

# Dystroglycan is a binding protein of laminin and merosin in peripheral nerve

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**Abstract**  $\alpha$ -Dystroglycan, a 156 kDa dystrophin-associated glycoprotein, binds laminin in skeletal muscle. Here we demonstrate that  $\alpha$ -dystroglycan is a binding protein of laminin (A/B1/B2) and merosin (M/B1/B2) in peripheral nerve. Immunocytochemical analysis demonstrates the localization of  $\alpha$ -dystroglycan and merosin surrounding myelin sheath of peripheral nerve fibers. Biochemical analysis demonstrates that the 120 kDa peripheral nerve  $\alpha$ -dystroglycan binds merosin as well as laminin. The binding of laminin and merosin is  $\text{Ca}^{2+}$  dependent and is inhibited by NaCl and heparin. Recently, merosin was shown to be deficient in the peripheral nerve of *dy* mice which have defects in myelination. The interaction between  $\alpha$ -dystroglycan and merosin may play a role in the regulation of Schwann cell myelination and/or maintenance of myelin sheath.

**Key words:** Dystroglycan; Laminin; Merosin; Schwann cell; Myelin sheath; Myelination

## 1. Introduction

$\alpha$ -Dystroglycan, a 156 kDa dystrophin-associated glycoprotein is presumed to be a proteoglycan bound to the external surface of striated muscle cell membrane [1-9]. Striated muscle  $\alpha$ -dystroglycan binds the basement membrane component laminin thus linking the sarcolemma and the basement membrane [6,8,9]  $\alpha$ -Dystroglycan also binds the basement membrane component agrin which mediates the clustering of acetylcholine receptor in the neuromuscular junction, suggesting that  $\alpha$ -dystroglycan may bind multiple forms of G domain-containing basement membrane molecules [10-12].  $\alpha$ -Dystroglycan is expressed in non-muscle tissues such as brain, lung and kidney [6-8] In peripheral nerve,  $\alpha$ -dystroglycan is expressed surrounding nerve fibers [13]. Thus far biological functions of  $\alpha$ -dystroglycan in peripheral nerve remain obscure.

The prototypical laminin molecule is a heterotrimer made up of three chains of classes A, B1 and B2 [14]. Each class of laminin chain has multiple isoforms and laminin exists in numerous trimeric isoforms in different tissues [14] In peripheral nerve merosin, a laminin heterotrimer comprised of the M (an isoform of the A chain), B1 and B2 chains [15-17], is expressed in the endoneurium surrounding Schwann cell and myelin sheath [15,18-20]. So far biological functions and the putative receptor of merosin remain elusive in peripheral nerve. These findings, all together, raise a possibility that  $\alpha$ -dystroglycan may be a hitherto-unidentified merosin receptor in peripheral nerve To begin to address this intriguing possibility, we investigated the status of expression and laminin-binding activities of  $\alpha$ -dystroglycan in peripheral nerve.

## 2. Materials and methods

### 2.1. Immunocytochemical analysis using confocal laser scanning microscopy (cLSM)

Immunostaining of cryosections (7 $\mu$ m) from rabbit peripheral nerve or teased peripheral nerve fibers [21] was performed as previously

described [13,22] Specimens were observed and fluorescent images were obtained on a Zeiss cLSM model LSM 310, employing an argon ion laser ( $\lambda = 488 \text{ nm}$ ).

### 2.2. Affinity chromatography

Crude membranes from bovine peripheral nerve were extracted at a protein concentration of 2 mg/ml in a buffer containing 1% digitonin as previously described [2,4,22,23]. 1.2 ml of the extracts were incubated at 4°C overnight with 0.3 ml of Sepharose or wheat germ agglutinin (WGA)-Sepharose (Pharmacia). The Sepharose matrices were separated from the voids by centrifugation, washed extensively and eluted with SDS-sample buffer. Mouse EHS sarcoma laminin (Biomedical Technologies) and human placenta merosin (Chemicon) were coupled to CNBr-activated Sepharose 4B (Pharmacia). Peripheral nerve membranes were extracted at a protein concentration of 2 mg/ml in 50 mM Tris-HCl pH 7.4, containing 1% digitonin, 0.5 M sucrose and 0.44 M NaCl [8]. The extracts were diluted 10-fold with 50 mM Tris-HCl, pH 7.4, 1.11 mM  $\text{CaCl}_2$ , and 1.11 mM  $\text{MgCl}_2$ , to reduce the digitonin, sucrose and NaCl concentrations to 0.1%, 50 mM and 44mM, respectively. 10 ml of the diluted extracts were incubated at 4°C overnight with 0.1 ml of laminin- or merosin-Sepharose. The Sepharose matrices were separated from the voids by centrifugation, washed extensively and were eluted with a buffer containing 10 mM EDTA. The results were analyzed by 3-12% SDS-PAGE, immunoblotting and blot overlay.

### 2.3. Others

Affinity-purified sheep antibody against  $\alpha$ -dystroglycan fusion protein D and monoclonal antibody IIH6 against  $\alpha$ -dystroglycan were characterized previously [6-8]. Monoclonal antibodies 2G9 against human M, 11D5 against human A, 4E10 against human B1 and 2E8 against human B2 chains were purchased from Gibco. Nitrocellulose transfer overlays with laminin or merosin were performed as previously described [8], except that the bound laminin and merosin were detected using affinity-purified rabbit antibody against mouse EHS laminin (Sigma).

## 3. Results and discussion

cLSM fluorescent images of  $\alpha$ -dystroglycan and laminin in rabbit peripheral nerve are shown in Fig. 1.  $\alpha$ -Dystroglycan, the M and B1 chains of laminin were localized surrounding myelin sheath of nerve fibers (Fig. 1a). Anti-B2 chain antibody 2E8 did not react with rabbit tissues but stained a similar region in human peripheral nerve (not shown). The A chain was not

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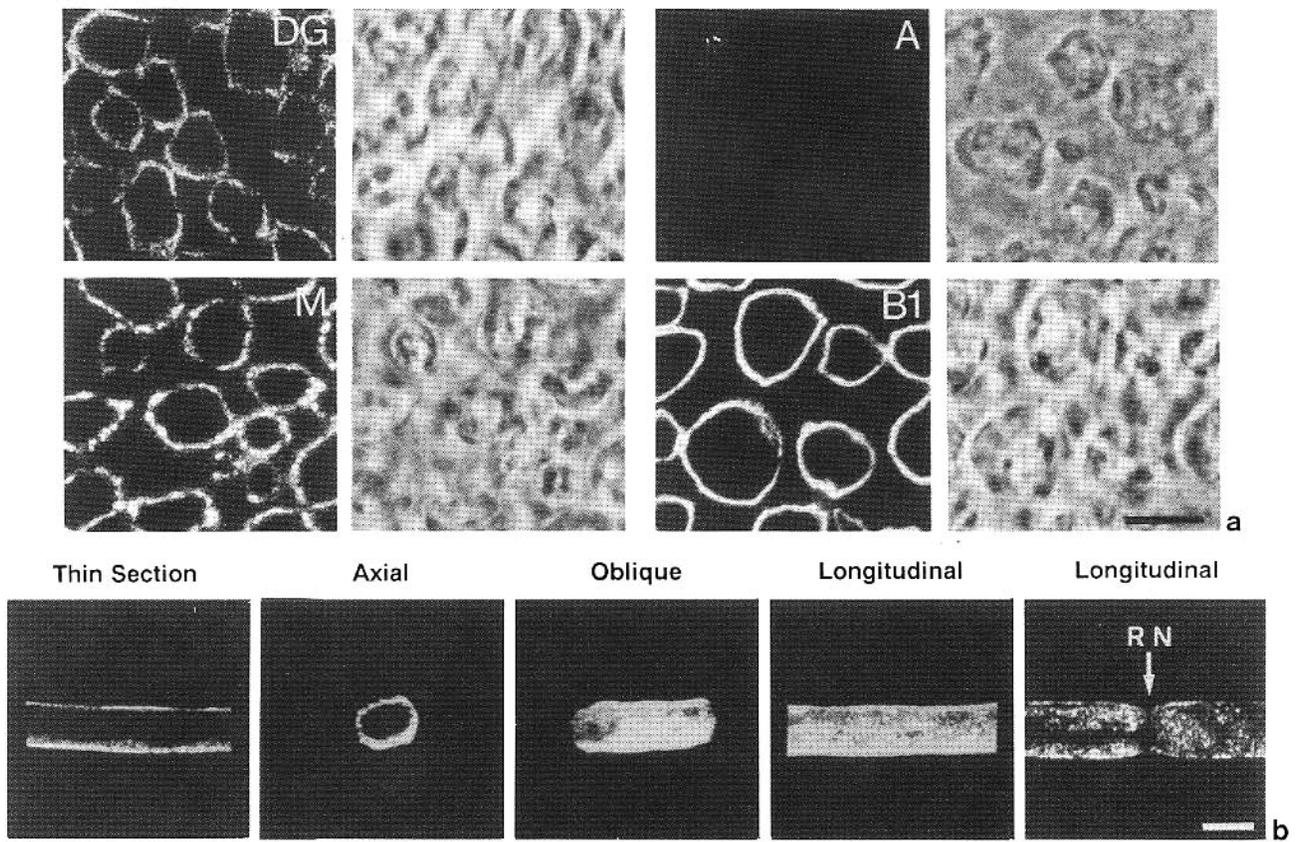
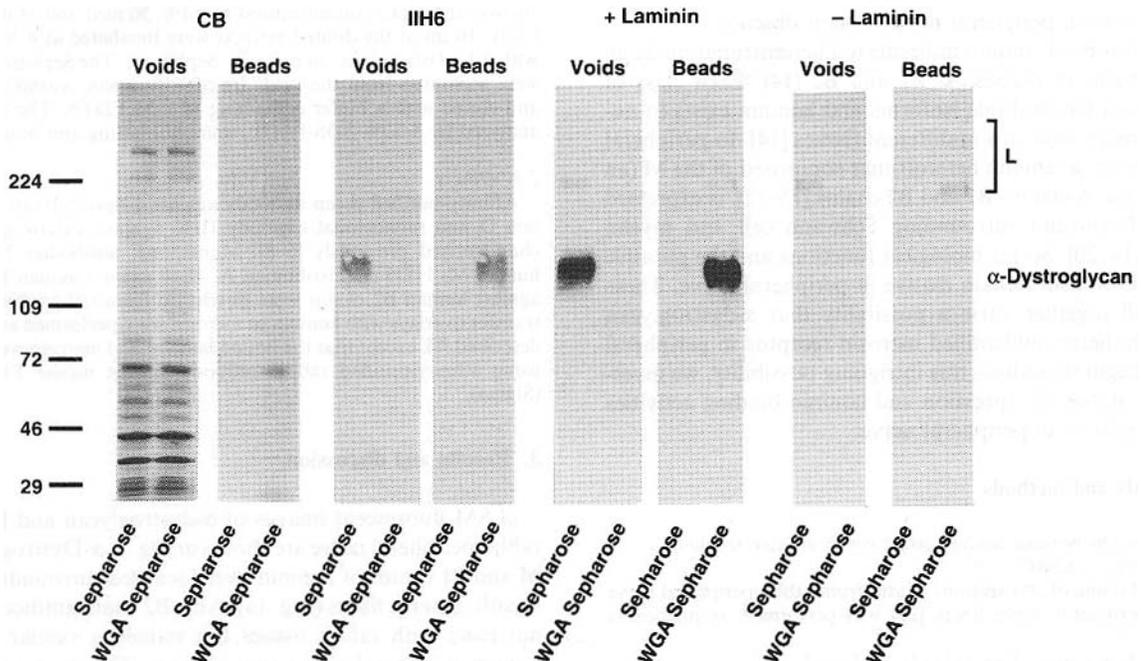


Fig. 1. (a) cLSM analysis of  $\alpha$ -dystroglycan and merosin in peripheral nerve. cLSM fluorescent and the corresponding phase-contrast images are shown. DG, M, A and B1 indicate  $\alpha$ -dystroglycan, the M, A and B1 chains, respectively. Bar = 10/ $\mu$ m. (b) cLSM analysis of  $\alpha$ -dystroglycan in teased peripheral nerve fibers. Shown are the thin-slice (0.4  $\mu$ m) image obtained by optical knife and the axial, oblique and longitudinal views of the reconstructed three-dimensional images. Bar = 10  $\mu$ m.

detected in this site (Fig. 1a). Furthermore, double-immunostaining analysis demonstrated the co-localization of  $\alpha$ -dystroglycan and the M/B1 chains surrounding myelin sheath

(not shown). In order to further clarify the site of expression of  $\alpha$ -dystroglycan in peripheral nerve, we performed the immunocytochemical analysis of teased peripheral nerve fibers



using cLSM. Immunoreactivity for  $\alpha$ -dystroglycan was detected homogeneously on the surface of myelin sheath of teased peripheral nerve fibers and was not detectable in the node of Ranvier (Fig. 1b), suggesting the localization of  $\alpha$ -dystroglycan in the outer membrane of myelin sheath.

Immunoblot analysis demonstrated that  $\alpha$ -dystroglycan was enriched in peripheral nerve membranes (not shown) and was extracted by 1% digitonin (Fig. 2). As reported previously [13], peripheral nerve  $\alpha$ -dystroglycan had a molecular mass of 120 kDa (Fig. 2). The 120 kDa  $\alpha$ -dystroglycan was quantitatively absorbed by WGA-Sepharose (Fig. 2), indicating that it is a membrane-associated glycoprotein with *N*-acetylneuraminic acid/*N*-acetylglucosamine residues.

Laminin- and merosin-Sepharose absorbed, in the presence of 1 mM  $\text{CaCl}_2$ , and 1 mM  $\text{MgCl}_2$ , the 120 kDa  $\alpha$ -dystroglycan from the digitonin extracts of peripheral nerve membranes, and the absorbed  $\alpha$ -dystroglycan was eluted by 10 mM EDTA (Fig. 3). When overlaid with laminin or merosin on the nitrocellulose transfers, the eluted  $\alpha$ -dystroglycan bound both these proteins (Fig. 3). In order to confirm laminin and merosin binding of peripheral nerve  $\alpha$ -dystroglycan, the nitrocellulose transfers of the digitonin extracts of peripheral nerve membranes were overlaid with laminin or merosin. The 120 kDa  $\alpha$ -dystroglycan bound laminin and merosin in the presence of 1 mM  $\text{CaCl}_2$  and 1 mM  $\text{MgCl}_2$  (Figs. 2 and 4). The binding of merosin was inhibited by the inclusion of 10 mM EDTA or EGTA in the overlay medium (Fig. 4). Addition of 20 mM  $\text{CaCl}_2$ , but not 20 mM  $\text{MgCl}_2$ , in the overlay medium containing 10 mM EDTA restored merosin binding (Fig. 4). The absence of  $\text{CaCl}_2$ , but not  $\text{MgCl}_2$ , in the overlay medium also inhibited merosin binding (not shown). These results demonstrate the  $\text{Ca}^{2+}$ -dependency of merosin binding. The binding of merosin was inhibited by the presence of 0.5 M NaCl or 1,000-fold excess (w/w) of heparin in the overlay medium (Fig. 4). The binding properties of laminin to peripheral nerve  $\alpha$ -dystroglycan were identical to those of merosin (not shown).

Here we demonstrated the localization of  $\alpha$ -dystroglycan and merosin surrounding myelin sheath of peripheral nerve fibers. Furthermore, immunocytochemical analysis of teased peripheral nerve fibers suggests the localization of  $\alpha$ -dystroglycan in the outer membrane of myelin sheath. We also demonstrated that the 120 kDa peripheral nerve  $\alpha$ -dystroglycan bound merosin as well as laminin. All together, our results indicate that  $\alpha$ -dystroglycan is a novel merosin-binding protein in peripheral nerve, most likely in the myelin sheath membrane. The size of  $\alpha$ -dystroglycan varies among different tissues [6,8]. The molecular mass of peripheral nerve  $\alpha$ -dystroglycan is comparable to that of the brain form (120 kDa), and is smaller by about 40 kDa than that of the striated muscle or lung form (156 kDa) [6,8,13,24]. This is presumed to be due to differences in the post-translational modification, such as glycosylation, of  $\alpha$ -dystroglycan [6-8,13]. However, the binding activities of laminin and merosin to peripheral nerve  $\alpha$ -dystroglycan were sim-

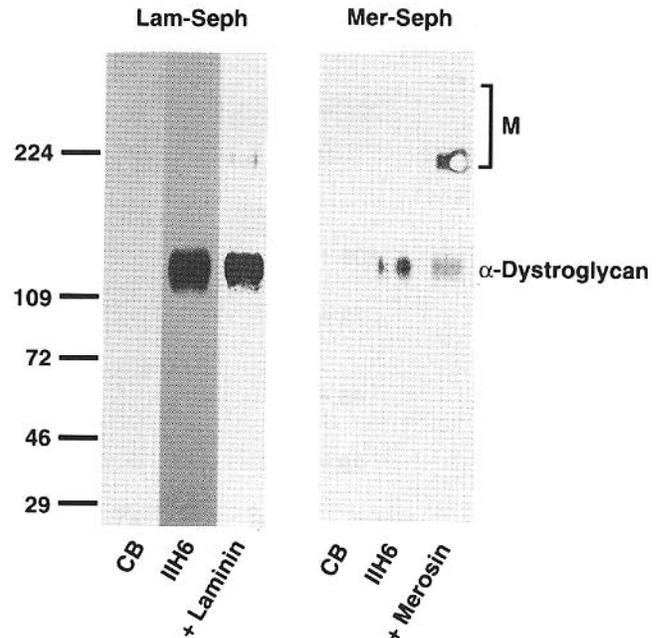


Fig. 3. Laminin and merosin affinity column chromatography of peripheral nerve membranes. After incubation of the digitonin extracts of peripheral nerve membranes with laminin- or merosin-Sepharose (Lam-Seph and Mer-Seph, respectively), proteins absorbed by the matrices were eluted with 10 mM EDTA and analyzed by SDS-PAGE. Shown are the gel stained with Coomassie blue (CB), the nitrocellulose transfer immunostained with monoclonal antibody IIH6 against  $\alpha$ -dystroglycan (IIH6), and the nitrocellulose transfers overlaid with laminin (+Laminin) or merosin (+Merosin). A small amount of laminin (L) and merosin (M) were detached from the Sepharose matrices by 10 mM EDTA. The 120 kDa peripheral nerve  $\alpha$ -dystroglycan was not stained with Coomassie blue as in the skeletal muscle form [2,4]. Laminin-Sepharose was apparently more efficient than merosin-Sepharose in absorbing  $\alpha$ -dystroglycan. This could be due to partial degradation of commercial merosin preparation as previously described [8], or the affinity of mouse EHS laminin to bovine peripheral nerve  $\alpha$ -dystroglycan might be higher than that of human placenta merosin. Molecular weight standards (Dax  $10^{-3}$ ) are shown on the left.

ilar to the laminin-binding activities of skeletal muscle  $\alpha$ -dystroglycan [8].

Recently, the specific reduction of merosin M chain was demonstrated in skeletal muscle of patients with Fukuyama-type congenital muscular dystrophy (FCMD), a disease characterized by brain anomaly and muscular dystrophy [25]. The abnormality in the expression of  $\alpha$ -dystroglycan in the sarcolemma of FCMD patients implicates the disturbance of the interaction between  $\alpha$ -dystroglycan and merosin in the molecular pathogenesis of FCMD [26]. The M chain is also deficient in skeletal muscle and peripheral nerve of *dy* mice [27-29], which have muscular dystrophy and peripheral nerve dysmyelination [27-31]. Intriguingly, laminin is known to promote Schwann cell myelination [32,33]. Furthermore, the expression

Fig. 2. WGA column chromatography of peripheral nerve membranes. After incubation of the digitonin extracts of peripheral nerve membranes with Sepharose or WGA-Sepharose, proteins remaining in the voids (Voids) or absorbed by the matrices (Beads) were analyzed by SDS-PAGE. Shown are the gel stained with Coomassie blue (CB), the nitrocellulose transfer immunostained with monoclonal antibody IIH6 against  $\alpha$ -dystroglycan (IIH6), and the nitrocellulose transfers incubated with the overlay medium containing laminin (+Laminin) or lacking laminin (-Laminin). The bands indicated by L are the endogenous peripheral nerve laminin. The 120 kDa peripheral nerve  $\alpha$ -dystroglycan was not stained with Coomassie blue as in the skeletal muscle form [2,4]. Molecular weight standards (Da  $\times 10^{-3}$ ) are shown on the left.

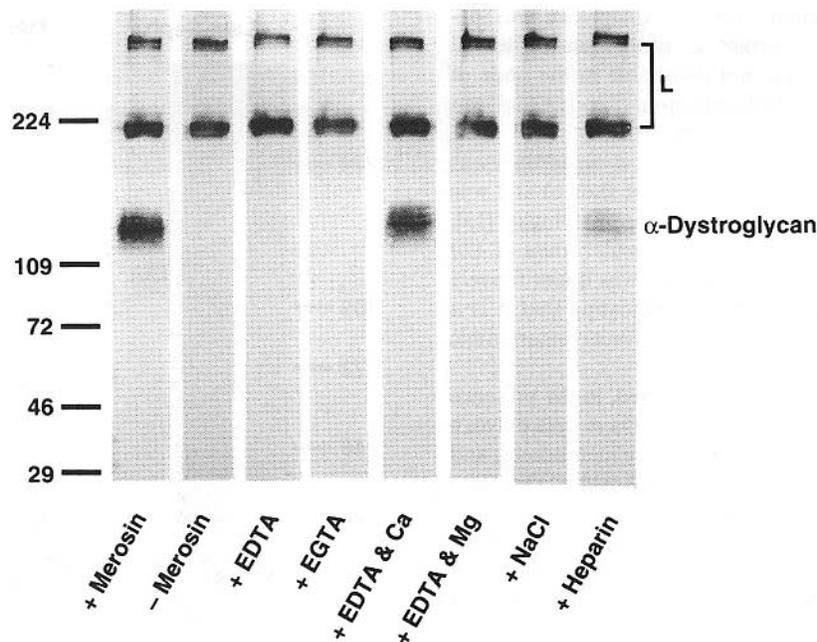


Fig. 4. Merosin-binding properties of peripheral nerve  $\alpha$ -dystroglycan. Nitrocellulose transfers of the digitonin extracts of peripheral nerve membranes were incubated, in the presence of 1 mM  $\text{CaCl}_2$ , and 1 mM  $\text{MgCl}_2$ , with the overlay medium containing merosin (+Merosin) or lacking merosin (-Merosin). The bands indicated by L are the endogenous peripheral nerve laminin. The binding of merosin to the 120 kDa peripheral nerve  $\alpha$ -dystroglycan was inhibited by the inclusion of 10 mM EDTA (+EDTA) or EGTA (+EGTA) in the overlay medium. Addition of 20 mM  $\text{CaCl}_2$  (+EDTA & Ca), but not 20 mM  $\text{MgCl}_2$ , (+EDTA & Mg), in the overlay medium containing 10 mM EDTA restored merosin binding. The binding of merosin was significantly reduced by the presence of 0.5 M Nad (+NaCl) or 1,000-fold excess (w/w) of heparin (+Heparin) in the overlay medium. Molecular weight standards ( $\text{Da} \times 10^{-3}$ ) are shown on the left.

of merosin is up-regulated during development of peripheral nerve [15], and merosin is expressed in differentiated Schwann cell neoplasms but not detectable in undifferentiated malignant schwannomas [34]. These findings indicate that the expression of laminin/merosin in the basement membrane is associated with Schwann cell differentiation and myelination. Taken together, our results raise an intriguing possibility that  $\alpha$ -dystroglycan may be involved in the regulation of Schwann cell myelination and/or maintenance of myelin sheath. The disturbance of the interaction between  $\alpha$ -dystroglycan and merosin may play a role in the pathogenesis of peripheral neuropathy in *dy* mice.

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## References

- [1] Campbell, K.P. and Kahl, S.D. (1989) *Nature* 338, 259-262.
- [2] Ervasti, J.M., Ohndieck, K., Kahl, S.D., Gaver, M.G. and Campbell, K.P. (1990) *Nature* 345, 315-319.
- [3] Yoshida, M. and Ozawa, E. (1990) *J. Biochem.* 108, 748-752.
- [4] Ervasti, J.M., Kahl, S.D. and Campbell, K.P. (1991) *J. Biol. Chem.* 266, 9161-9165.
- [5] Ervasti, J.M. and Campbell, K.P. (1991) *Cell* 66, 1121-1131.
- [6] Ibraghimov-Beskrovnaya, O., Ervasti, J.M., Leveille, C.J., Slaughter, C.A., Sernett, S.W. and Campbell, K.P. (1992) *Nature* 355, 696-702.
- [7] Ibraghimov-Beskrovnaya, O., Milatovich, A., Ozcelik, T., Yang, B., Franke, U. and Campbell, K.P. (1993) *Hum. Mol. Genet.* 2, 1651-1657.
- [8] Ervasti, J.M. and Campbell, K.P. (1993) *J. Cell Biol.* 122, 809-823.
- [9] Klietsch, R., Ervasti, J.M., Arnold, W., Campbell, K.P. and Jorgensen, A.O. (1993) *Circ. Res.* 72, 349-360.
- [10] Bove, M.A., Deyst, K.A., Leszyk, J.D. and Fallen, J.R. (1994) *Neuron* 12, 1173-1180.
- [11] Campanelli, J.T., Roberds, S.L., Campbell, K.P. and Scheller, R.H. (1994) *Cell* 77, 663-674.
- [12] Gee, S.H., Montanaro, F., Lindenbaum, M.H. and Carbonetto, S. (1994) *Cell* 77, 675-686.
- [13] Matsumura, K., Yamada, H., Shimizu, T. and Campbell, K.P. (1993) *FEBS Lett.* 334, 281-285.
- [14] Tryggvason, K. (1993) *Curr. Opin. Cell Biol.* 5, 877-882.
- [15] Leivo, I. and Engvall, E. (1988) *Proc. Natl. Acad. Sci. USA* 85, 1544-1548.
- [16] Ehrig, K., Leivo, I., Argraves, W.S., Rouslahti, E. and Engvall, E. (1990) *Proc. Natl. Acad. Sci. USA* 87, 3264-3268.
- [17] Vuolteenaho, R., Nissinen, M., Sainio, K., Byers, M., Eddy, R., Hirvonen, H., Shows, T.B., Sariola, H., Engvall, E. and Tryggvason, K. (1994) *J. Cell Biol.* 124, 381-394.
- [18] Sanes, J.R., Engvall, E., Butkowski, R. and Hunter, D.D. (1990) *J. Cell Biol.* 113, 1685-1699.
- [19] Engvall, E., Earwicker, D., Haaparanta, T., Ruoslahti, E. and Sanes, J.R. (1990) *Cell Regul.* 1, 731-740.
- [20] Jaakkola, S., Savunen, O., Haime, T., Uitto, J. and Peltonen, J. (1993) *J. Neurocytol.* 22, 215-230.
- [21] Toyota, B., Carbonetto, S. and David, S. (1990) *Proc. Natl. Acad. Sci. USA* 87, 1319-1322.

- [22] Matsumura, K., Ervasti, J.M., Ohiendieck, K., Kahl, S.D. and Campbell, K.P. (1992) *Nature* 360, 588-591.
- [23] Matsumura, K., Shasby, M.D. and Campbell, K.P. (1993) *FEBS Lett.* 326, 289-293.
- [24] Gee, S.H., Blacher, R.W., Douville, P.J., Provost, P.R., Yurchenco, P.D. and Carbonetto, S. (1993) *J. Biol. Chem.* 268, 14972-14980.
- [25] Hayashi, Y.K., Engvall, E., Arikawa-Hirasawa, E., Goto, K., Koga, R., Nonaka, I., Sugita, H. and Arahata, K. (1993) *J. Neurol. Sci.* 119, 53-64.
- [26] Matsumura, K., Nonaka, I. and Campbell, K.P. (1993) *Lancet* 341, 521-522.
- [27] Arahata, K., Hayashi, Y.K., Koga, R., Goto, K., Lee, J.H., Miyagoe, Y., Ishii, H., Tsukahara, T., Takeda, S., Woo, M., Nonaka, I., Matsuzaki, T. and Sugita, H. (1993) *Proc. Japan Acad B* 69, 259-264.
- [28] Sunada, Y., Bernier, S.M., Kozak, C.A., Yamada Y and Campbell, K.P. (1994) *J. Biol. Chem.* 269, 13729-13732.
- [29] Xu, H., Christmas, P., Wu, X.-R., Wewer, U.M., and Engvall, E. (1994) *Proc. Natl. Acad. Sci. USA* 91, 5572-5576.
- [30] Bradley, W.G. and Jenkinson, M. (1973) *J. Neurol. Sci.* 25 249-255.
- [31] Madrid, R.E., Jaros, E., Cullen, M.J. and Bradley, W.G. (1975) *Nature* 257, 319-321.
- [32] Eldridge, C.F., Bunge, M.B. and Bunge, R.P. (1989) *J. Neurosci* 9, 625-628.
- [33] Reichardt, L.F. and Tomaselli, K.J. (1991) *Annu. Rev. Neurosci.* 14, 531-570.
- [34] Leivo, I., Engvall, E., Laurila, P. and Miettinen, M. (1989) *Lab Invest.* 61, 426-432.