 155, 51–56.

[5] Fox, G.Q. and Richardson, G.P. (1978) J. Comp. Neurol. 179, 677–698.

[6] Koenig, M., Hoffman, E.P., Bertelson, C.J., Monaco, A.P., Feener, C. and Kunkel, L.M. (1987) Cell 50, 509–517.

[7] Feener, C.A., Koenig, M. and Kunkel, L.M. (1989) Nature 338, 509–511.

[8] Nudel, U., Zuk, D., Einat, P., Zeelon, E., Levy, Z., Neuman, S. and Yaffe, D. (1989) Nature 337, 76–78.

[9] Klamut, H.J., Gangopadhyay, S.B., Worton, R.G. and Ray, P.N. (1990) Mol. Cell. Biol. 10, 193–205.

[10] Gòrecki, D.C., Monaco, A.P., Derry, J.M.J., Walker, A.P., Barnard, E.A. and Barnard, P.J. (1992) Hum. Mol. Genet. 1, 505–51.

[11] Bar, S., Barnea, E., Levy, Z., Neuman, S., Yaffe, D. and Nudel, U. (1990) Biochem. J. 272, 557–560.

[12] Byers, T.J., Lidov, H.G.W. and Kunkel, L.M. (1993) Nature Genet. 4, 77–81

[13] Bloom, T. (1995) Curr. Biol. 5, 338–341.

[14] Lidov, H.G.W., Selig, S., Kunkel, L.M. (1995) Hum. Mol. Genet. 4, 329–335.

[15] D'Souza, V.N., Man, N.T., Morris, G.E., Karges, W., Pillers, D.A.M. and Ray, P.N. (1995) Hum. Mol. Genet. 4, 837–842.

[16] Ahn, A. and Kunkel, L.M. (1993) Nature Genet. 3, 283–291.

[17] Fabbrizio, E., Pons, F., Robert, A., Hugon, G., Bonet-Kerrache, A. and Mornet, D. (1994) J. Muscle Res. Cell Motil. 15, 595–606.

[18] Suzuki, A., Yoshida, M., Hayashi, K., Mizuno, Y., Hagiwara, Y. and Ozawa, E. (1994) Eur. J. Biochem. 220, 283–292.

[19] Tinsley, J.M., Blake, D.J., Zuellig, R.A. and Davies, K.E. (1994) Proc. Natl. Acad. Sci. USA 91, 8307–8313.

[20] Ibraghimov-Beskrovnaya, O., Ervasti, J.M., Leveille, C.J., Slaughter, C.A., Sernett, S.W. and Campbell, K.P. (1992) Nature 355, 696–702.

[21] Ervasti, J.M. and Campbell, K.P. (1993) J. Cell. Biol. 122, 809–823.

[22] Love, D.R., Hill, D.F., Dickson, G., Spurr, N.K., Byth, B.C., Marsden, R.F., Walsh, F.S., Edwards, Y.H. and Davies, K.E. (1989) Nature 339, 55–58.

[23] Pons, F., Augier, N., Léger, J., Robert, A., Tomé, F.M.S., Fardeau, M., Voit, T., Nicholson, L.V.B., Mornet, D. and Léger, J.J. (1991) FEBS Lett. 282, 161–165.

[24] Sealock, R. and Froehner, S.C. (1994) Cell 77, 617–619.

[25] Campanelli, J.T., Roberds, S.L., Campbell, K.P. and Scheller R.H. (1994) Cell 77, 663–674.

[26] Gee, S.H., Montanaro, F., Lindenbaum, M.H. and Carbonetto, S. (1994) Cell 77, 675–686.

[27] Sugiyama, J., Bowen, D.C. and Hall, Z.W. (1994) Neuron 13, 103–115.

[28] Bowe, M.A., Deyst, K.A., Leszyk, J.D. and Fallon, J.R. (1994) Neuron 12, 1173–1180.

[29] Matsumura, K., Yamada, H., Shimizu, T. and Campbell, K.P. (1993) FEBS Lett. 334, 281–285.

[30] Fabbrizio, E., Latouche, J., Rivier, F., Hugon, G. and Mornet, D. (1995) Biochem. J. 311, in press.

[31] Fabbrizio, E., Léger, J., Anoal, M., Léger, J.J. and Mornet, D. (1993) FEBS Lett. 322, 10–14.

[32] Yeadon, J.E., Lin, H., Dyer, S.M. and Burden, S.J. (1991) J. Cell Biol. 115, 1069–1076.

[33] Blake, D.J., Schofield, J.N., Zuellig, R.A., Gòrecki, D.C., Phelps, S.R., Barnard, E.A., Edwards, Y.H. and Davies, K.E. (1995) Proc. Natl. Acad. Sci. USA 92, 3697–3701.

[34] Yamada, H., Shimizu, T., Tanaka, T., Campbell, K.P. and Matsumura, K. (1994) FEBS Lett. 352, 49–53.

[35] Sunada, Y., Bernier, S.M., Kozak, C.A., Yamada, Y. and Campbell, K.P. (1994) J. Biol. Chem. 269, 13729–13732.