

Secretion of laminin $\alpha 2$ chain in cerebrospinal fluid

Hiroki Yamada^a, Hisae Hori^b, Takeshi Tanaka^c, Sachiko Fujita^a, Hiroko Fukuta-Ohi^a, Shuntaro Hojo^d, Akira Tamura^d, Teruo Shimizu^a, Kiichiro Matsumura^{a,*}

^aDepartment of Neurology and Neuroscience, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173, Japan

^bDepartment of Tissue Physiology, Medical Research Institute, Tokyo Medical and Dental University, 2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101, Japan

^cSaitama Red Cross Blood Center, 903 Kamiochiai, Yono 338, Japan

^dDepartment of Neurosurgery, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173, Japan

Received 4 October 1995; revised version received 16 October 1995

Abstract The absence of laminin $\alpha 2$ chain causes muscle cell degeneration and peripheral dysmyelination in congenital muscular dystrophy patients and *dy* mice, suggesting its role in the maintenance of sarcolemmal architecture and peripheral myelogenesis. Here we demonstrate the secretion of laminin $\alpha 2$ chain in cerebrospinal fluid (CSF). Laminin $\alpha 2$ chain was detected as a minor component of the total CSF proteins or glycoproteins. Laminin $\alpha 2$ chain was localized in the cytoplasm of epithelial cells of choroid plexus, suggesting active secretion. Our results suggest that immunochemical analysis of CSF laminin $\alpha 2$ chain could be useful as an aid for the diagnosis of congenital muscular dystrophy.

Key words: Laminin $\alpha 2$ chain; Laminin-2; Cerebrospinal fluid; Congenital muscular dystrophy; *dy* mouse

1. Introduction

Laminin, a heterotrimer made up of three chains of classes α , β and γ , is a major component of the basal lamina (for reviews see [1,2]). Laminin has numerous biological functions in cell differentiation, adhesion, migration and proliferation, in addition to being a true structural component of the basal lamina meshwork [2]. Each class of laminin chains has multiple isoforms and laminin exists in numerous trimeric isoforms in different tissues [1,2]. Laminin-2, comprised of the $\alpha 2$, $\beta 1$ and $\gamma 1$ chains, is expressed in the basal lamina of striated muscle cells, Schwann cells and trophoblasts [2–5]. Recently, the absence of laminin $\alpha 2$ chain was identified as the cause of muscle cell degeneration and peripheral dysmyelination in congenital muscular dystrophy (CMD) and its animal model *dy* mice [6–12]. Since laminin-2 is a ligand of dystroglycan in both striated muscle and Schwann cell membranes [5,7,13], these findings implicate the laminin-2–dystroglycan interaction in not only the maintenance of sarcolemmal architecture but also peripheral myelogenesis. Thus far, it is unknown if laminin $\alpha 2$ chain is secreted into body fluids such as cerebrospinal fluid (CSF). If this is the case, immunochemical analysis of laminin $\alpha 2$ chain in body fluids could be useful as an aid for the diagnosis of CMD, because it is reasonable to suspect that laminin $\alpha 2$ chain is absent in the body fluids of CMD patients. We have addressed this issue in the present study.

2. Materials and methods

2.1. Immunochemical analysis of CSF proteins

CSF was obtained from several humans who had no abnormalities in the CSF composition as determined by routine laboratory analyses, including cell count and total protein concentration. After particulates were cleared by brief centrifugation, 2 ml of CSF from each individual was concentrated by centrifugal concentrator (Tomy CC-101). CSF concentrates were separated by 3–12% SDS-PAGE and analyzed by immunoblotting.

2.2. Isolation of wheat germ agglutinin (WGA)-binding glycoproteins from CSF

100 μ l of WGA-Sepharose (Pharmacia) was equilibrated with buffer A (50 mM sodium phosphate pH 7.4, 0.5 M NaCl, 0.1 mM phenylmethylsulfonyl fluoride and 0.75 mM benzamidine) and then incubated, overnight at 4°C, with 2 ml of human CSF in the presence of 0.5 M NaCl. After extensive wash with buffer A, WGA-Sepharose was eluted with 300 μ l of buffer A containing 0.3 M *N*-acetylglucosamine (GlcNAc). The eluates were separated by 3–12% SDS-PAGE and analyzed by immunoblotting.

2.3. SDS and EDTA extraction of proteins from choroid plexus and spinal roots

Bovine choroid plexus and spinal roots were obtained at a local abattoir and quick frozen in isopentane cooled in liquid nitrogen. For SDS extraction, cryosections from choroid plexus and spinal roots were homogenized in 20 vols. of SDS extraction buffer (10% SDS, 80 mM Tris-HCl pH 6.8, 115 mM sucrose, 1% β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine) and then incubated for 15 min at 60°C. For EDTA extraction, cryosections from choroid plexus and spinal roots were homogenized in 20 vols of EDTA extraction buffer (10 mM EDTA, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and 0.75 mM benzamidine) and then incubated for 2 h at 4°C. After insoluble debris were cleared by brief centrifugation, supernatants were separated by 3–12% SDS-PAGE and analyzed by immunoblotting.

2.4. Others

Immunohistochemical analysis, 3–12% SDS-PAGE and immunoblotting were performed as previously described [5]. Monoclonal antibody 2D9 against the proximal portion of the G domain of human laminin $\alpha 2$ chain was characterized previously [14]. Antibody against mouse EHS sarcoma laminin was purchased from Sigma. Peroxidase-labeled WGA was purchased from Boehringer-Mannheim.

3. Results and discussion

Human CSF was analyzed by SDS-PAGE and immunoblotting. Antibody against mouse EHS sarcoma laminin detected two bands in the range of 200 kDa corresponding to laminin $\beta 1$ and $\gamma 1$ chains, but did not detect laminin $\alpha 1$ chain (Fig. 1). The results suggest that laminin $\alpha 1$ chain is not present in CSF, although a possibility can not be excluded that the antibody did not react with human laminin $\alpha 1$ chain. Instead, monoclonal

*Corresponding author. Fax: (81) (3) 3964-6397.

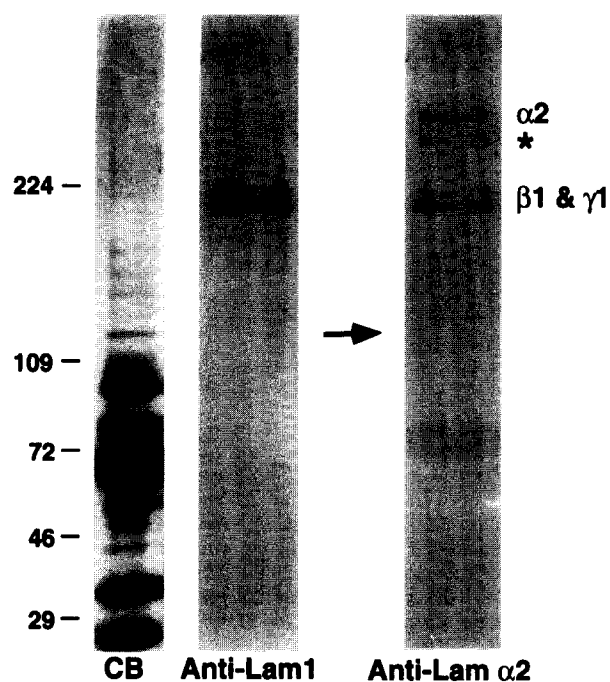


Fig. 1. Immunoblot analysis of laminin $\alpha 2$ chain in CSF. Human CSF concentrate was analyzed by immunoblotting. The nitrocellulose transfer was first immunoreacted with antibody against mouse EHS sarcoma laminin (anti-Lam1) and then with monoclonal antibody 2D9 against human laminin $\alpha 2$ chain (anti-Lam $\alpha 2$). CB indicates the SDS gel stained with Coomassie blue. Asterisk indicates the 300 kDa C-terminal fragment of laminin $\alpha 2$ chain [7,9]. Molecular weight standards ($\text{Da} \times 10^{-3}$) are shown on the left.

antibody 2D9 detected laminin $\alpha 2$ chain of 400 kDa (Fig. 1). The bands corresponding to laminin $\alpha 2$, $\beta 1$ and $\gamma 1$ chains could not be identified in the SDS gel stained with Coomassie blue (Fig. 1), indicating that these proteins were not major components of the total CSF proteins. The same results as shown in Fig. 1 were obtained for all the CSF samples tested.

To know the relative abundance of laminin $\alpha 2$ chain in the CSF glycoproteins, we isolated WGA-binding glycoproteins from human CSF by WGA chromatography. The GlcNAc eluates of WGA-Sepharose were analyzed by SDS-PAGE and immunoblotting. Although immunoblot analysis demonstrated that CSF laminin $\alpha 2$ chain was recovered in the GlcNAc eluates, the band corresponding to laminin $\alpha 2$ chain was not identified in the SDS gel stained with Coomassie blue or the nitrocellulose transfer stained with peroxidase-labeled WGA (Fig. 2), indicating that laminin $\alpha 2$ chain was not a major component of the total CSF glycoproteins.

To see if laminin $\alpha 2$ chain is secreted by choroid plexus, we performed immunohistochemical analysis. Immunoreactivity for laminin $\alpha 2$ chain was clearly detected in the cytoplasm of epithelial cells of choroid plexus (Fig. 3). To confirm the expression of laminin $\alpha 2$ chain in choroid plexus, we performed immunoblot analysis. Previously, laminin $\alpha 2$ chain was shown to be extracted from skeletal muscle by EDTA [7,9]. Similar to skeletal muscle, laminin $\alpha 2$ chain was detected in not only the SDS but also EDTA extracts of choroid plexus (Fig. 4). All together, these findings suggest that laminin $\alpha 2$ chain is actively secreted by choroid plexus.

It is unlikely that CSF laminin $\alpha 2$ chain originates from cells

or connective tissue debris present in CSF for the following reasons: (1) CSF contains very few cells; (2) the vast majority of CSF cells are leukocytes which do not produce laminin $\alpha 2$ chain; and (3) immunoreactivity for laminin $\alpha 2$ chain was not detected in the leptomeninges and connective tissues surrounding subarachnoid space by immunohistochemistry (not shown). Although immunoreactivity for laminin $\alpha 2$ chain was detected in the basal lamina of Schwann cells of spinal roots (Fig. 3), the fact that leptomeninges and connective tissues densely surround bundles of spinal root nerve fibers in vivo makes it unlikely that Schwann cells secrete laminin $\alpha 2$ chain into CSF. Interestingly, the molecular weight of laminin $\alpha 2$ chain differed between choroid plexus and spinal roots (Fig. 4). Differential splicing of mRNA or protein glycosylation may be responsible for this finding. The latter possibility is of interest in view of the hypothesis that differential glycosylation of proteins may play a role in the sorting mechanism for apical or basolateral secretory pathway [15].

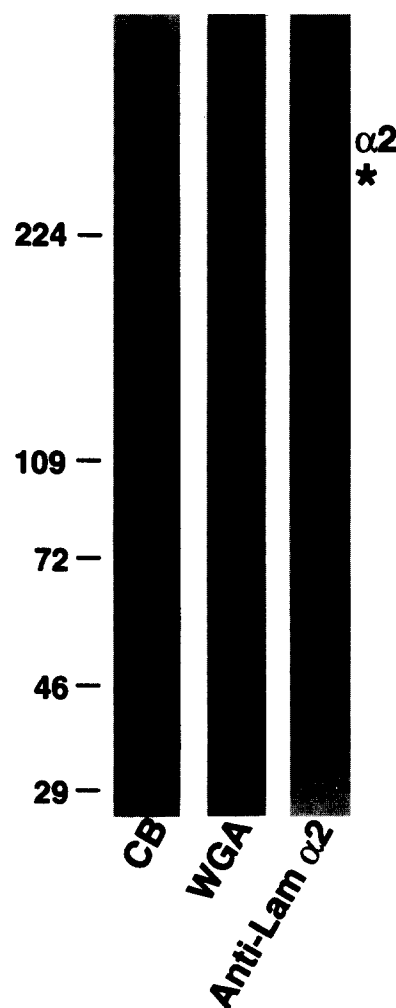


Fig. 2. Immunoblot analysis of laminin $\alpha 2$ chain in CSF glycoproteins. The GlcNAc eluates of WGA-Sepharose were analyzed by lectin staining and immunoblotting. CB, WGA and anti-Lam $\alpha 2$ indicate the SDS gel stained with Coomassie blue, the nitrocellulose transfer stained with peroxidase-labeled WGA or with monoclonal antibody 2D9, respectively. Asterisk indicates the 300 kDa C-terminal fragment of laminin $\alpha 2$ chain [7,9]. Molecular weight standards ($\text{Da} \times 10^{-3}$) are shown on the left.

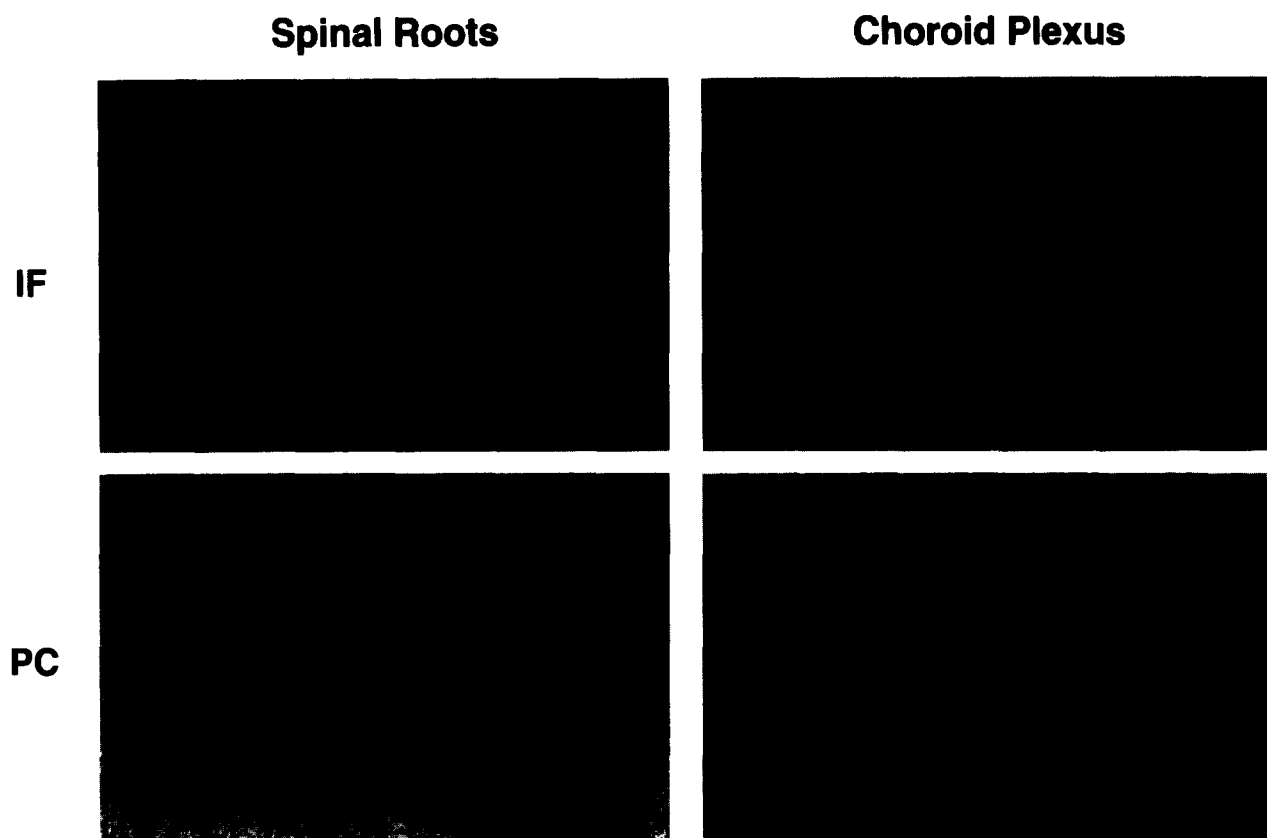


Fig. 3. Immunohistochemical analysis of laminin $\alpha 2$ chain in spinal roots and choroid plexus. Bovine cryosections were immunostained with monoclonal antibody 2D9. IF, PC, Rt, SpC, Endo and Epi indicate immunofluorescence, phase contrast, spinal roots, spinal cord, endothelial and epithelial cells, respectively. Bar, 50 μ m.

Laminin assembles to form a reversible polymer that contributes to the network structure of the basal lamina (for a review see [16]). Currently, the three-arm interaction model for laminin network formation proposes that the N-terminal moieties of each of the short arms of laminin form the basic bond of laminin polymers [16]. Once incorporated into the basal lamina, laminin has numerous biological functions in cell differentiation, adhesion, migration and proliferation (for a review see [2]). In particular, laminin-2, comprised of the $\alpha 2$, $\beta 1$ and $\gamma 1$ chains, plays important roles in the maintenance of sarcolemmal architecture and peripheral myelinogenesis [3–13,17,18]. In the present study, we have demonstrated the secretion of laminin $\alpha 2$ chain, most likely in the form of laminin-2 heterotrimer, into CSF. Our results thus indicate that laminin also exists in a free form, not incorporated into polymers, in vivo. At present, the biological functions of laminin $\alpha 2$ chain secreted into body fluids such as CSF are unclear and their elucidation awaits future research. It would be interesting to see if secreted laminin $\alpha 2$ chain has inhibitory, and potentially regulatory, effects on the binding of basal lamina laminin-2 with cell surface receptors such as dystroglycan and integrins.

Recent evidence indicates that the absence of laminin $\alpha 2$ chain is the primary cause of CMD [9–12]. Thus, it is reasonable to suspect that laminin $\alpha 2$ chain is also absent in the CSF of CMD patients. Currently, definite diagnosis of CMD is possible only by immunochemical demonstration of the absence of laminin $\alpha 2$ chain in skeletal muscle obtained through biopsy [9–12], which is a stressful procedure for young children. Our

results suggest that immunochemical analysis of CSF laminin $\alpha 2$ chain could be useful as a less stressful means of diagnosis of this devastating disease.

Acknowledgements: This work was supported by grants from Ciba-Geigy Foundation (Japan) for the Promotion of Science, Nitoh Foundation and Uehara Memorial Foundation, the Science Research Promotion Fund from Japan Private School Promotion Foundation, Research Grant (5A-2) for Nervous and Mental Disorders from the Ministry of Health and Welfare, and Grants-in-Aid for Scientific Research on Priority Areas, for Scientific Research (05454262, 06454280, 06670670 and 06770463) and for Scientific Research on Developmental areas (05557037) from the Ministry of Education, Science, Sports and Culture.

References

- [1] Burgeson, R.E., Chiquet, M., Deutzmann R., Ekblom, P., Engel, J., Kleinman, H., Martin, G.R., Meneguzzi, G., Paulsson, M., Sanes, J., Timpl, R., Tryggvason, K., Yamada, Y. and Yurchenco, P.D. (1994) *Matrix Biol.* 14, 209–211.
- [2] Tryggvason, K. (1993) *Curr. Biol.* 5, 877–882.
- [3] Leivo, I. and Engvall, E. (1988) *Proc. Natl. Acad. Sci. USA* 85, 1544–1548.
- [4] Sanes, J.R., Engvall, E., Butkowski, R. and Hunter, D.D. (1990) *J. Cell Biol.* 111, 1685–1699.
- [5] Yamada, H., Shimizu, T., Tanaka, T., Campbell, K.P. and Matsumura, K. (1994) *FEBS Lett.* 352, 49–53.
- [6] 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. Jpn. Acad. B* 69, 259–264.

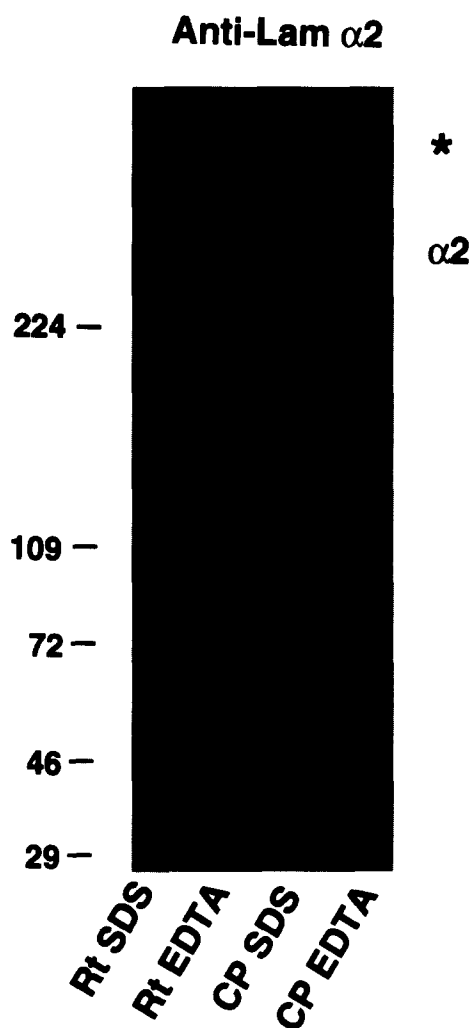


Fig. 4. Immunoblot analysis of laminin $\alpha 2$ chain in spinal roots and choroid plexus. The SDS or EDTA extracts of bovine spinal roots (Rt) and choroid plexus (CP) were analyzed by immunoblotting with monoclonal antibody 2D9 (anti-Lam $\alpha 2$). Asterisk indicates laminin-2 heterotrimer not dissociated by the SDS sample buffer [7,9]. Molecular weight standards ($\text{Da} \times 10^{-3}$) are shown on the left.

- [7] Sunada, Y., Bernier, S.M., Kozak, C.A., Yamada, Y. and Campbell, K.P. (1994) *J. Biol. Chem.* 269, 13729–13732.
- [8] Xu, H., Christmas, P., Wu, X.R., Wewer, U.M. and Engvall, E. (1994) *Proc. Natl. Acad. Sci. USA* 91, 5572–5576.
- [9] Tomé, F.M.S., Evangelista, T., Leclerc, A., Sunada, Y., Manole, E., Estournet, B., Barois, A., Campbell, K.P. and Fardeau, M. (1994) *CR Acad. Sci. [III]* 317, 351–357.
- [10] Hillaire, D., Leclerc, A., Faure, S., Topaloglu, H., Chiannikulchai, N., Guicheney, P., Grinas, L., Legos, P., Philpot, J., Evangelista, T., Routon, M.C., Mayer, M., Pellisier, J.F., Estournet, B., Barois, A., Hentati, F., Feingold, N., Beckmann, J.S., Dubovitz, V., Tomé, F.M.S. and Fardeau, M. (1994) *Hum. Mol. Genet.* 3, 1657–1661.
- [11] Bloom, T. (1995) *Curr. Biol.* 5, 338–341.
- [12] Campbell, K.P. (1995) *Cell* 80, 675–679.
- [13] Ervasti, J.M. and Campbell, K.P. (1993) *J. Cell Biol.* 122, 809–823.
- [14] Hori, H., Kanamori, T., Mizuta, T., Yamaguchi, N., Liu, Y. and Nagai, Y. (1994) *J. Biochem.* 116, 1212–1219.
- [15] Fiedler, K. and Simons, K. (1995) *Cell* 81, 309–312.
- [16] Yurchenco, P.D. and O'Rear, J.L. (1994) *Curr. Opin. Cell Biol.* 6, 674–681.
- [17] Xu, H., Wu, X.R., Wewer, U.M. and Engvall, E. (1994) *Nature Genet.* 8, 297–302.
- [18] Sunada, Y., Bernier, S.M., Utani, A., Yamada, Y. and Campbell, K.P. (1995) *Hum. Mol. Genet.* 4, 1055–1061.