cannot generally be added to the culture medium, although it is essential to maintain the correct biological conditions for cell culture. This is because the plasmid DNA carrier complex often interacts with serum components, leading to suppression of the ability of gene transfection.

In this study, a new transfection method is introduced to enable cell culture under better conditions in the presence of serum. Pullulan of polysaccharide was chemically cationized using spermine to prepare a non-viral carrier for gene transfection. It has been demonstrated that the cationized spermine-pullulan enhanced *in vitro* gene expression for various types of cells.[12] The spermine-pullulan complex of a plasmid DNA was coated onto a culture substrate together with a cell-adhesion substance, and then cells were cultured on the complex-coated substrate using different culture methods for gene transfection. The level and duration of gene expression using the new reverse transfection method were evaluated and compared with those of the conventional transfection method. We examine cytotoxicity after the reverse transfection.

MATERIALS AND METHODS

Materials

Pullulan with an average molecular weight of 47,300 was purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). Spermine was purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals were obtained from Nacalai Tesque. Inc. (Kyoto, Japan) and used without further purification.

Preparation of cationized pullulan derivative

Spermine was introduced to the hydroxyl groups of pullulan using an N,N′-carbonyldiimidazole (CDI) activation method.[13] Spermine (1.87×10^3 mg) and CDI (2.25×10^2 mg) were added to 50 mL of dehydrated dimethyl sulfoxide containing 50 mg of pullulan. Following agitation at 35°C for 20 h, the reaction mixture was dialyzed against ultra-pure double-distilled water (DDW) for 2 days with a dialysis membrane (cut-off molecular weight = 12,000–14,000, Viskase Companies, Inc, Willowbrook, IL). Then, the dialyzed solution was freeze-dried to obtain the spermine-introduced pullulan (spermine-pullulan). When determined from the conventional elemental analysis and expressed by the molar extent of spermine introduced to the hydroxyl groups of pullulan, the percentage of spermine introduced was 12.3 mole.

Preparation of plasmid DNA

The plasmid DNA used was the pGL3 vector (5.26 kb) coding for a firefly luciferase gene (Luciferase Reporter Vectors-pGL3, Promega, Madison, WI). The plasmid DNA was propagated in *E. coli* (strain DH5α) and purified using QIAGEN plasmid Mega kit (Qiagen K.K., Tokyo, Japan) according to the manufacturers' instructions. The yield and purity of the plasmid DNA were evaluated using ultraviolet spectroscopy (Ultrospec 2000, Pharmacia Biotech, Cambridge, England). The absorbance ratio at wavelengths of 260 to 280 nm for plasmid DNA solution was measured to be between 1.8 and 2.0.

Preparation of polyion complexes

Polyion complexes were prepared by mixing an aqueous solution of spermine-pullulan with that of plasmid DNA. Briefly, various amounts of spermine-pullulan were dissolved in 50 μL of DDW, mixed with 50 μL of phosphate-buffered saline solution (PBS, 10 mM, pH 7.4) containing 100 μL of plasmid DNA, and left for 15 min at room temperature to obtain various polyion complexes (PICs) of spermine-pullulan and plasmid DNA. The PIC composition was calculated on the basis of the nitrogen number of spermine-pullulan (N) per the phosphorus number of plasmid DNA (P) and expressed as the N/P ratio.

Preparation and culture of mesenchymal stem cells

Mesenchymal stem cells (MSCs) were isolated from the bone shaft of femurs of 3-wk-old male Wister rats according to the technique reported by Lennon *et al.*[14] Briefly, both ends of rat femurs were cut away from the epiphysis, and the bone marrow was flushed out using a syringe (21 gauge needle) with 1 mL of alpha minimum essential medium (αMEM) supplemented with 15 vol% fetal calf serum (FCS), penicillin (50 U/mL), and streptomycin (50 U/mL). The cell suspension (5 mL) was placed into two 25 cm^2 flasks (Iwaki Glass, Funabashi, Chiba, Japan) and cultured at 37°C in a 95% air, 5% carbon dioxide atmosphere. The medium was changed on day 4 of culture and every 3 days thereafter. When the cells of the first passage became sub-confluent, usually 7 to 10 days after seeding, the cells were detached from the flask using treatment for 5 min at 37°C with PBS solution containing 0.25 wt% trypsin and 0.02 wt% ethylenediaminetetraacetic acid. Cells were normally subcultured at a density of 2×10^4 cells/cm^2. Second-passage cells at sub-confluence were used for all experiments.

Conventional 2-dimensional transfection in the static method

Cells were seeded on each well of a 12 well multi-dish culture plate (Corning, NY) at a density of 5×10^4 cells/well and cultured in 1 mL of αMEM medium with 15 vol% FCS for 24 h. PICs were formed by mixing 50 μL of DDW containing spermine-pullulan and 50 μL of PBS containing 2.5 μg of pGL3-luciferase plasmid DNA at different N/P ratios. Immediately after the medium was exchanged using FCS-free αMEM medium, 100 μL of the PIC solution was added, and transfection culture was performed for 6 h (conventional transfection). After the medium was changed to

αMEM with FCS, cells were incubated further for 1, 3, 5, and 7 days.

Two-dimension reverse transfection in the static method

Succinic anhydride (90.1 mg) was added to 20 mL of 100 mg/mL gelatin solution in dimethyl sulfoxide, followed by agitation at room temperature for 18 h to allow the carboxyl groups to be introduced into the amino groups of gelatin for anionization. When determined as the extent of amino groups decreased using the tri-nitrobenzene sulfonic acid method,[15] the molar amount of carboxylic groups introduced was 100 mole%.

The aqueous solution of the anionized gelatin (100 μg/mL) and different amounts of Pronectin was placed into each well of a 12 well culture plate and left at 37°C for 1 h for coating. After PBS washing, the well was coated with the plasmid DNA-spermine-pullulan complex containing 2.5 μg of plasmid DNA. After 30 min incubation, the wells were washed with PBS. Then MSCs (5×10^4 cells/well) were seeded on the complex-coated well, followed by cell culture in the αMEM medium with or without 15 vol% FCS for 1, 3, 5, and 7 days.

Cells were washed with PBS once, lysed in 100 μL of cell culture lysis reagent (Promega Corp., Madison, WI), transferred into a micro reaction tube, and the cell debris separated using centrifugation (14,000 rpm, 20 min). Then 100 μL of luciferase assay reagent (Promega Corp., Madison, WI) was added to 20 μL of supernatant, and the relative light unit (RLU) of the sample was determined using a luminometer (MicroLumatPlus LB 96V, Berthold, Tokyo, Japan). The total protein of each well was determined using a bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL) according to the manufacturers' instructions. The RLU was divided by the protein amount to normalize the influence of variance of number of cells on luciferase activity. Each experimental group was carried out 3 times independently.

Three-dimensional reverse transfection using different culture methods

Static, agitated, and stirring methods were used to culture MSCs in a non-woven fabric of polyethylene terephthalate (PET, fiber diameter 26 μm, 6 mm×3 mm) for their plasmid DNA transfection. A similar coating procedure with the complex and Pronectin was performed for the PET non-woven fabric. MSCs were seeded into the complex-coated 3-dimensional PET fabric using the agitation method reported previously.[16] Briefly, the non-woven fabric was placed in 0.5 mL of cell suspension (1×10^6 cells/mL), followed by agitation with an orbital shaker (Bellco Glass, Vineland, NJ) at 300 rpm for 6 h at 37°C. The cell-seeded non-woven fabric was thoroughly washed with the medium to exclude non-adherent cells. The MSC-attached PET fabric was incubated for 2, 5, and 8 days under the conventional static condition (static culture).

For the agitated culture method, the prepared MSC-attached PET fabric was placed in each well of a 6 well culture plate containing 6 mL of αMEM with 15 vol% FCS while the culture plate was agitated using the orbital shaker (Bellco Glass, Vineland, NJ) at 50 rpm for 2, 5, and 8 days. In addition, the MSC-attached fabric was fixed with a needle immobilized in the spinner flask, and 150 mL of medium was stirred at 50 rpm for 2, 5, and 8 days (stirring culture). To measure the level of gene expression for MSCs cultured using the static, agitated, and stirring methods, the PET fabrics were collected, and gene expression was assessed using a procedure similar to the one described above.

Cell viability

Cytotoxicity was assayed using a cell counting kit (Nacalai tesque, Inc., Kyoto, Japan). MSCs were transfected with 0.50 μg of free plasmid DNA or lipofectamine 2000 (0.50 μg) and the spermine-pullulan complexing 0.50 μg of plasmid DNA using the conventional and reverse methods for 2 days. Then the medium was changed to αMEM with FCS, and 100 μL of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H- tetrazolium (WST-8) solution was added and the cells incubated for another 3 h. The absorbance of samples was measured at 450 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale CA). The percentage cell viability was expressed as 100% for non-transfected, control cells.

Statistical analysis

Data were expressed as means ± standard deviations. Data were analyzed using 2-way analysis of variance, and then differences between means were analyzed using Fisher's protected least significant difference multiple comparison test, and significance was accepted at $p < 0.05$.

RESULTS

Gene expression for MSC using the conventional and reverse transfection methods

Table 1 and Fig. 1 show the level and duration period of gene expression after the reverse transfection of MSCs together with those of the conventional transfection. For the conventional method, in which the complex is added to the culture medium, the presence of serum suppressed the level of gene expression. Alternatively, a high expression level of MSCs transfected using the reverse method was observed even in the presence of serum. Moreover, the time period of gene expression was longer than with the conventional method. When reverse transfected in the absence of FCS, MSCs were detached from the culture substrate during the transfection culture (data not shown). Free plasmid DNA did not enhance gene expression, irrespective of the presence of serum. Fig. 2 shows the level of gene expression of MSCs

TABLE 1. LUCIFERASE EXPRESSION LEVEL OF MSC TRANSFECTED BY THE CONVENTIONAL AND REVERSE METHODS IN THE PRESENCE OR ABSENCE OF SERUM[a]

Transfection method		The level of gene expression (RLU/mg protein)	
		Free plasmid DNA	Plasmid DNA-spermine-pullulan complex
Conventional	FCS (−)	$(3.0 \pm 0.02) \times 10^3$	$(4.2 \pm 0.62) \times 10^6$
	FCS (+)	$(3.0 \pm 0.98) \times 10^3$	$(3.3 \pm 0.66) \times 10^5$
Reverse	FCS (−)	–	ND[b]
	FCS (+)	–	$(6.2 \pm 1.4) \times 10^7$

The level of gene expression for non-transfected, original MSC was 3.0×10^3 RLU/mg protein.
[a]The MSC were transfected in the static culture.
[b]The level could not be measured because of cell death.
Mean ± S.D.

using the reverse transfection method at different coating concentrations of Pronectin. Expression level was enhanced with greater coating concentration of Pronectin of adhesion substance.

Cell viability

Fig. 3 shows the cell viability of MSCs transfected using the conventional and reverse methods. Vell viability decreased significantly using the conventional transfection culture with the spermine-pullulan and Lipofectamine 2000

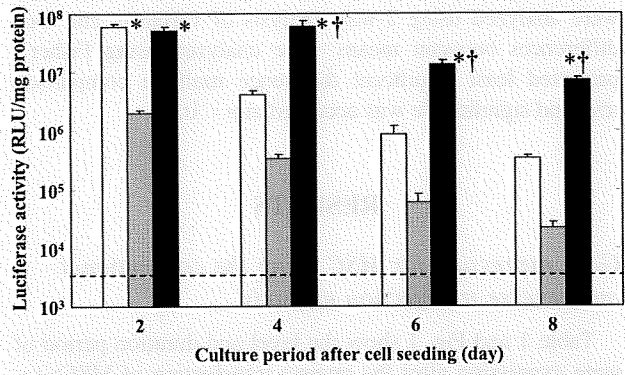

FIG. 1. Time course of luciferase expression level of mesenchymal stem cells (MSCs) transfected using the conventional (open and light gray columns) and reverse methods (solid columns) in static culture: (open columns) the plasmid DNA-spermine-pullulan complex in the absence of fetal calf serum (FCS) (dark gray columns), the plasmid DNA-spermine-pullulan complex in the presence of FCS, and (solid columns) the plasmid DNA-cationized pullulan complex in the presence of FCS. The dotted line indicates the level of non-transfected, original MSCs. $*p < 0.05$ versus the level in the presence of FCS using the conventional method at the corresponding time. $^{\dagger}p < 0.05$ versus the level in the absence of FCS using the conventional method at the corresponding time. RLU, relative light unit.

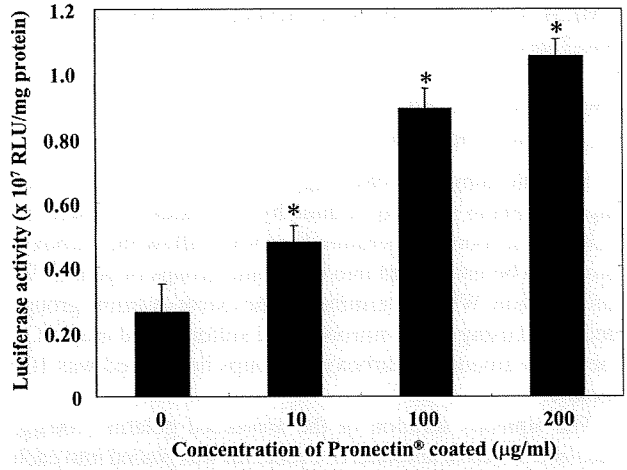

FIG. 2. Effect of Pronectin coating concentration on the luciferase expression level of mesenchymal stem cells (MSCs) transfected using the reverse method in the static culture. $*p < 0.05$ versus the luciferase activity of MSCs in the coating concentration of 0 μg/mL. RLU, relative light unit.

complexing plasmid DNA. On the contrary, the viability of cells after reverse transfection culture was similar to that of non-transfected, original cells.

Gene expression using 3-dimensional reverse transfection in different culture methods

Fig. 4 shows the time profile of gene expression of MSCs after reverse transfection using different culture methods.

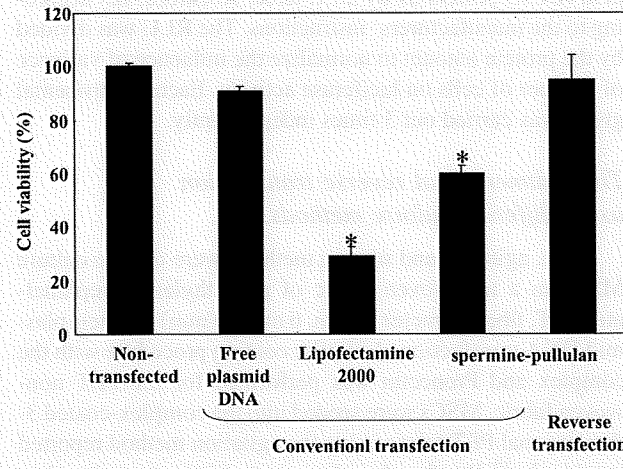

FIG. 3. Cell viability of mesenchymal stem cells (MSCs) 2 days after conventional and reverse transfection cultures. The cells were transfected using the conventional method with free plasmid deoxyribonucleic acid (DNA) or complexed with Lipofectamine 2000 and spermine-pullulan in the absence of serum. The cells were transfected using the reverse method with plasmid DNA-spermine-pullulan complex in the presence of serum. $*p < 0.05$ versus the cell viability of non-transfected, original MSCs.

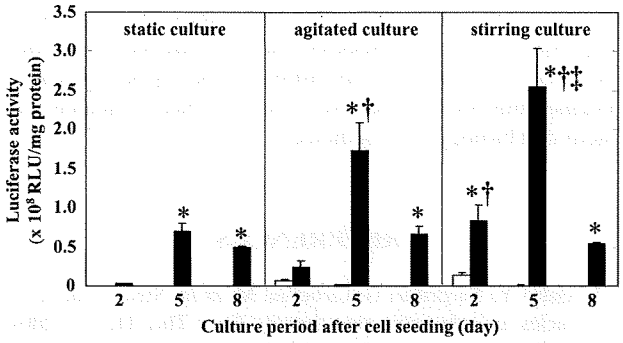

FIG. 4. Time course of luciferase expression level of mesenchymal stem cells (MSCs) transfected using the conventional (open columns) and reverse methods (solid columns) in static, agitation, and stirring cultures in polyethylene terephthalate non-woven fabric: (open columns) the plasmid deoxyribonucleic acid-cationized pullulan complex in the absence of fetal calf serum (FCS) and (solid columns) the complex in the presence of FCS. $^{*}p < 0.05$ versus the level in the presence of FCS using the conventional method at the corresponding time. $^{\dagger}p < 0.05$ versus the level in the absence of FCS using the reverse method in static culture at the corresponding time. $^{\ddagger}p < 0.05$ versus the level in the absence of FCS using the reverse method in agitation culture at the corresponding time. RLU, relative light unit.

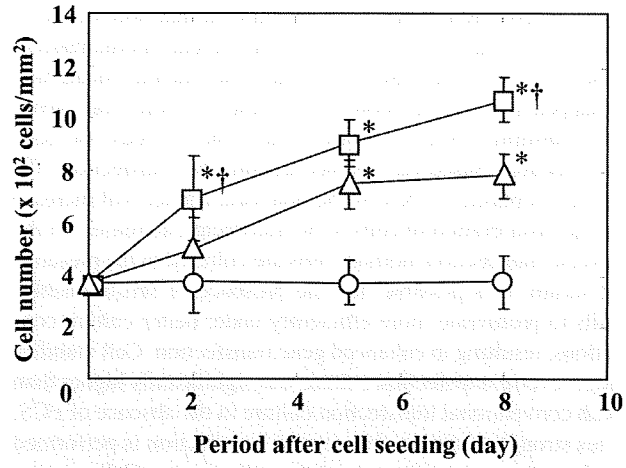

FIG. 5. Time course of proliferation of mesenchymal stem cells (MSCs) transfected using the reverse method in polyethylene terephthalate-non-woven fabric transfection in static (○), agitation (△), and stirring cultures (□). $^{*}p < 0.05$ significant against the cell number in static culture at the corresponding time. $^{\dagger}p < 0.05$ significant against the cell number in agitation culture at the corresponding time.

Irrespective of the culture method, the level and duration of gene expression were enhanced significantly more using the reverse transfection than the conventional one. The enhanced and prolonged extents of gene expression depended on the type of culture method, although the time profile of transfected level was similar. The extent increased from the static to the agitated to the stirring culture method. Using reverse transfection culture, the level of gene expression increased for 5 days after cell seeding but thereafter decreased. This was different from the time profile of gene expression using the conventional transfection culture, with which there was a maximum level on the second day for any culture method. The agitated and stirring cultures tended to increase the level of gene expression. Fig. 5 shows the time course of MSC proliferation during the reverse transfection culture. The number of MSCs that proliferated in the PET fabrics became larger from the static to the agitated to the stirring culture methods, although little cell proliferation was observed in the static culture.

DISCUSSION

The present study clearly demonstrates that the reverse transfection method was more effective in enhancing the level and duration time of gene expression than the conventional method. Considering the positioning of cells and the complex, it is likely that the complex always exists near the cells to be transfected. In addition, Pronectin achieves cell adhesion to the surface of complex-coated substrate. It is highly conceivable that the continuous exposure of complex to cells and the adhesion between cells and the substrate, which minimizes the serum influence on the transfection activity of complex, results in the enhanced and prolonged gene expression.

There are 5 key steps for expression of plasmid DNA: (1) the attachment of plasmid DNA onto the cell surface, (2) the internalization of plasmid DNA into the cell, (3) the endosomal escape of plasmid DNA, (4) the transfer of plasmid DNA to the nucleus, and (5) the internalization of plasmid DNA into the nucleus. Different from the viral vector, the non-viral carrier essentially does not have any specific mechanisms to accelerate and facilitate the above steps. Several trials have been performed to enhance the efficiency of each step. For steps (1) and (2), it has been attempted to use receptor-mediated endocytosis mechanism.[17–38] In this study, pullulan was selected as the material for a non-viral carrier because it is thought that it is internalized into cells by way of the sugar-recognizable receptor. We have demonstrated that the receptor internalizes the pullulan carrier and enhances the *in vitro* level of gene expression more than the cationized polymer carrier, which the receptor-independent mechanism is known to internalize.[12] Spermine was used for cationization of pullulan for 2 reasons. One is the need for the amine groups to allow the plasmid DNA to complex ionically. The other is an inherent property of the "buffering effect" for better endosomal escape of plasmid DNA than other amino compounds.[39] It is conceivable that the spermine-pullulan-plasmid DNA complex travels in the cytosol by simple diffusion, because it does not have any nucleus transfer mechanisms. Once the complex reaches the nuclear membrane, internalization into the nucleus will be necessary for

gene transfection. It has been recognized that non-viral carriers do not have any mechanism for nuclear internalization. Therefore, it is possible that, when the nuclear membrane disappears in cell division, the complex of non-viral carrier and plasmid DNA eventually enters the nucleus for gene transfection. Based on this mechanism, the transfection efficiency of plasmid DNA by the non-viral carrier will increase as the proliferation of cells to be transfected increases. In the reverse transfection method, cells are cultured in the presence of serum. It is possible that the presence of serum enables cells to proliferate more efficiently under better culture conditions, resulting in enhanced gene transfection. Cell viability after reverse transfection culture was significantly higher than with conventional transfection culture in the absence of FCS. This strongly indicates that reverse transfection is performed under culture conditions good for cell activity. Cells always make contact with the complex in reverse transfection culture, which is different from the contact time of 6 h in conventional transfection culture.

To allow cells to proliferate under *in vitro* culture conditions, it is necessary to contrive the local environment of cells, including the medium, the substrate of cell attachment and proliferation, the supply of oxygen and nutrients, and the excretion of waste. Some trials have been performed to accelerate the *in vitro* proliferation of MSCs. MSCs proliferated more efficiently with the addition of basic fibroblast growth factor to the culture medium[40,41] and the surface modification of culture substrates.[41] Because MSC proliferation is substrate-dependent, it is preferable to increase the surface area of culture substrate. Several 3-dimensional substrates, so-called scaffolds, have been designed to demonstrate their feasibility in proliferation enhancement.[42–44] In adddition, culture methods have been designed from the viewpoint of the supply of oxygen and nutrients and the excretion of waste. Stirring and perfusion culture methods were effective in enhancing the rate of MSC proliferation compared with the static culture method.[16,39]

For the agitated and stirring culture methods, because the culture medium is circulated, oxygen and nutrients are supplied to MSCs, and cellular wastes are excreted more efficiently than with the static culture method without active medium circulation. The plasmid DNA-carrier complex does not have any inherent potential to allow the plasmid DNA to integrate positively into the genome of cells. Considering the gene transfection mechanism, it is likely that the plasmid DNA has a chance to internalize into the nucleus of cells for gene expression only when the nuclear membrane of cells disappears in cell division. The medium-circulated culture method promoted cell proliferation (Fig. 5). Taken together, it is highly conceivable that more-efficient proliferation of MSCs under better biological conditions promotes the internalization of complex without any lethal damage to cells, resulting in enhanced gene expression. In conclusion, the reverse transfection method combined with the stirring cell culture method in the presence of the adhesion substance is a promising technology to enhance the efficiency of gene expression for stem cells. This technology is applicable to any type of cell. It is expected that this transfection method with the non-viral pullulan carrier can be applied to genetic engineering for cell therapy as well as basic research into stem cell biology and medicine.

REFERENCES

1. Gafni Y, Turgeman G, Liebergal M, *et al.* Stem cells as vehicles for orthopedic gene therapy. Gene Ther **11,** 417, 2004.
2. Chang S.C., Chuang H.L., Chen YR, *et al.* Ex vivo gene therapy in autologous bone marrow stromal stem cells for tissue-engineered maxillofacial bone regeneration. Gene Ther **10,** 2013, 2003.
3. Cho K.C., Kim S.H., Jeong J.H., *et al.* Folate receptor-mediated gene delivery using folate-poly(ethylene glycol)-poly(L-lysine) conjugate. Macromol Biosci **5,** 512, 2005.
4. Turgeman G, Pittman D.D., Muller R, *et al.* Engineered human mesenchymal stem cells: a novel platform for skeletal cell mediated gene therapy. J Gene Med **3,** 240, 2001.
5. Mangi A.A., Noiseux N, Kong D, *et al.* Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med **9,** 1195, 2003.
6. Sato H, Kuwashima N, Sakaida T, *et al.* Epidermal growth factor receptor-transfected bone marrow stromal cells exhibit enhanced migratory response and therapeutic potential against murine brain tumors. Cancer Gene Ther **12,** 757, 2005.
7. Studeny M, Marini F.C., Dembinski J.L., *et al.* Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents. J Natl Cancer Inst **96,** 1593, 2004.
8. De Smedt S.C., Demeester J, Hennink W.E. Cationic polymer based gene delivery systems. Pharm Res **17,** 113, 2000.
9. Ewert K, Slack N.L., Ahmad A, *et al.* Cationic lipid-DNA complexes for gene therapy: understanding the relationship between complex structure and gene delivery pathways at the molecular level. Curr Med Chem **11,** 133, 2004.
10. Pedroso de Lima M.C., Neves S, Filipe A, *et al.* Cationic liposomes for gene delivery: from biophysics to biological applications. Curr Med Chem **10,** 1221, 2003.
11. Hirko A, Tang F, Hughes J.A. Cationic lipid vectors for plasmid DNA delivery. Curr Med Chem **10,** 1185, 2003.
12. Jo J, Yamamoto M, Okazaki A, *et al.* Enhancement of *in vitro* gene transfection efficiency of adult stem cells by cationized polysaccharide derivatives. Polymer Preprints, Japan **54,** 2142, 2005.
13. Hermanson G.T. Bioconjugate Techniques. San Diego, CA: Academic Press, 1996.
14. Lennon D.P., Haynesworth S.E., Young R.G., *et al.* A chemically defined medium supports *in vitro* proliferation and maintains the osteochondral potential of rat marrow-derived mesenchymal stem cells. Exp Cell Res **219,** 211, 1995.
15. Habeeb A.F. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal Biochem **14,** 328, 1966.
16. Takahashi Y, Tabata Y. Homogeneous seeding of mesenchymal stem cells into nonwoven fabric for tissue engineering. Tissue Eng **9,** 931, 2003.
17. Sudimack J, Lee R.J. Targeted drug delivery via the folate receptor. Adv Drug Deliv Rev **41,** 147, 2000.

18. Lu Y, Low P.S. Folate-mediated delivery of macromolecular anticancer therapeutic agents. Adv Drug Deliv Rev **54,** 675, 2002.
19. Zhao X.B., Lee R.J. Tumor-selective targeted delivery of genes and antisense oligodeoxyribonucleotides via the folate receptor. Adv Drug Deliv Rev **56,** 1193, 2004.
20. Mislick K.A., Baldeschwieler J.D., Kayyem J.F., *et al.* Transfection of folate-polylysine DNA complexes: evidence for lysosomal delivery. Bioconjug Chem **6,** 512, 1995.
21. Hashida M, Nishikawa M, Yamashita F, *et al.* Cell-specific delivery of genes with glycosylated carriers. Adv Drug Deliv Rev **52,** 187, 2001.
22. Bies C, Lehr C.M., Woodley J.F. Lectin-mediated drug targeting: history and applications. Adv Drug Deliv Rev **56,** 425, 2004.
23. Choi Y.H., Liu F, Choi J.S., *et al.* Characterization of a targeted gene carrier, lactose-polyethylene glycol-grafted poly-L-lysine and its complex with plasmid DNA. Hum Gene Ther **10,** 2657, 1999.
24. Mahato R.I., Takemura S, Akamatsu K, *et al.* Physicochemical and disposition characteristics of antisense oligonucleotides complexed with glycosylated poly(L-lysine). Biochem Pharmacol **53,** 887, 1997.
25. Ferkol T, Perales J.C., Mularo F, *et al.* Receptor-mediated gene transfer into macrophages. Proc Natl Acad Sci U S A **93,** 101, 1996.
26. Chowdhury N.R., Wu C.H., Wu G.Y., *et al.* Fate of DNA targeted to the liver by asialoglycoprotein receptor-mediated endocytosis *in vivo*. Prolonged persistence in cytoplasmic vesicles after partial hepatectomy. J Biol Chem **268,** 11265, 1993.
27. Parkes R.J., Hart S.L. Adhesion molecules and gene transfer. Adv Drug Deliv Rev **44,** 135, 2000.
28. Erbacher P, Remy J.S., Behr J.P. Gene transfer with synthetic virus-like particles via the integrin-mediated endocytosis pathway. Gene Ther **6,** 138, 1999.
29. Hart S.L., Harbottle R.P., Cooper R, *et al.* Gene delivery and expression mediated by an integrin-binding peptide. Gene Ther **2,** 552, 1995.
30. Colin M, Maurice M, Trugnan G, *et al.* Cell delivery, intracellular trafficking and expression of an integrin-mediated gene transfer vector in tracheal epithelial cells. Gene Ther **7,** 139, 2000.
31. Schiffelers R.M., Ansari A, Xu J, *et al.* Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. Nucleic Acids Res **32,** e149, 2004.
32. Wagner E, Cotten M, Foisner R, *et al.* Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. Proc Natl Acad Sci U S A **88,** 4255, 1991.
33. Qian Z.M., Li H, Sun H, *et al.* Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacol Rev **54,** 561, 2002.
34. Kircheis R, Wightman L, Schreiber A, *et al.* Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application. Gene Ther **8,** 28, 2001.
35. Sosnowski B.A., Gonzalez A.M., Chandler LA, *et al.* Targeting DNA to cells with basic fibroblast growth factor (FGF2). J Biol Chem **271,** 33647, 1996.
36. Blessing T, Kursa M, Holzhauser R, *et al.* Different strategies for formation of pegylated EGF-conjugated PEI/DNA complexes for targeted gene delivery. Bioconjug Chem **12,** 529, 2001.
37. Kircheis R, Kichler A, Wallner G, *et al.* Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery. Gene Ther **4,** 409, 1997.
38. Shimizu N, Chen J, Gamou S, *et al.* Immunogene approach toward cancer therapy using erythrocyte growth factor receptor-mediated gene delivery. Cancer Gene Ther **3,** 113, 1996.
39. Hosseinkhani H, Inatsugu Y, Hiraoka Y, *et al.* Perfusion culture enhances osteogenic differentiation of rat mesenchymal stem cells in collagen sponge reinforced with poly (glycolic Acid) fiber. Tissue Eng **11,** 1476, 2005.
40. Tsutsumi S, Shimazu A, Miyazaki K, *et al.* Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. Biochem Biophys Res Commun **288,** 413, 2001.
41. Hori Y, Inoue S, Hirano Y, *et al.* Effect of culture substrates and fibroblast growth factor addition on the proliferation and differentiation of rat bone marrow stromal cells. Tissue Eng **10,** 995, 2004.
42. Yasuda K, Inoue S, Tabata Y. Influence of culture method on the proliferation and osteogenic differentiation of human adipostromal cells in nonwoven fabrics. Tissue Eng **10,** 1587, 2004.
43. Takahashi Y, Tabata Y. Effect of the fiber diameter and porosity of non-woven PET fabrics on the osteogenic differentiation of mesenchymal stem cells. J Biomater Sci Polym Ed **15,** 41, 2004.
44. Takahashi Y, Yamamoto M, Tabata Y. Osteogenic differentiation of mesenchymal stem cells in biodegradable sponges composed of gelatin and beta-tricalcium phosphate. Biomaterials **26,** 3587, 2005.

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Plectin 1 links intermediate filaments to costameric sarcolemma through β-synemin, α-dystrobrevin and actin

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Summary

In skeletal muscles, the sarcolemma is possibly stabilized and protected against contraction-imposed stress by intermediate filaments (IFs) tethered to costameric sarcolemma. Although there is emerging evidence that plectin links IFs to costameres through dystrophin-glycoprotein complexes (DGC), the molecular organization from plectin to costameres still remains unclear. Here, we show that plectin 1, a plectin isoform expressed in skeletal muscle, can interact with β-synemin, actin and a DGC component, α-dystrobrevin, in vitro. Ultrastructurally, β-synemin molecules appear to be incorporated into costameric dense plaques, where they seem to serve as actin-associated proteins rather than IF proteins. In fact, they can bind actin and α-dystrobrevin in vitro. Moreover, in vivo immunoprecipitation analyses demonstrated that β-synemin- and plectin-immune complexes from lysates of muscle light microsomes contained α-dystrobrevin, dystrophin, nonmuscle actin, metavinculin, plectin and β-synemin. These findings suggest a model in which plectin 1 interacts with DGC and integrin complexes directly, or indirectly through nonmuscle actin and β-synemin within costameres. The DGC and integrin complexes would cooperate to stabilize and fortify the sarcolemma by linking the basement membrane to IFs through plectin 1, β-synemin and actin. Besides, the two complexes, together with plectin and IFs, might have their own functions as platforms for distinct signal transduction.

Supplementary material available online at
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Key words: Costamere, Dystrobrevin, Dystrophin-glycoprotein complex, Plectin, Synemin

Introduction

Skeletal muscle fibers possess heteropolymeric intermediate filaments (IFs) comprised of desmin, synemin, and paranemin (Bilak et al., 1998; Granger and Lazarides, 1980; Price and Lazarides, 1983; Schweitzer et al., 2001). These IFs are located at the periphery of myofibrillar Z-discs, interlink adjacent myofibrils at the Z-line level, and anchor onto costameres beneath the sarcolemma (Craig and Pardo, 1983; Fujimaki et al., 1986; Lazarides, 1980; Tokuyasu et al., 1983). The importance of IF-interlinkages between adjacent Z-discs and IF-anchorage on costameric sarcolemma in muscle function and integrity is supported by investigations of desmin-knockout mice, in which muscle fibers lacking desmin IFs develop reduced maximum force and their sarcolemmae are more susceptible to damage during contraction (Balogh et al., 2003; Li et al., 1996; Li et al., 1997; Sam et al., 2000). However, molecular organization of the linkages from IFs to Z-discs and costameres still remains unclear.

Within costameres, dystrophin-glycoprotein complex (DGC) provides structural support to the sarcolemma by linking the actin-based cytoskeleton with the extracellular matrix (ECM) (for a review, see Ozawa, 2006). It is comprised of dystrophin, α- and β-dystroglycan, sarcoglycans, sarcospan, α-dystrobrevin and syntrophin. Dystrophin is associated with β-dystroglycan and extracellular α-dystroglycan, which in turn binds laminin-2 in the basement membrane (BM) (Ervasti and Campbell, 1993), while it is indirectly associated with a group of five integral membranous proteins, the sarcoglycans and sarcospan (Ozawa et al., 1998; Yoshida et al., 1994). Other subsarcolemmal proteins in the complex include α-dystrobrevin and syntrophin, which directly interact with dystrophin (Ahn et al., 1996; Sadoulet-Puccio et al., 1997).

β-Synemin is a constituent of heteropolymeric IF in muscles, and has a very short N-terminal head domain, a central α-helical rod domain conserved in all IF proteins, and a very long C-terminal tail domain (Titeux et al., 2001). β-synemin molecules are assumed to link IFs to DGC, based on the in vitro findings that they can bind α-dystrobrevin, dystrophin and desmin (Bhosle et al., 2006; Mizuno et al., 2001). However, their binding sites for desmin, α-dystrobrevin and dystrophin are all confined to their rod domains, thereby raising a possibility that heteropolymerization of β-synemin with desmin may prevent β-synemin from binding α-dystrobrevin and/or dystrophin and therefore from linking IFs to DGC due to their competition for the rod domains.

Another candidate for the linker between IFs and costameres is the versatile crosslinker protein plectin. Plectin harbors a binding site for IF proteins at its C-terminus (Foisner et al., 1988; Nikolic et al., 1996; Reipert et al., 1999), whereas its N-terminal parts include binding domains for actin and integrin β4 (Andra et al., 1998; Geerts et al., 1999; Rezniczek et al., 1998). With these binding properties, plectin links cytokeratin IFs to the plasma membrane at hemidesmosomes of epidermal cells (Hieda et al., 1992; Litjens et al., 2006). By analogy, it is conceivable that plectin links desmin IFs to costameric sarcolemma. In fact, our previous immunoelectron microscopic study ultrastructurally revealed that plectin-labeled fine threads linked IFs to dystrophin- or vinculin-containing subsarcolemmal dense plaques, or costameres (Hijikata at al., 2003). In that study, however, we did not identify plectin-binding partners within costameres and, therefore, could not fully explore molecular organization from IFs to costameres through plectin.

To address these unexplored subjects, the present study was undertaken just to identify β-synemin as a protein binding plectin N-terminal fragments. The present immuno-EM analyses revealed that β-synemin was incorporated into costameric dense plaques associated with plectin threads. Furthermore, our in vitro analyses showed that β-synemin could bind actin as well as α-dystrobrevin, whereas plectin 1 – a plectin isoform expressed in skeletal muscles – could interact with α-dystrobrevin, β-synemin and actin. Based on the results obtained here, we propose a model of molecular organization from IF to costameres that includes DGC and integrin complexes.

Results

Identification of β-synemin as a plectin-1-binding protein

Our previous immunoelectron microscopic analysis of rat skeletal muscle fibers revealed that plectin-labeled fine threads extended from desmin IFs to subsarcolemmal dense plaques containing dystrophin (Hijikata et al., 2003). Given this and the desmin-binding property of plectin at its C-terminus (Reipert et al., 1999), N-terminal parts of plectin would be expected to interact with as yet unidentified molecules within dystrophin-containing dense plaques. Thus, N-terminal fragments of plectin 1, an isoform of plectin expressed in skeletal muscles (Fuchs et al., 1999), were used as a probe for blot overlay assay to identify plectin-1-binding proteins within the subsarcolemmal dense plaques. The crude IF fraction prepared from muscle light microsome (LM) with detergents was separated by SDS-PAGE and blotted onto PVDF membranes. The membranes were overlaid with Myc-tagged N-terminal plectin-1-recombinant proteins (PleN1, 1-1273 aa), followed by detection of plectin-binding proteins with anti-Myc antibody. This blot overlay assay detected some Myc-positive bands representing possible plectin-1-binding proteins (data not shown).

Of these possible plectin-1-binding proteins, we focused our analysis on a protein of ~160 kDa (supplementary material Fig. S1A). We determined its partial amino acid sequence and found that it contained PHEFH and VQLQRMVDQRS sequences. These amino acid sequences were used to search the GenBank database using BLASTP for similar sequences. The two sequences were identical to those of KIAA0353, an incomplete cDNA sequence isolated from a human brain cDNA library (GenBank™ accession number AB002351). To obtain the full-length cDNA, cloning of the 160 kDa plectin-1-binding protein was performed in a cDNA library prepared from skeletal muscle mRNA of newborn rats. By determining the complete DNA sequences of the obtained clones, we identified the plectin-1-binding protein as desmuslin (GenBank accession number AB091769). Desmuslin, a novel member of IF proteins, was found as a α-dystrobrevin-binding protein by the yeast-two-hybrid system (Mizuno et al., 2001). Comparison of the previously reported human and the present rat sequences revealed an overall identity of 72.4%. Desmuslin has subsequently been referred to as β-synemin (Mizuno et al., 2004; Titeux et al., 2001), and this name is also used in this article. The interactions of plectin with β-synemin and then α-dystrobrevin, a dystrophin-associated protein, agreed with our previous findings of the association of plectin threads with dystrophin-containing dense plaques.

Plectin 1 interacts with β-synemin in vitro and in vivo

In vitro interactions between plectin and β-synemin were further verified by pull-down assays using recombinant plectin 1 and β-synemin proteins. GST-fused full-length β-synemin was incubated with either Myc-tagged N-terminal plectin 1 (PleN1) fragments or Myc-tagged β-galactosidase (LacZ) and then immunoprecipitated by using anti-Myc antibody and protein L-agarose. The results indicated that PleN1 fragments coimmunoprecipitated with β-synemin, but neither LacZ nor PleN1 did this in combination with control IgG (supplementary material Fig. S1B). A reciprocal pull-down assay was performed using glutathione beads. GST-fused β-synemin pulled down PleN1, but not LacZ, whereas GST protein alone did not precipitate PleN1 (supplementary material Fig. S1C).

For in vivo analysis, plectin or β-synemin immune complex was immunoprecipitated from lysates of muscle LM using anti-plectin or anti-β-synemin antibody, respectively. Subsequent immunoblotting for β-synemin or plectin indicated that plectin and β-synemin formed protein complexes in vivo as well (supplementary material Fig. S1D). In control experiments, neither plectin nor β-synemin was immunoprecipitated by control IgG.

Localization of β-synemin with plectin and α-dystrobrevin at costameric sarcolemma

To assess the localization of β-synemin relative to plectin and α-dystrobrevin in skeletal muscle, rat diaphragm cryosections were doubly immunolabeled with anti-β-synemin and anti-plectin or anti-α-dystrobrevin antibodies, and observed by confocal laser scanning microscopy. As shown in Fig. 1A-C, β-synemin completely colocalized with plectin, which was found around Z-discs and beneath the sarcolemma (Hijikata et al., 1999; Hijikata et al., 2003; Schröder et al., 1997; Schröder et al., 1999). On longitudinal sections, both β-synemin and plectin displayed striated staining of Z-lines and subsarcolemmal intermittent staining confined to areas overlying Z-lines or costameres (Fig. 1D-F). By contrast, α-dystrobrevin was more diffusely distributed along the sarcolemma (Fig. 1H,K,N,Q). Doubly immunostained tangential sections including the sarcolemma clearly delineated costameric striations coinciding between β-synemin and α-dystrobrevin (Fig. 1J-L), whereas the longitudinal sections displayed intermittent superimposition of β-synemin-staining on diffuse α-dystrobrevin-staining along the sarcolemma (Fig. 1M-O). Consistent with the present observation of its complete colocalization with β-synemin, plectin also colocalized with α-dystrobrevin at costameres (Fig. 1P-R). These results indicated the colocalization of β-synemin and plectin with α-dystrobrevin at the costameric sarcolemma.

To further explore the precise localization of β-synemin beneath the sarcolemma, immunogold electron microscopy (EM) analysis was performed in rat skeletal muscle fibers chemically skinned with saponin. In this EM analysis, specific gold particles indicative of

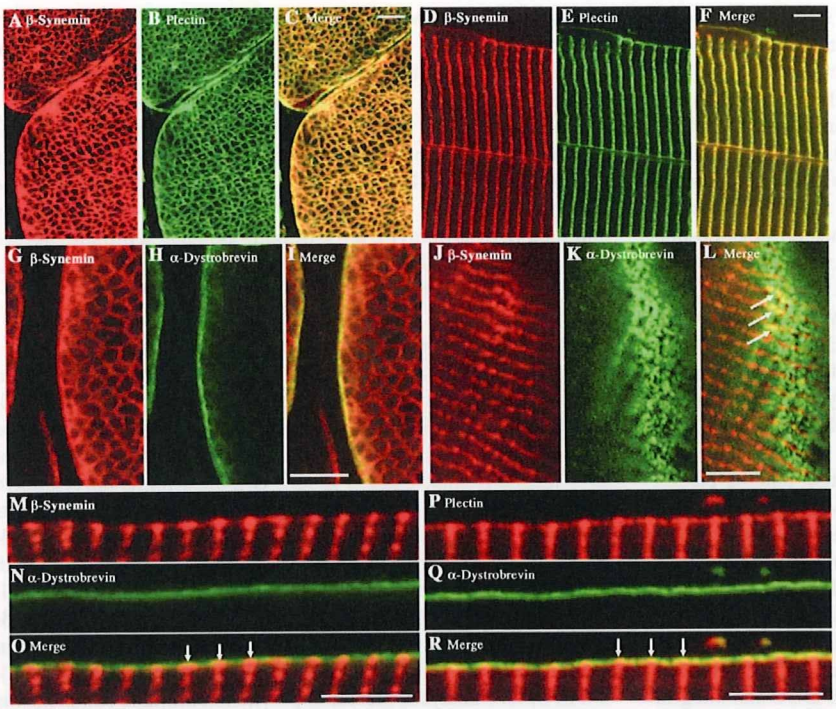

Fig. 1. (A-R) Colocalization of β-synemin with plectin and α-dystrobrevin at the costameric sarcolemma. β-Synemin colocalized precisely with plectin around Z-discs and beneath the sarcolemma (A-F), whereas it colocalized with α-dystrobrevin beneath the sarcolemma (G-O), especially at costameric regions (L,O, arrows). Plectin also colocalized with α-dystrobrevin at costameres (P-R, arrows in R). Bars, 5 μm.

β-synemin were found along IFs, on the cytoplasmic side of subsarcolemmal dense plaques overlying Z-lines, namely costameres, and between IFs and costameric dense plaques (Fig. 2). The association of β-synemin-specific gold particles along IFs was consistent with the notion that β-synemin, together with desmin and paranemin, formed heteropolymeric IF (Hemken et al., 1997; Hirako et al., 2003). Extensive observations disclosed that IFs were interlinked with costameric dense plaques by fine threads, possibly plectin molecules, on both sides of which β-synemin labels were localized (Fig. 2C). Costameric dense plaques without association of IF were also immunolabeled with the gold particles (Fig. 2B). These results indicated discrete incorporation of β-synemin molecules into two distinct sites: IFs and costameric dense plaques.

Mapping of subdomains interacting between plectin 1 and β-synemin

Pull-down assay was carried out to define more precisely the β-synemin subdomains involved in the interaction with PleN1. Based on the β-synemin domain structure consisting of head, rod and tail domains, ten β-synemin mutant recombinant proteins were generated by subcloning rat β-synemin cDNA into a GST expression system (Fig. 3A). These mutant β-synemin proteins were incubated with Myc-tagged PleN1 and pulled down using anti-Myc antibody and protein L-agarose. The mutant proteins (Tail N1, N2 and Rod C), including the N-terminal part of β-synemin tail domains, were pulled down using PleN1. In addition, mutant proteins (Rod Ms and Ml) including the C-terminal part of the rod domain (rod domain 2B) were also precipitated, but in smaller amounts. Without anti-Myc antibody, however, none of the β-synemin mutant proteins were pulled down. These results indicated that the part of β-synemin around the boundary between its rod and tail domains preferentially interacted with PleN1.

Next, to refine β-synemin-interacting sites on PleN1, reciprocal pull-down experiments were performed using GST-fused N-terminal tail fragments of β-synemin (Tail N1) and a variety of mutant plectin recombinant fragments, as shown in Fig. 3B. β-Synemin Tail N1 fragments precipitated mutant plectin 1 fragments, including the

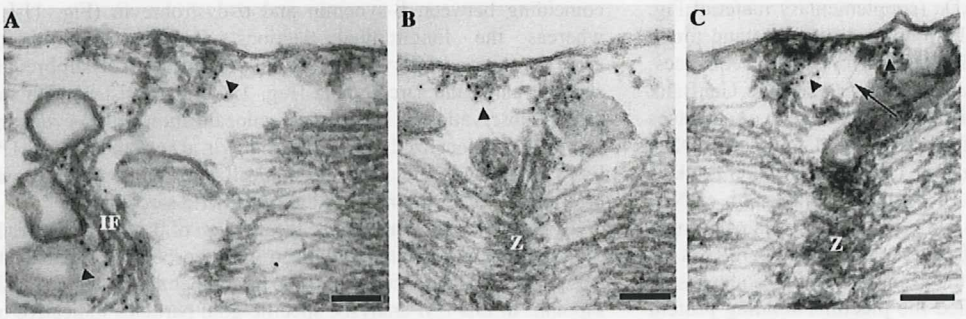

Fig. 2. Immunoelectron microscopic analyses indicate the localization of β-synemin along IFs and in costameres. (A,B) Gold particles labeling β-synemin (arrowheads) were distributed along IFs and on subsarcolemmal dense plaques overlying Z-lines, or costameres. (C) A thin thread (arrow), possibly plectin molecules, projects from IF to a dense plaque labeled with gold particles. Bars, 0.1 μm.

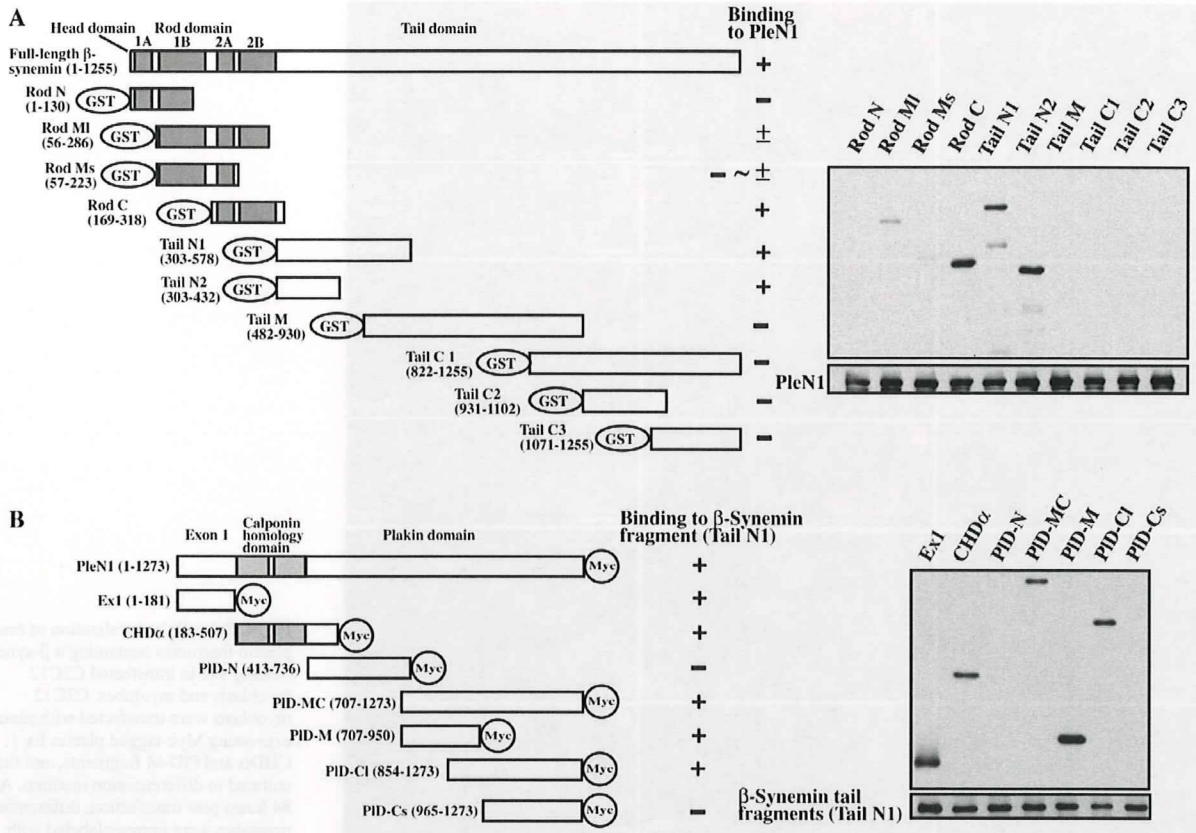

Fig. 3. Mapping of the interacting sites between N-terminal plectin 1 (PleN1) fragments and full-length β-synemin by in vitro pull-down assay. (A) Schematic representations of the domain structures of full-length β-synemin and GST-fused recombinant β-synemin fragments used in the pull-down assay. The in vitro PleN1-binding phenotypes of mutant β-synemin fragments are summarized as + (strong binding) or ± (weak binding). GST-fused β-synemin fragments were incubated with Myc-tagged PleN1 and immunoprecipitated by anti-Myc antibody and protein L-agarose. These immunoprecipitates were subjected to immunoblotting with detection by anti-GST antibody. Each lane contained equivalent amounts of the immunoprecipitate, as represented by immunoblots of PleN1. (B) Domain structures of PleN1 and Myc-tagged recombinant plectin fragments, together with a summary of in vitro binding ability of plectin fragments to β-synemin tail fragments (Tail N1). Plectin fragments were incubated with GST-fused Tail N1 and pulled down using glutathione beads, followed by immunoblotting with anti-Myc antibody. Each lane contained equivalent amounts of the immunoprecipitate, as represented by immunoblots of Tail N1.

first exon product, calponin-homology domains and the middle portion of plakin domain (Fig. 3B), indicating that PleN1 fragments included three β-synemin-binding sites.

Subcellular localization of mutant plectin fragments relative to β-synemin in C2C12 cells

The present mapping revealed that three mutant plectin 1 fragments, Ex1, CHDα and PlD-M could interact with β-synemin in vitro. To further assess these interactions within cells, we expressed the three Myc-tagged plectin fragments in C2C12 myoblasts and myotubes, and examined their subcellular localization relative to β-synemin by double immunostaining with anti-Myc and anti-β-synemin antibodies. Before the transfection experiments, we examined subcellular localization of β-synemin by immunohistochemistry using anti-β-synemin antibody and fluorescent phallotoxins in C2C12 myoblasts and myotubes. In most of C2C12 myoblasts, β-synemin was expressed in a diffuse dotted pattern throughout the cytoplasm, whereas in C2C12 myotubes and some myoblasts, it was localized along stress-fiber-like structures (SFLSs) or immature myofibrils in an intermittent pattern, in addition to its dotted sarcoplasmic distribution (Fig. 4A-F).

The three plectin fragments colocalized at least partly with β-synemin in C2C12 cells (Fig. 4J-X), suggesting their intracellular interactions with β-synemin. Plectin Ex1 fragments were primarily localized in the nucleus and did not colocalize with β-synemin in most of the transfectants (Fig. 4G-I). In some transfectants, however, they were weakly distributed throughout cytoplasm with faint staining of their colocalization with β-synemin (Fig. 4J-L). These results could be interpreted as follows: overexpression of Ex1 fragments resulted in their distribution at the cytoplasm, where they were preferentially distributed along β-synemin-associated SFLSs, although the fragments were mostly localized in the nucleus because of a putative nuclear localization signal included in them (Rezniczek et al., 2003). Plectin CHDα fragments appeared to colocalize with β-synemin along SFLS and in the sarcoplasm, where they were distributed in a pattern somewhat more patchy than a dotted pattern of β-synemin (Fig. 4M-R). Plectin PlD-M fragments were locally distributed in an irregularly intermittent pattern along a subset and some parts of β-synemin-associated SFLSs, and almost lack their sarcoplasmic distribution, indicating their partial colocalization with β-synemin along some portions of SFLSs (Fig. 4S-X).

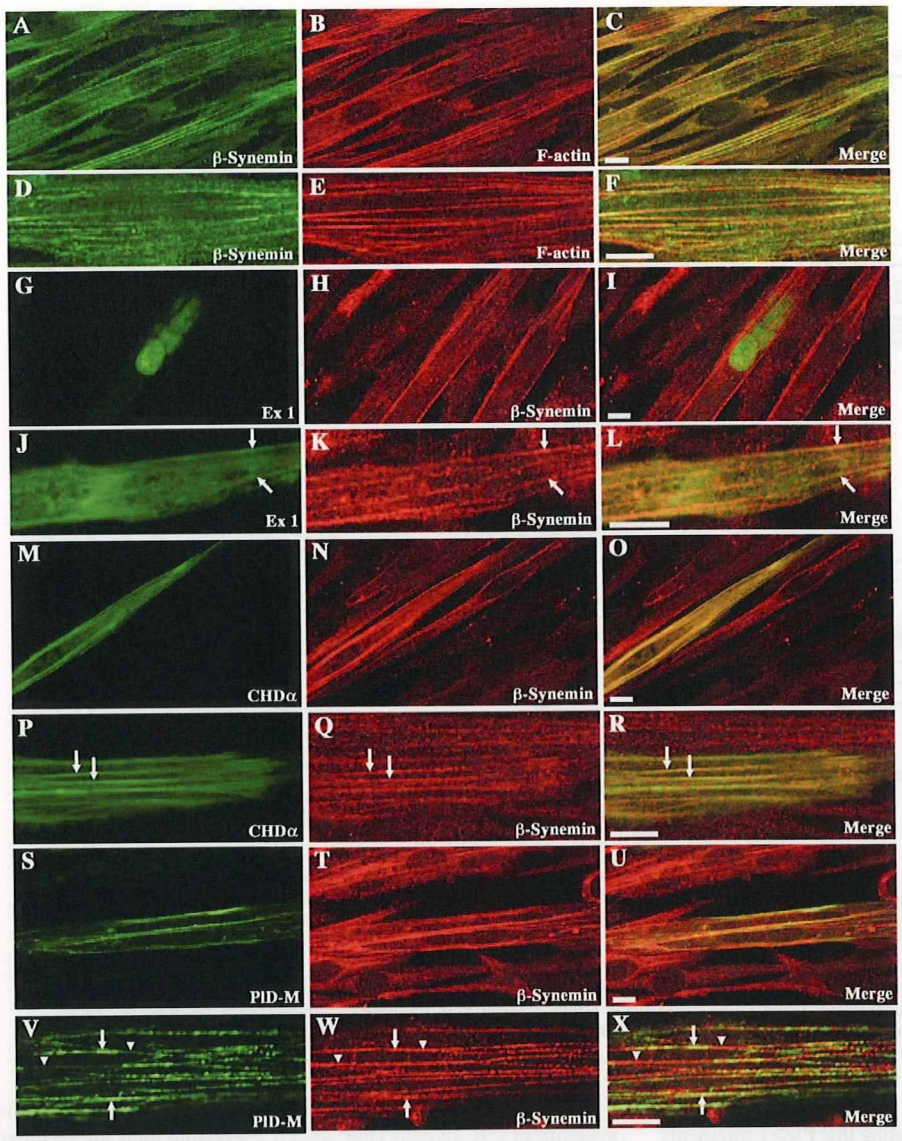

Fig. 4. Subcellular localization of mutant plectin fragments containing a β-synemin-binding site in transfected C2C12 myoblasts and myotubes. C2C12 myoblasts were transfected with plasmids expressing Myc-tagged plectin Ex 1, CHDα and PlD-M fragments, and then cultured in differentiation medium. At 60-84 hours post transfection, differentiated myotubes were immunolabeled with antibodies specific for Myc and β-synemin. Localization of β-synemin along SFLSs and throughout the sarcoplasm in a dotted pattern was found in control myotubes doubly immunolabeled with Alxea-Fluor-594-conjugated phallotoxins and anti-β-synemin pAb (A-F). The three mutant plectin fragments more or less colocalized with β-synemin along SFLS (arrows in J-L,P-R,V-X). Arrowheads in V and X indicate sites associated predominantly with β-synemin, but scarcely with PlD-M. Bars, 10 μm.

In vitro interactions of plectin 1 and β-synemin with F-actin

The present pull-down assay showed that plectin CHDα, including actin-binding sites, interacted with β-synemin tail fragments (Tail N1). This prompted us to examine the effect of β-synemin Tail N1 on the actin-binding of plectin CHDα. First, we confirmed in vitro interaction of plectin CHDα with F-actin. Consistent with previous reports (Fontao et al., 2001; Geerts et al., 1999), plectin CHDα co-sedimented with F-actin, whereas it was found in the soluble fraction in the absence of F-actin (Fig. 5, lanes 2 and 3). Next, we tested in vitro interaction of β-synemin Tail N1 with F-actin. Unexpectedly, β-synemin Tail N1 fragments slightly co-sedimented with F-actin, but were hardly found in the pellet fraction in the absence of F-actin (Fig. 5, lanes 4-6). These results indicated a direct, but weak association of β-synemin tail fragments with F-actin. In a co-sedimentation assay to assess the effect of β-synemin Tail N1, F-actin was polymerized in the presence of plectin CHDα, incubated with a tenfold molar excess of β-synemin Tail N1, and then sedimented by centrifugation. Even in the presence of β-synemin Tail N1, plectin CHDα was found to be associated with F-actin and almost absent in the soluble fraction (Fig. 5, lanes 7 and 8). These results indicated that β-synemin Tail N1 did not inhibit interactions of plectin CHDα with F-actin under this experimental condition.

In vitro interactions of plectin 1 with β-synemin and α-dystrobrevin

The present pull-down assays demonstrated that plectin 1 bound the rod domain 2B and N-terminal tail domain of β-synemin, whereas a previous study reported that α-dystrobrevin interacted with the β-synemin rod domain (Mizuno et al., 2001). These findings implied that plectin 1 and α-dystrobrevin compete with each other for β-synemin owing to the partial overlap of their interacting sites on the rod domain – although we initially presumed that plectin 1 interacted with β-synemin, which in turn bound

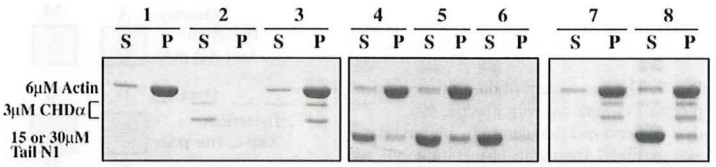

Fig. 5. In vitro interactions of plectin 1 and β-synemin with F-actin. Bindings of plectin CHDα and β-synemin Tail N1 fragments to F-actin were demonstrated by actin co-sedimentation assay. After centrifugation, most of CHDα fragments (3 μM) were found in the pellet (P) in the presence of F-actin (6 μM), whereas they remained in the supernatant (S) in the absence of actin (lanes 2 and 3). Small amounts of β-synemin Tail N1 fragments were sedimented in the presence of F-actin (15 μM and 30 μM β-synemin in lanes 4 and 5, respectively), while in the absence of F-actin, the Tail N1 fragments (30 μM) were observed mostly in the supernatant (lanes 6). Even in the presence of a 10-fold molar excess of Tail N1 fragments (30 μM), CHDα fragments were still found mostly in the pellet together with F-actin (compare lanes 7 with lanes 8).

α-dystrobrevin. This presumptive molecular array of plectin 1, β-synemin and α-dystrobrevin was examined in immunoprecipitation experiments using Myc-tagged PleN1 fragments, GST-fused full-length β-synemin and α-dystrobrevin fragments including products of exons 8 to 16, which were indispensable for interactions with β-synemin (Mizuno et al., 2001). Prior to this experiment, interactions of β-synemin with α-dystrobrevin were ascertained by pull-down assay. Using glutathione beads, GST-fused β-synemin pulled down α-dystrobrevin fragments, whereas GST protein alone did not (Fig. 6A). Next, interactions of plectin 1 with α-dystrobrevin were also tested by immunoprecipitation. In this experiment, Myc-tagged LacZ recombinant proteins and N-terminal fragments of plectin 1f (PleN1f) were used as control and comparison samples, respectively. Plectin 1f is an alternative variant differing from plectin 1 only in the first exon and is most likely to interact with α-dystrobrevin, because it is preferentially distributed along the sarcolemma of skeletal muscle fibers (Rezniczek et al., 2007). In contrast to this prediction, only PleN1 coimmunoprecipitated with α-dystrobrevin fragments, whereas neither PleN1f nor LacZ did (Fig. 6B). These results indicated that plectin 1 directly interacts with α-dystrobrevin through its unique exon 1 part.

To assess the formation of the presumptive molecular array, full-length β-synemin, α-dystrobrevin fragments and Myc-tagged PleN1, PleN1f or LacZ were incubated and then coimmunoprecipitated using anti-Myc antibody and protein L-agarose beads. As shown in Fig. 6C, PleN1 fragments coimmunoprecipitated with α-dystrobrevin fragments as well as β-synemin, whereas PleN1f fragments coimmunoprecipitated with β-synemin but not α-dystrobrevin fragments. Taking account of β-synemin- and α-dystrobrevin-binding sites on plectin 1 and plectin 1f, these results suggest that plectin 1 and plectin 1f can bind β-synemin at their CH and/or plakin domains, thereby probably preventing the association of β-synemin with α-dystrobrevin, whereas plectin 1 can interact further with α-dystrobrevin or both α-dystrobrevin and β-synemin through its exon 1 part. In control experiments, neither LacZ fragments nor PleN1 in combination with control IgG significantly precipitated α-dystrobrevin and β-synemin.

In vitro interactions of plectin 1 with α-dystrobrevin, F-actin and β-synemin

The results presented so far indicate that plectin PleN1 fragments can interact with α-dystrobrevin, F-actin and β-synemin. To assess whether plectin 1 forms a molecular complex with all of these three proteins, we performed a blot overlay assay. PleN1 fragments were immobilized on nitrocellulose membrane, overlaid and incubated with one of the three proteins or the mixture of all the proteins, followed by detection of bound proteins using antibody specific for each protein. As shown in Fig. 7, comparison of resulting signals indicated no significant differences between overlays of each protein alone and of the three proteins. These results suggest a possibility that plectin 1 can form molecular complexes with all of α-dystrobrevin, F-actin and β-synemin.

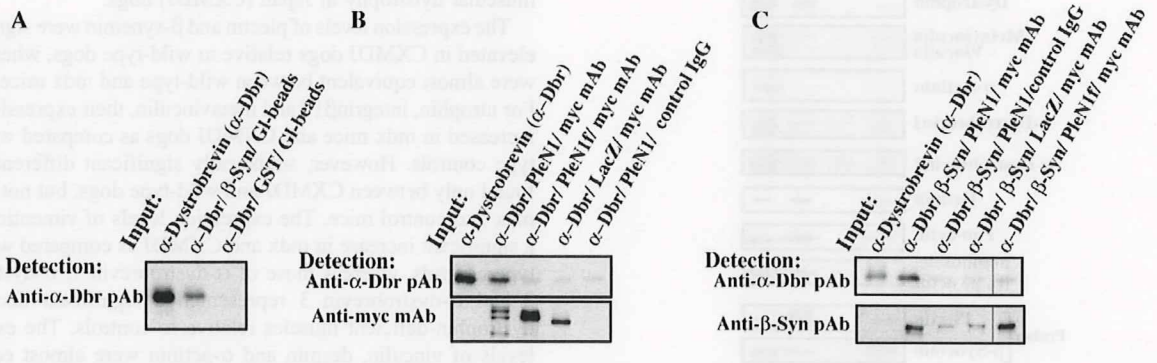

Fig. 6. In vitro interactions of plectin 1 with α-dystrobrevin and β-synemin. (A) α-Dystrobrevin fragments (α-Dbr, 0.8 μg) were incubated with GST-fused β-synemin (β-Syn, 10 μg) or GST (1.8 μg) in the incubation buffer (1 ml) and then precipitated by glutathione beads (Gl-beads, 50 μl). GST-fused β-Syn precipitated α-Dbr, but GST alone did not. (B) α-Dbr (0.5 μg) were incubated with Myc-tagged plectin 1 (PleN1, 9.6 μg) or plectin 1f fragments (PleN1f, 3.2 μg) or β-galactosidase (LacZ, 7.6 μg) in the incubation buffer (1.4 ml) including 0.4% BSA and then immunoprecipitated with anti-Myc antibody (4 μg) and protein L-agarose (40 μl). PleN1 coimmunoprecipitated with α-Dbr, but neither PleN1f nor LacZ did. (C) α-Dbr (0.4 μg) and GST-fused β-Syn (2 μg) were incubated with Myc-tagged PleN1 (9 μg) or PleN1f (3 μg) or LacZ (9 μg) in the incubation buffer (1.2 ml) including 0.4% BSA and then immunoprecipitated with anti-Myc antibody (4 μg) and protein L-agarose (40 μl). PleN1 coimmunoprecipitated with both β-Syn and α-Dbr, whereas PleN1f pulled down only β-Syn. However, LacZ and PleN1 in combination with control IgG pulled down none of them.

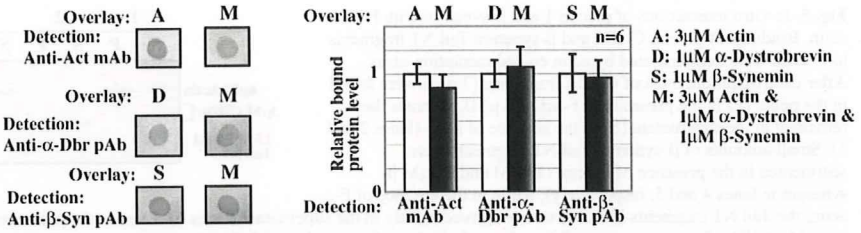

Fig. 7. The assessment of the formation of plectin 1 complexes with F-actin, α-dystrobrevin and β-synemin by blot overlay assays. PleN1 fragments (1 μg) immobilized on nitrocellulose were incubated with 3 μM F-actin (A) or 1 μM α-dystrobrevin fragments (D) or 1 μM GST-fused full-length β-synemin (S) or the mixture of the three proteins (M), followed by detection of bound proteins using antibody specific for each protein. The signals resulting from the overlay with the three proteins were almost equivalent to those from the overlay with each protein alone. Values represent the mean ± s.d. (error bars) of two spots in three independent experiments.

In vivo association of β-synemin and plectin with costameric and cytoskeletal proteins

To explore in vivo association of β-synemin and plectin with costameric components including DGC and cytoskeletal proteins, β-synemin and plectin immune complexes were immunoprecipitated from lysates of muscle LM by anti-β-synemin and anti-plectin antibodies. Subsequently, the presence or absence of proteins in the immune complexes was determined by immunoblotting using antibodies against α-dystrobrevin, dystrophin, pan-actin, (meta)vinculin, desmin and α-actinin. All of the proteins examined, with the exception of α-actinin, were detected in both β-synemin and plectin immune complexes, but control IgG immune complex did not significantly contain any of these proteins (Fig. 8). Desmin coimmunoprecipitated more abundantly with β-synemin than plectin, whereas other proteins, such as dystrophin, metavinculin, α-dystrobrevin 1, α-dystrobrevin 2 and actin, coimmunoprecipitated in greater amounts with plectin. As reported previously (Hijikata et al., 2003), metavinculin, rather than vinculin, was preferentially immunoprecipitated by either of the antibodies.

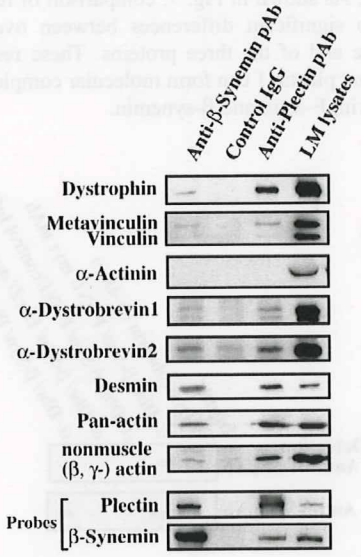

Fig. 8. In vivo interactions of plectin and β-synemin with costameric proteins including DGC. Plectin- and β-synemin-immune complexes were immunoprecipitated from LM lysates by anti-plectin or anti-β-synemin antibodies, respectively. Both the immune complexes contained dystrophin, α-dystrobrevin 1 and α-dystrobrevin 2, meta-vinculin, desmin and actin, including nonmuscle actin, but not α-actinin. In control experiments, control IgG did not significantly coimmunoprecipitate with any proteins.

Next, anti-nonmuscle actin (β- and γ-actin) antibody was used to determine whether costameric nonmuscle actin is associated with β-synemin and plectin in the immune complexes. Both the immune complexes included nonmuscle actin, but its levels were higher in plectin immune complexes. These results suggest that nonmuscle actin, possibly costameric γ-actin, is involved in the interactions of β-synemin and plectin with DGC and other costameric components beneath the sarcolemma. In fact, γ-actin was found to localize predominantly to the costameric sarcolemma, where it seemed to be associated with DGC through dystrophin (Rybakova et al., 2000).

Expression and distribution of IF, IF-associated and DGC proteins in animal models of dystrophin-deficient muscular dystrophy

Since plectin and β-synemin indirectly interacted with dystrophin through α-dystrobrevin, as described above, we postulated that their expression and/or distribution might be affected by the deficiency of dystrophin. Therefore, the expression and distribution of plectin and β-synemin in dystrophin-deficient skeletal muscles were explored by immunoblotting and immunohistochemistry, and compared with those in control muscles. In addition, those of other costameric proteins including α-dystrobrevin were also examined. The specimens were obtained from anterior tibial muscles of wild-type mice and dogs, and dystrophin-deficient X-chromosome-linked muscular dystrophy (mdx) mice and canine X-chromosome-linked muscular dytstrophy in Japan (CXMDJ) dogs.

The expression levels of plectin and β-synemin were significantly elevated in CXMDJ dogs relative to wild-type dogs, whereas they were almost equivalent between wild-type and mdx mice (Fig. 9). For utrophin, integrinβ1d and metavinculin, their expressions were increased in mdx mice and CXMDJ dogs as compared with wild-type controls. However, statistically significant differences were found only between CXMDJ and wild-type dogs, but not between mdx and control mice. The expression levels of vimentin showed a significant increase in mdx and CXMDJ as compared with wild-type controls, whereas those of α-dystrobrevin 1, α-dystrobrevin 2 and α-dystrobrevin 3 represented a significant decrease in dystrophin-deficient muscles relative to controls. The expression levels of vinculin, desmin and α-actinin were almost equivalent between wild-type and mdx or CXMDJ muscles.

The distributions of plectin and β-synemin were slightly altered in dystrophin-deficient muscles of mdx mice and CXMDJ dogs as compared with normal controls (supplementary material Fig. S2A-F). Dystrophin-deficient muscle fibers showed more intense staining for β-synemin and plectin along the sarcolemma as compared with normal muscles. More conspicuous phenotypic alterations were found

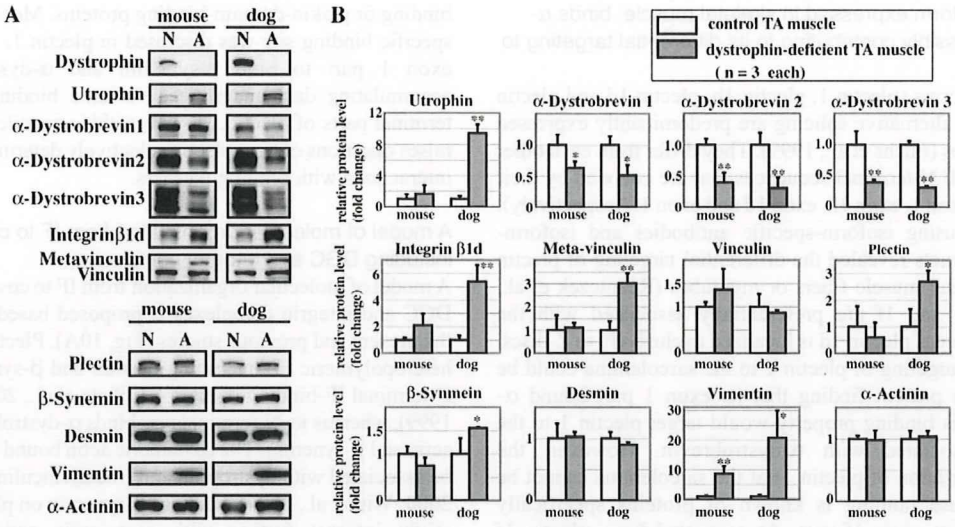

Fig. 9. Immunoblots of costameric, IF-associated and IF proteins from wild-type (N) and dystrophin-deficient (A) mdx mice (5-6 weeks old) and CXMDJ dogs (6-10 months old). (A,B) Reduced expression of α-dystrobrevin 1, α-dystrobrevin 2 and α-dystrobrevin 3, and increased expression of utrophin, integrin β1d, metavinculin and vimentin were detected in dystrophin-deficient mice and dogs when compared with wild-type controls. Note slightly elevated expression of plectin and β-synemin in dystrophin-deficient dogs. Values represent the mean ± s.d. (error bars) of muscle specimens obtained from three mice and dogs. $^{**}P<0.01$ and $^{*}P<0.05$, compared with control mice or dogs.

in IF networks of CXMDJ dogs, but not in those of mdx mice. Aberrant IF networks and/or abnormal sarcoplasmic deposits or aggregates were observed in CXMDJ muscle fibers. Aberrant IF networks appeared as larger and more irregular honeycomb structures when compared with normal controls. The sarcoplasmic deposits were positive for β-synemin, plectin, vimentin and desmin, but negative for utrophin, α-dystrobrevin and integrin β1d. These aberrant IF networks and sarcoplasmic deposits were found mainly in regenerating fibers that were positive for vimentin or developmental myosin staining (supplementary material Fig. S2G-I).

Discussion

It has been long assumed that desmin IFs anchor onto the costameric sarcolemma. Consistent with this assumption, our previous immuno-EM study ultrastructurally demonstrated that plectin linked desmin IFs to dystrophin- and vinculin-containing subsarcolemmal dense plaques, or costameres (Hijikata et al., 2003). Except for plectin, however, proteins involved in the linkages from IF to costameres and their molecular organization remained largely unknown. Recently, direct association of plectin 1f to dystrophin and β-dystroglycan was shown as a linkage between IFs and DGC (Rezniczek et al., 2007). Extending this knowledge further, this study provides new insights into the molecular organization from IF to costameres, by demonstrating that plectin 1 can bind actin, α-dystrobrevin and β-synemin, whereas β-synemin can also bind actin as well as α-dystrobrevin.

β-synemin is a peculiar IF protein involved in molecular organization of actin-based costameres

Unlike other IF proteins, β-synemin can bind actin and actin-associated proteins. These abilities are supported by several lines of evidence, provided by our results and those of others: (1) β-synemin tail fragments slightly co-sedimented with F-actin in the actin co-sedimentation assay; (2) β-synemin was found along SFLS, including actin, in C2C12 myotubes; (3) β-synemin coimmunoprecipitated with sarcomeric and nonmuscle actin from muscle LM lysates; (4) previous yeast-two-hybrid analyses using avian synemin cDNA as a bait demonstrated direct interactions of synemin with α-actinin and vinculin (Bellin et al., 1999; Bellin et al., 2001). However, mammalian β-synemin is unlikely to interact with α-actinin, although it seems to bind vinculin. In the present immunoprecipitation experiment, β-synemin immuno-complexes from LM lysates contained metavinculin but not α-actinin. This absence of β-synemin-interaction with α-actinin was possibly due to the lack of the approximately 300-residue-long C-terminal sequences of avian synemin that are required for binding α-actinin in rat β-synemin molecules.

In costameres, β-synemin seems to serve as an actin-associated protein rather than an IF protein to form heteropolymeric IFs, and is unlikely to link IFs to costameric components. As demonstrated by our immuno-EM analysis, β-synemin appeared to be incorporated into costameric dense plaques without associating with IF. Similar β-synemin-association with the sarcolemma, independent of IF, was found in muscle fibers of desmin knockout mice that lacked desmin IFs (Carlsson et al., 2000). Such β-synemin-associations with the sarcolemma possibly occur through its interactions with α-dystrobrevin, actin, and/or metavinculin within costameres, where β-synemin might contribute to the integration of actin-based molecular architectures. However, β-synemin molecules were incorporated into heteropolymeric IFs as well. These β-synemin molecules, which are heteropolymerized with desmin, would not interact with α-dystrobrevin or dystrophin anymore and, therefore would not link IFs to costameres, because desmin, α-dystrobrevin and dystrophin confine their binding sites onto β-synemin rod domain (Bhosle et al., 2006; Mizuno et al., 2001). This notion is supported by the present immunoprecipitation results, indicating that β-synemin associated with plectin 1f through a part of rod domain could not further co-precipitate with α-dystrobrevin.

Plectin 1, an isoform expressed in skeletal muscle, binds α-dystrobrevin, possibly contributing to its differential targeting to the sarcolemma

Four plectin isoforms (plectin 1, plectin 1b, plectin 1d and plectin 1f) generated by alternative splicing are predominantly expressed in skeletal muscles (Fuchs et al., 1999). They differ from each other only in their small N-terminal sequences that are encoded by their own first exon (exon 1, exon 1b, exon 1d and exon 1f, respectively). A recent study using isoform-specific antibodies and isoform-expression constructs revealed the differential targeting of plectin isoforms in skeletal muscle fibers or myotubes (Rezniczek et al., 2007). Plectin 1 and 1f are preferentially associated with the sarcolemma, whereas plectin 1d is localized exclusively to Z-discs. The differential targeting of plectin 1 to the sarcolemma could be explained by the present finding that its exon 1 part bound α-dystrobrevin. This binding property would target plectin 1 to the sarcolemma associated with α-dystrobrevin. However, the differential localization of plectin 1f at the sarcolemma cannot be explained, because nothing is known of proteins specifically interacting with its exon 1f part. As presented here, plectin 1f fragments did not interact with α-dystrobrevin. Nevertheless, their unique exon 1f parts may interact with other costameric proteins or different domains of α-dystrobrevin, e.g. its exon parts 1 to 7, ultimately leading to its localization at the sarcolemma.

A versatile binding property of the N-terminal part of plectin

At its N-terminus, plectin possesses multifunctional CH domains to interact with multiple proteins, in addition to the plakin domains. The CH domains contain binding sites for actin (Andra et al., 1998), integrin β4 (Geerts et al., 1999; Rezniczek et al., 1998), vimentin (Sevcík et al., 2004), nesprin-3 (Wilhelmsen et al., 2005), the nonreceptor tyrosine kinase Fer (Lunter and Wiche, 2002), dystrophin and utrophin (Rezniczek et al., 2007), whereas the plakin domain was found to bind β-dystroglycan (Rezniczek et al., 2007). The present study has added β-synemin to the lists of CH-domain-binding or plakin-domain-binding proteins. Moreover, an isoform-specific binding site was disclosed in plectin 1, which has unique exon 1 part to bind β-synemin and α-dystrobrevin. These accumulating data underline a versatile binding property of N-terminal parts of plectin. However, this versatile binding property raises questions of how plectin selectively determines and regulates interactions with binding partners.

A model of molecular organization from IF to costameres including DGC and integrin complexes

A model of molecular organization from IF to costameres including DGC and integrin complexes is proposed based on the results of the present and previous studies (Fig. 10A). Plectin 1 interacts with heteropolymeric IFs including desmin and β-synemin through its C-terminal IF-binding domain (Hijikata et al., 2003; Reipert et al., 1999), whereas its N-terminal part binds α-dystrobrevin, costameric actin and β-synemin. The costameric actin bound by plectin 1 might be associated with dystrophin and (meta)vinculin (Rybakova et al., 2000; Witt et al., 2004), whereas β-synemin on plectin 1 molecules might interact further with costameric actin. Moreover, α-dystrobrevin associated with plectin 1 might also bind β-synemin. In addition to this model, another one has been recently proposed (see Fig. 10B), in which plectin 1f links IFs directly to dystrophin and β-dystroglycan within DGC (Rezniczek et al., 2007). This direct binding of plectin to and its indirect interactions through actin with dystrophin well agreed with our previous findings that treatment of plectin immune complexes from LM lysates with gelsolin decreased dystrophin within the immune complexes, but did not completely remove it (Hijikata et al., 2003).

Costameres are structurally and functionally analogous to hemidesmosomes

With respect to linking IFs to the BM, costameres in skeletal muscles are quite analogous to hemidesmosomes in epidermal cells, although costameric components are much more numerous and organized in

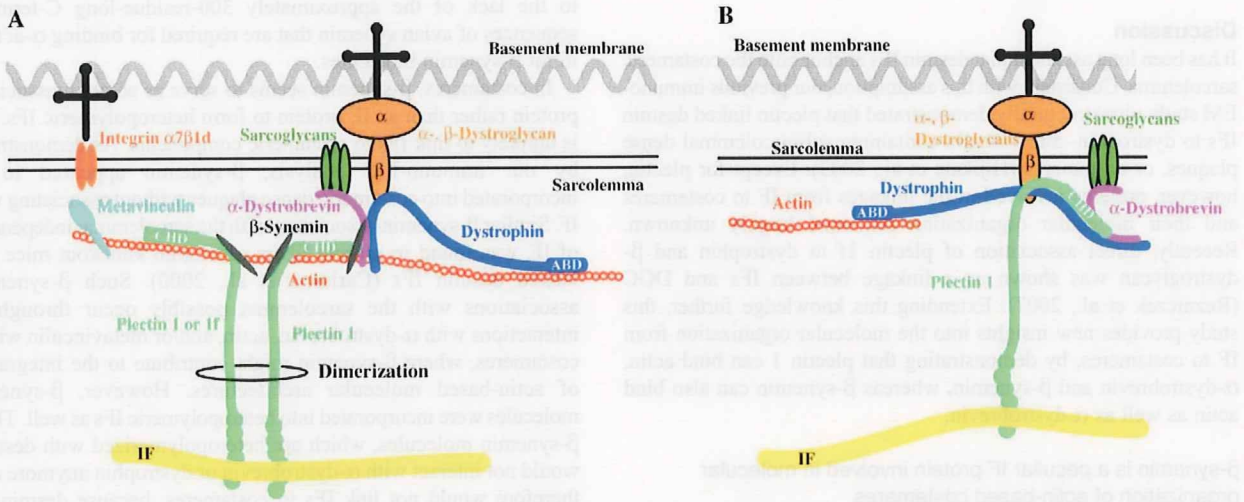

Fig. 10. Models of molecular organization from IF to costameres including DGC and integrin complexes. (A) The N-terminal part of plectin 1 is associated with costameres by binding β-synemin, costameric actin and α-dystrobrevin, whereas its C-terminal part is linking to IFs. Exon 1 part of plectin 1 might interact with not only α-dystrobrevin but also β-synemin, whereas β-synemin on plectin molecules might be further associated with costameric actin linking to plectin, dystrophin and metavinculin. (B) As an alternative model, direct interactions of plectin with dystrophin and β-dystroglycan were proposed by Rezniczek et al. (Rezniczek et al., 2007). This model is partly modified to represent the interaction of plectin 1 with α-dystrobrevin through its exon 1 part.

more complicated manner than hemidesmosomal ones. Costameres including DGC and integrin complexes are extracellularly associated with the BM through α-dystroglycan and integrin α7β1d (Belkin et al., 1996; Eravsti and Campbell, 1993), whereas they are intracelluarly linked to IFs by plectin (Hijikata et al., 2003; Rezniczek et al., 2007; Schröder et al., 2002). Both the analogous structures are assumed mechanically to stabilize the plasma membrane and to protect cell against mechanical stress. Attesting this assumption, similar disruptions of plasma membranes and cell structures were reported to occur in skin or skeletal muscle by the deficiency or defects of constituents of hemidesmosomes or costameres, such as plectin (Andra et al., 1997; Gache et al., 1996; McMillan et al., 2007), IF proteins (desmin and keratin) (Chan et al., 1994; Li et al., 1997) and other membranous or membrane-associated proteins (integrin α7, dystrophin, α-dystrobrevin, sarcoglycan, integrin α6, integrin β4, BP180) (Dowling et al., 1996; Duclos et al., 1998; Grady et al., 1999; Hack et al., 1998; Hoffman et al., 1987; Huber et al., 2002; Mayer et al., 1997; Pulkinnen et al., 1997; van der Neut et al., 1996).

Quantitative alterations of costameric components in dystrophin-deficient muscles

Quantitative alterations of costameric components were found in dystrophin-deficient muscles. In contrast to the reduction of DGC components (Ozawa, 2006; Straub and Campbell, 1997), other costameric components, such as plectin, β-synemin, (meta)vinculin, talin and integrins, increase their expressions in dystrophin-deficient muscles, as demonstrated in the present and previous studies (Hodges et al., 1997; Law et al., 1994; Rezniczek et al., 2007). Increased synemin- and plectin-staining along the dystrophin-deficient sarcolemma were also noted by the present and previous immunohistochemical studies (Schröder et al., 1997). Moreover, costameric γ-actin is also more abundantly expressed in mdx muscles lacking dystrophin, compared with control muscles (Hanft et al., 2006). These increased expressions of costameric components, including integrin complexes, might be a compensatory or an adaptive cellular response to unstable costameres and unstable anchorages of IF on costameres.

As a compensatory response in dystrophin-deficient muscles, increased β-synemin and plectin 1, together with costameric γ-actin, might preserve the subsarcolemmal localization of α-dystrobrevin, which loses a main binding partner dystrophin beneath the sarcolemma. As presented in this study, β-synemin and plectin 1 can bind α-dystrobrevin. Another possibility is that sarcoglycan-sarcospan complex also contributes to recruitment of α-dystrobrevin to the sarcolemma, because the complex interacts with the N-terminal portion of α-dystrobrevin and is still present in the dystrophin-deficient sarcolemma (Hack et al., 2000; Ozawa et al., 2000; Yoshida et al., 2000). In this context, it is worth noting that utrophin, a structurally related protein that can compensate for lack of dystrophin, might not retain α-dystrobrevin at the sarcolemma. α-Dystrobrevin 2 localized at the extrasynaptic sarcolemma was found not to interact with utrophin (Peters et al., 1998).

Functional significances of costameres connecting to IF through plectin

DGC and integrin complexes within costameres would have the similar function of linking the BM to IFs through plectin, costameric actin and β-synemin. This would result in stabilizing the sarcolemma and protecting it against contraction-imposed stress. Their functional similarity would be reasonably supposed, given that the reduction of DGC induced a compensatory response of increased expression of integrin complexes. However, the two complexes would also have their own functional roles, because dystrophin-deficient muscles still undergo degeneration despite increased expression of integrin complexes. This fact implies that increased integrin complexes are not sufficient, either quantitatively or qualitatively, to compensate for functional roles of DGC. The two complexes in combination with plectin and IFs might serve as platforms for distinct signal transduction, because DGC and integrin complexes are associated with distinct proteins involved in different signaling, i.e. nNOS and FAK, respectively (Brenman et al., 1996; Pham et al., 2000).

Materials and Methods

Cloning of rat β-synemin, plectin isoforms and α-dystrobrevin cDNA

To clone cDNA of a plectin-binding protein, a rat skeletal muscle 5′-stretch plus cDNA library (Clontech) was screened with DNA probes, which were prepared by PCR using the cDNA library and a pair of primers predicted from partial amino acid sequences (5′-CCNCAYGARTTYCAY-3′ for PHEFH and 5′-RTCNACCATYCT-YTG-3′ for QRMVD). This screening identified 47 positive clones that did not contain a 5′ sequence with an ATG start codon. Therefore, to clone the full-length cDNA, we constructed a cDNA library from single-stranded DNAs generated by reverse transcription using mRNA obtained from skeletal muscles of newborn rats, random hexamers and gene-specific primers based on the sequences of incomplete cDNAs (5′-CATGCGCTCAGGCAACGTTTCCTCCAGCTG-3′ and 5′-TTGACTCTGT-CTGGTCAGGGCAGACAGCTG-3′). This cDNA library was screened with a new probe that had been generated from a positive cDNA clone obtained in the previous screening to identify ten overlapping cDNA clones including the full-length clone.

To obtain cDNA clones encoding the N-terminal parts of various plectin isoforms (exon 1~exon 30), a partial cDNA library was constructed (as described above) with random hexamers, and rat plectin-specific primers (5′-TTCATCCAGAGCC-TGCAGGCCCGCTGCTT-3′, 5′-TCTCTGCCTCGGCTTGGCACAGCCACCGTT-3′, and 5′-TTCTCCAGCTCCTGCTCAGCCAGCTCTCGC-3′). This library was screened with DNA probes generated using rat plectin cDNA fragments (plectin 1, 1-1311bp), which were obtained by RT-PCR using total RNA of new-born rat skeletal muscles and a pair of rat plectin 1-specific primers (5′-ATGGTGGCTGG-CATGCTCATG-3′ and 5′-GTGGTGCCGGATCCACTHTAG-3′). Thirty-three positive clones were identified and found to include various plectin isoforms, such as plectin 1, 1a, 1b, 1d, 1f and 1e.

α-Dystrobrevin cDNA encoding exon 8 to exon 16 was amplified from the rat skeletal muscle 5′-stretch plus cDNA library by PCR using a pair of primers (5′-GCNAAYGTNGARAAYGTNTTYCAYCCNGTNG-3′ and 5′-RTAYTCYTCCAT-YTGNAGYTCRTTYTCNAG-3′). These primers were designed based on the published sequences of mouse and human α-dystrobrevin 1. A cDNA of approximately 1.5 kb was obtained and sequenced to confirm that it was an α-dystrobrevin cDNA fragment encoding amino acid residues 232-669 of α-dystrobrevin isoform1 isoform11 (XP_001054793).

Expression and purification of GST or 6xHis recombinant proteins

β-Synemin constructs encoding the following segments of the protein were subcloned into pGEX-6p-1 glutathione S-transferase (GST) bacterial expression vector (GE Healthcare): full-length (amino acids 1-1255), Rod N (1-130), Rod Nl (56-286), Rod Ms (57-223), Rod C (169-318), Tail N1 (303-578), Tail N2 (303-432), Tail M (482-930), Tail C1 (822-1255), Tail C2 (931-1102), and Tail C3 (1071-1255). Similarly, cDNA fragments encoding full-length β-galactosidase (LacZ), the N-terminal portion of plectin 1 including exon 1 (PleN1, amino acids 1-1273), the N-terminal part of plectin 1f including exon 1f (PleN1f, 1-1121), exon 1 (Ex1, 1-181), calponin homology domain (CHDα, 183-507), various plakin domain fragments (PlD-N, 413-736; PlD-MC, 707-1273; PlD-M, 707-950; PlD-Cl, 854-1273; PlD-Cs, 965-1273) were subcloned into the pGEX-6p-1. All of these plectin fragments and β-galactosidase contained the Myc-tag epitope at their C-termini. α-Dystrobrevin cDNA fragment obtained from the rat skeletal muscle cDNA library was subcloned into the pET-19b vector carrying an N-terminal 6xHis tag sequence (Novagen). Escherichia coli BL21 (DE3) was transformed with the constructs described above. Expression of GST or 6xHis fusion proteins was induced by 0.2 mM IPTG for 3 h at 28°C. Purification of GST or 6xHis fusion proteins was performed as described in the respective manufacturer's protocols. GST was removed from plectin recombinant fragments and LacZ by digestion with PreScission protease (GE Healthcare) on glutathione bead columns.

Antibodies

Rabbit β-synemin antisera were raised against recombinant rat synemin tail fragments (amino acids 822-1255). The antisera were purified by passage over affinity columns

conjugated with the synemin tail fragments. Other antibodies used for immunohistochemistry, immunoblotting, and immunoprecipitation were as follows: polyclonal and monoclonal anti-plectin antibody [(Hijikata et al., 2003) clone 7A8; Sigma-Aldrich], monoclonal and polyclonal anti-α-dystrobrevin antibody [Clone 23; BD Transduction Laboratories (Yoshida et al., 2000)], monoclonal anti-pan-actin antibody (C4; Novus Biological, Inc.), polyclonal anti-nonmuscle (β- and γ-) actin antibody (Cosmo Bio Co. Ltd.), monoclonal anti-integrin β1d antibody (clone 2B1; Chemicon International), monoclonal anti-α-actinin antibody (sarcomeric EA-53; Sigma-Aldrich), polyclonal and monoclonal anti-desmin antibody (Progen Biotechnik GmbH, DE-U-10; Sigma-Aldrich), monoclonal anti-Myc antibody (clone 9E10; Roche), monoclonal anti-vinculin antibody (clone hVin-1; Sigma-Aldrich), monoclonal anti-vimentin antibody (clone VIM13.2; Sigma-Aldrich), polyclonal anti-utrophin antibody (Imamura and Ozawa, 1998), monoclonal anti-dystrophin antibody (clone Dy8/6C5; Novocastra, clone mandy8; Sigma-Aldrich), monoclonal anti-developmental myosin heavy-chain antibody (clone RNMy2/9D2; Novocastra).

The secondary antibodies used in the present study were as follows: Alexa Fluor 488 goat anti-mouse IgG(H+L), Alexa Fluor 488 or Alexa Fluor 594 goat anti-rabbit IgG(H+L) (Invitrogen), peroxidase-conjugated goat anti-mouse IgG(H+L), goat anti-rabbit IgG(H+L) (Pierce), and rabbit anti-chicken IgY antibody (Promega), goat anti-rabbit IgG(H+L) conjugated to 5-nm gold particles (BioCell Research Laboratories).

Cell culture and transfection

C2C12 cells (2.0×10^5 cells) were cultured on collagen-coated Aclar coverslips within 35-mm dishes in growth medium (DMEM containing 20% FCS, 100 U/ml penicillin G and 100 μg/ml streptomycin). Plasmid DNA was prepared by subcloning exon 1 (Ex1), calponin homology domains (CHDα), plakin domain (PlD-M) cDNA fragments including Myc sequence into pZac expression vector (kindly provided by James M. Wilson. Plasmid DNA (4 μg) was transfected into the cells within each dish by using Lipofectamine™ 2000 (Invitrogen). After washing out plasmid DNA, the transfected cells were cultured in growth medium overnight, and then their differentiation was initiated by switching the medium to the DMEM medium containing 5% horse serum, 10 μg/ml insulin, and the antibiotics. After 2 or 3 days, C2C12 myotubes were fixed with chilled (–20°C) methanol and processed for immunostaining (Hijikata et al., 1997).

Immunofluorescence microscopy and immunoelectron microscopy

Cryosections of rat skeletal muscles (diaphragm and tibialis anterior) were prepared and immunostained as described previously (Hijikata et al., 1999). These sections were observed under a confocal scanning laser microscope (Fluoview FV1000, Olympus). For F-actin staining in C2C12 cells, Alexa Fluor 594-conjugated phallotoxins (Invitrogen) were utilized.

For immunoelectron microscopy, small bundles of muscle fibers were carefully teased from glycerinated muscle strips, chemically skinned with 50 μg/ml saponin in EGTA rigor solution, immunolabeled, processed for thin-section EM using tannic acid enhancement, and observed, as described previously (Hijikata et al., 2003).

Protein pull-down assay and immunoprecipitation

For pull-down assay, purified GST-synemin recombinant fragments were incubated with Myc-tagged PleN1 in the incubation buffer (50 mM HEPES pH 7.0, 10% glycerin, 1 mM DTT, 0.5% NP-40) for 3-4 hours at 4°C. The reaction mix was further incubated with anti-Myc mAb for 3-4 hours and then with protein L-agarose beads (Pierce) with rocking at 4°C overnight. The beads carrying the immune complexes were washed six times with the same buffer. The immune complexes were eluted by addition of SDS sample buffer. The Myc-tagged plectin recombinant fragments were also incubated with and pulled down by GST-synemin fragments Tail N1 and glutathione beads. Prior to the incubation, Myc-tagged plectin recombinant fragments were incubated with glutathione beads and precleared by centrifugation to remove contaminating plectin fragments still fused to GST. The glutathione beads carrying GST-synemin fragments associated with plectin fragments were washed six times with the incubation buffer and eluted with SDS sample buffer at 95°C.

For in vivo immunoprecipitation, light microsomes (LM), prepared from rat skeletal muscles according to the procedure described by Ohlendieck et al. (Ohlendieck et al., 1991), were lysed with solubilization buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 15% glycerol, 50 mM Tris-HCl pH 7.5) containing protease inhibitors (100 μg/ml PMSF, 2 μg/ml leupeptin). After centrifugation, the supernatant was precleared with protein G-Sepharose (Sigma-Aldrich). The precleared supernatant was incubated with either polyclonal anti-plectin or anti-synemin antibody and then with protein G-Sepharose overnight at 4°C. The beads carrying the immune complexes were washed three times with solubilization buffer, three times with solubilization buffer without SDS, and once with TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5), followed by incubation with SDS sample buffer at 95°C.

Actin co-sedimentation assay

Actin was purified from rabbit skeletal muscle as described previously (Matsumura et al., 1983). The purity of actin was more than 97%, determined by SDS-PAGE. The actin was allowed to polymerize in the presence of recombinant plectin and synemin fragments in actin polymerization buffer (20 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 100 mM KCl, 0.5 mM ATP, 0.1 mM β-mercaptoethanol) for 1 h at room temperature. Actin filaments with bound proteins were sedimented by centrifugation for 1 hour at 100,000 *g* and 20°C, and corresponding amounts of pellet and supernatant were analyzed by SDS-PAGE.

Blot overlay assay

Plectin PleN1 fragments (1 μg) were immobilized on nitrocellullose membranes, which were blocked in TBS containing 5% BSA and 0.2% Tween-20 for 5 hours at 4°C. Subsequently, membranes were overlaid and incubated with 3 μM actin or 1 μM α-dystrobrevin or 1 μM β-synemin or the mixture of the three proteins in 120 μl overlay buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, and 3.5% BSA) overnight at 4°C. Bound proteins were detected by using the protein-specific antibody, HRP-conjugated secondary antibody and ECL system (GE Healthcare).

Densitometric analyses of overlay blots and immunoblots

Blot membranes treated with ECL solutions were scanned and evaluated using luminescent image analyzer LAS-3000 and Multi Gauge software (Fuji film). The mean value of spot intensities measured in the overlay with a single protein was calculated, and then each spot intensity was represented relative to this mean value by calculating the ratio of measured value per the mean value. Similarly, intensity of each band obtained in immunoblottings of control and dystrophin-deficient muscles was represented relative to the mean value of intensities measured in control muscles.

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References

Ahn, A. H., Freener, C. A., Gussoni, E., Yoshida, M., Ozawa, E. and Kunkel, L. M. (1996). The three human syntrophin genes are expressed in diverse tissues, have distinct chromosomal locations, and each bind to dystrophin and its relatives. *J. Biol. Chem.* **271**, 2724-2730.

Andra, K., Lassmann, H., Bittner, R., Shorny, S., Fassler, R., Propst, F. and Wiche, G. (1997). Targeted inactivation of plectin reveals essential function in maintaining the integrity of skin, muscle, and heart cytoarchitecture. *Genes Dev.* **11**, 3143-3156.

Andra, K., Nikolic, B., Stocher, M., Drenckhahn, D. and Wiche, G. (1998). Not just scaffolding: plectin regulates actin dynamics in cultured cells. *Genes Dev.* **12**, 3442-3451.

Balogh, J., Li, Z., Paulin, D. and Arner, A. (2003). Lower active force generation and improved fatigue resistance in skeletal muscle from desmin deficient mice. *J. Muscle Res. Cell Motil.* **24**, 453-459.

Belkin, A. M., Zhidkova, N. I., Balzac, F., Altruda, F., Tomatis, D., Maier, A., Tarone, G., Koteliansky, V. E. and Burridge, K. (1996). Beta 1D integrin displaces the beta 1A isoform in striated muscles: localization at junctional structures and signaling potential in nonmuscle cells. *J. Cell Biol.* **132**, 211-226.

Bellin, R. M., Sernett, S. W., Becker, B., Ip, W., Huiatt, T. W. and Robson, R. M. (1999). Molecular characteristics and interactions of the intermediate filament protein synemin. Interactions with alpha-actinin may anchor synemin-containing heterofilaments. *J. Biol. Chem.* **274**, 29493-29499.

Bellin, R. M., Huiatt, T. W., Critchley, D. R. and Robson, R. M. (2001). Synemin may function to directly link muscle cell intermediate filaments to both myofibrillar Z-lines and costameres. *J. Biol. Chem.* **276**, 32330-32337.

Bhosle, R. C., Michele, D. E., Campbell, K. P., Li, Z. and Robson, R. M. (2006). Interactions of intermediate filament protein synemin with dystrophin and utrophin. *Biochem. Biophys. Res. Commun.* **346**, 768-777.

Bilak, S. R., Sernett, S. W., Bilak, M. M., Bellin, R. M., Stromer, M. H., Huiatt, T. W. and Robson, R. M. (1998). Properties of the novel intermediate filament protein synemin and its identification in mammalian muscle. *Arch. Biochem. Biophys.* **355**, 63-76.

Brenman, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Wu, Z., Huang, F., Xia, H., Peters, M. F. et al. (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. *Cell* **84**, 757-767.

Carlsson, L., Li, Z. L., Paulin, D., Price, M. G., Breckler, J., Robson, R. M., Wiche, G. and Thornell, L. E. (2000). Differences in the distribution of synemin, paranemin, and plectin in skeletal muscles of wild-type and desmin knock-out mice. *Histochem. Cell Biol.* **114**, 39-47.

Chan, Y., Anton-Lamprecht, I., Yu, Q. C., Jäckel, A., Zabel, B., Ernst, J. P. and Fuchs, E. (1994). A human keratin 14 "knockout": the absence of K14 leads to severe epidermolysis bullosa simplex and a function for an intermediate filament protein. *Genes Dev.* **8**, 2574-2587.

Craig, S. W. and Pardo, J. V. (1983). Gamma actin, spectrin, and intermediate filament proteins colocalize with vinculin at costameres, myofibril-to-sarcolemma attachment sites. *Cell Motil.* **3**, 449-462.

Dowling, J., Yu, Q. C. and Fuchs, E. (1996). Beta4 integrin is required for hemidesmosome formation, cell adhesion and cell survival. *J. Cell Biol.* **134**, 559-572.

Duclos, F., Straub, V., Moore, S. A., Venzke, D. P., Hrstka, R. F., Crosbie, R. H., Durbeej, M., Lebakken, C. S., Ettinger, A. J., van der Meulen, J. et al. (1998). Progressive muscular dystrophy in alpha-sarcoglycan-deficient mice. *J. Cell Biol.* **142**, 1461-1471.

Ervasti, J. M. and Campbell, K. P. (1993). A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. *J. Cell Biol.* **122**, 809-823.
Foisner, R., Leichtfried, F. E., Herrmann, H., Small, J. V., Lawson, D. and Wiche, G. (1988). Cytoskeleton-associated plectin: in situ localization, in vitro reconstitution, and binding to immobilized intermediate filament proteins. *J. Cell Biol.* **106**, 723-733.
Fontao, L., Geerts, D., Kuikman, I., Koster, J., Kramer, D. and Sonnenberg, A. (2001). The interaction of plectin with actin: evidence for cross-linking of actin filaments by dimerization of the actin-binding domain of plectin. *J. Cell Sci.* **114**, 2065-2076.
Fuchs, P., Zorer, M., Rezniczek, G. A., Spazierer, D., Oehler, S., Castanon, M. J., Hauptmann, R. and Wiche, G. (1999). Unusual 5′ transcript complexity of plectin isoforms: novel tissue-specific exons modulate actin binding activity. *Hum. Mol. Genet.* **8**, 2461-2472.
Fujikamki, N., Kano, Y. and Ishikawa, H. (1986). The association of intermediate filaments with the sarcolemma in skeletal muscle fibers. In Proc XIth Int Cong on Electron Microscopy, Kyoto, p2657-2658.
Gache, Y., Chavanas, S., Lacour, J. P., Wiche, G., Owaribe, K., Meneguzzi, G. and Ortonne, J. P. (1996). Defective expression of plectin/HD1 in epidermolysis bullosa simplex with muscular dystrophy. *J. Clin. Invest.* **97**, 2289-2298.
Geerts, D., Fontao, L., Nievers, M. G., Schaapveld, R. Q., Purkis, P. E., Wheeler, G. N., Lane, E. B., Leigh, I. M. and Sonnenberg, A. (1999). Binding of integrin alpha6beta4 to plectin prevents plectin association with F-actin but does not interfere with intermediate filament binding. *J. Cell Biol.* **147**, 417-434.
Grady, R. M., Grange, R. W., Lau, K. S., Maimone, M. M., Nichol, M. C., Stull, J. T. and Sanes, J. R. (1999). Role for alpha-dystrobrevin in the pathogenesis of dystrophin-dependent muscular dystrophies. *Nat. Cell Biol.* **1**, 215-220.
Granger, B. L. and Lazarides, E. (1980). Synemin: a new high molecular weight protein associated with desmin and vimentin filaments in muscle. *Cell* **22**, 727-738.
Hack, A. A., Ly, C. T., Jiang, F., Clendenin, C. J., Sigrist, K. S., Wollmann, R. L. and McNally, E. M. (1998). Gamma-sarcoglycan deficiency leads to muscle membrane defects and apoptosis independent of dystrophin. *J. Cell Biol.* **142**, 1279-1287.
Hack, A. A., Lam, M. Y., Cordier, L., Shorturma, D. I., Ly, C. T., Hadhazy, M. A., Hadhazy, M. R., Sweeney, H. L. and MaNally, E. M. (2000). Differential requirement for individual sarcoglycans and dystrophin in the assembly and function of the dystrophin-glycoprotein complex. *J. Cell Sci.* **113**, 2535-2544.
Hanft, L. M., Rybakova, I. N., Patel, J. R., Rafael-Fortney, J. A. and Ervasti, J. M. (2006). Cytoplasmic gamma-actin contributes to a compensatory remodeling response in dystrophin-deficient muscle. *Proc. Natl. Acad. Sci. USA* **103**, 5385-5390.
Hemken, P. M., Bellin, R. M., Sernett, S. W., Becker, B., Huiatt, T. W. and Robson, R. M. (1997). Molecular characteristics of the novel intermediate filament protein paranemin. Sequence reveals EAP-300 and IFAPa-400 are highly homologous to paranemin. *J. Biol. Chem.* **272**, 32489-32499.
Hieda, Y., Nishizawa, Y., Uematsu, J. and Owaribe, K. (1992). Identification of a new hemidesmosomal protein, HD1: a major, high molecular mass component of isolated hemidesmosomes. *J. Cell Biol.* **116**, 1497-1506.
Hijikata, T., Lin, Z. X., Holtzer, S., Choi, J., Sweeney, H. L. and Holtzer, H. (1997). Unanticipated temporal and spatial effects of sarcomeric alpha-actinin peptides expressed in PtK2 cells. *Cell Motil. Cytoskeleton* **38**, 54-74.
Hijikata, T., Murakami, T., Imamura, M., Fujimaki, N. and Ishikawa, H. (1999). Plectin is a linker of intermediate filaments to Z-discs in skeletal muscle fibers. *J. Cell Sci.* **112**, 867-876.
Hijikata, T., Murakami, T., Ishikawa, H. and Yorifuji, H. (2003). Plectin tethers desmin intermediate filaments onto subsarcolemmal dense plaques containing dystrophin and vinculin. *Histochem. Cell Biol.* **119**, 109-123.
Hirako, Y., Yamakawa, H., Tsujimura, Y., Nishizawa, Y., Okumura, M., Usukura, J., Matsumoto, H., Jackson, K. W., Owaribe, K. and Ohara, O. (2003). Characterization of mammalian synemin, an intermediate filament protein present in all four classes of muscle cells and some neuroglial cells: co-localization and interaction with type III intermediate filament proteins and keratins. *Cell Tissue Res.* **313**, 195-207.
Hodges, B. L., Hayashi, Y. K., Nonaka, I., Wang, W., Arahata, K. and Kaufman, S. J. (1997). Altered expression of the alpha7beta1 integrin in human and murine muscular dystrophies. *J. Cell Sci.* **110**, 2873-2881.
Hoffman, E. P., Brown, R. H., Jr and Kunkel, L. M. (1987). Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* **51**, 919-928.
Huber, M., Floeth, M., Borradori, L., Schäcke, H., Rugg, E. L., Lane, E. B., Frenk, E., Hohl, D. and Bruckner-Tuderman, L. (2002). Deletion of the cytoplasmatic domain of BP180/collagen XVII causes a phenotype with predominant features of epidermolysis bullosa simplex. *J. Invest. Dermatol.* **118**, 185-192.
Imamura, M. and Ozawa, E. (1998). Differential expression of dystrophin isoforms and utrophin during dibutyryl-cAMP-induced morphological differentiation of rat brain astrocytes. *Proc. Natl. Acad. Sci. USA* **95**, 6139-6144.
Law, D. J., Allen, D. L. and Tidball, J. G. (1994). Talin, vinculin and DRP (utrophin) concentrations are increased at mdx myotendinous junctions following onset of necrosis. *J. Cell Sci.* **107**, 1477-1483.
Lazarides, E. (1980). Intermediate filaments as mechanical integrators of cellular space. *Nature* **283**, 249-256.
Li, Z., Colucci-Guyon, E., Pincon-Raymond, M., Mericskay, M., Pournin, S., Paulin, D. and Babinet, C. (1996). Cardiovascular lesions and skeletal myopathy in mice lacking desmin. *Dev. Biol.* **175**, 362-366.
Li, Z., Mericskay, M., Agbulut, O., Butler-Browne, G., Carlsson, L., Thornell, L. E., Babinet, C. and Paulin, D. (1997). Desmin is essential for the tensile strength and integrity of myofibrils but not for myogenic commitment, differentiation, and fusion of skeletal muscle. *J. Cell Biol.* **139**, 129-144.
Litjens, S. H., de Pereda, J. M. and Sonnenberg, A. (2006). Current insights into the formation and breakdown of hemidesmosomes. *Trends Cell Biol.* **16**, 376-383.
Lunter, P. C. and Wiche, G. (2002). Direct binding of plectin to Fer kinase and negative regulation of its catalytic activity. *Biochem. Biophys. Res. Commun.* **296**, 904-910.
Matsumura, F., Yamashiro-Matsumura, S. and Lin, J. J. (1983). Isolation and characterization of tropomyosin-containing microfilaments from cultured cells. *J. Biol. Chem.* **258**, 6636-6664.
Mayer, U., Saher, G., Fässler, R., Bornemann, A., Echtermeyer, F., von der Mark, H., Miosge, N., Pöschl, E. and von der Mark, K. (1997). Absence of integrin alpha 7 causes a novel form of muscular dystrophy. *Nat. Genet.* **17**, 318-323.
McMillan, J. R., Akiyama, M., Rouan, F., Mellerio, J. E., Lane, E. B., Leigh, I. M., Owaribe, K., Wiche, G., Fujii, N., Uitto, J. et al. (2007). Plectin defects in epidermolysis bullosa simplex with muscular dystrophy. *Muscle Nerve* **35**, 24-35.
Mizuno, Y., Thompson, T. G., Guyon, J. R., Lidov, H. G., Brosius, M., Imamura, M., Ozawa, E., Watkins, S. C. and Kunkel, L. M. (2001). Desmuslin, an intermediate filament protein that interacts with alpha-dystrobrevin and desmin. *Proc. Natl. Acad. Sci. USA* **98**, 6156-6161.
Mizuno, Y., Guyon, J. R., Watkins, S. C., Mizushima, K., Sasaoka, T., Imamura, M., Kunkel, L. M. and Okamoto, K. (2004). Beta-synemin localizes to regions of high stress in human skeletal myofibers. *Muscle Nerve* **30**, 337-346.
Nikolic, B., Mac Nulty, E., Mir, B. and Wiche, G. (1996). Basic amino acid residue cluster within nuclear targeting sequence motif is essential for cytoplasmic plectin-vimentin network junctions. *J. Cell Biol.* **134**, 1455-1467.
Ohlendieck, K., Ervasti, J. M., Snook, J. B. and Campbell, K. P. (1991). Dystrophin-glycoprotein complex is highly enriched in isolated skeletal muscle sarcolemma. *J. Cell Biol.* **112**, 135-148.
Ozawa, E. (2006). The functional biology of dystrophin: structural components and the pathogenesis of Duchenne muscular dystrophy. In *Duchenne Muscular Dystrophy Advances in Therapeutics* (ed. J. S. Chamberlain and T. A. Rando), pp. 21-53. New York, London: Taylor & Francis Group.
Ozawa, E., Noguchi, S., Mizuno, Y., Hagiwara, Y. and Yoshida, M. (1998). From dystrophinopathy to sarcoglycanopathy: evolution of a concept of muscular dystrophy. *Muscle Nerve* **21**, 421-438.
Ozawa, E., Imamura, M., Noguchi, S. and Yoshida, M. (2000). Dystrophinopathy and sarcoglycanopathy. *NeuroScience News* **3**, 13-19.
Peters, M. F., Sadoulet-Puccio, H. M., Grady, M. R., Kramarcy, N. R., Kunkel, L. M., Sanes, J. R., Sealock, R. and Froehner, S. C. (1998). Differential membrane localization and intermolecular associations of alpha-dystrobrevin isoforms in skeletal muscle. *J. Cell Biol.* **142**, 1269-1278.
Pham, C. G., Harpf, A. E., Keller, R. S., Vu, H. T., Shai, S. Y., Loftus, J. C. and Ross, R. S. (2000). Striated muscle-specific beta(1D)-integrin and FAK are involved in cardiac myocyte hypertrophic response pathway. *Am. J. Physiol. Heart Circ. Physiol.* **279**, H2916-H2926.
Price, M. G. and Lazarides, E. (1983). Expression of intermediate filament-associated proteins paranemin and synemin in chicken development. *J. Cell Biol.* **97**, 1860-1874.
Pulkkinen, L., Kimonis, V. E., Xu, Y., Spanou, E. N., McLean, W. H. and Uitto, J. (1997). Homozygous alpha6 integrin mutation in junctional epidermolysis bullosa with congenital duodenal atresia. *Hum. Mol. Genet.* **6**, 669-674.
Reipert, S., Steinbock, F., Fischer, I., Bittner, R. E., Zeold, A. and Wiche, G. (1999). Association of mitochondria with plectin and desmin intermediate filaments in striated muscle. *Exp. Cell Res.* **252**, 479-491.
Rezniczek, G. A., de Pereda, J. M., Reipert, S. and Wiche, G. (1998). Linking integrin alpha6beta4-based cell adhesion to the intermediate filament cytoskeleton: direct interaction between the beta4 subunit and plectin at multiple molecular sites. *J. Cell Biol.* **141**, 209-225.
Rezniczek, G. A., Abrahamsberg, C., Fuchs, P., Spazierer, D. and Wiche, G. (2003). Plectin 5'-transcript diversity: short alternative sequences determine stability of gene products, initiation of translation and subcellular localization of isoforms. *Hum. Mol. Genet.* **12**, 3181-3194.
Rezniczek, G. A., Konieczny, P., Nikolic, B., Reipert, S., Schneller, D., Abrahamsberg, C., Davies, K. E., Winder, S. J. and Wiche, G. (2007). Plectin 1f scaffolding at the sarcolemma of dystrophic (mdx) muscle fibers through multiple interactions with beta-dystroglycan. *J. Cell Biol.* **176**, 965-977.
Rybakova, I. N., Patel, J. R. and Ervasti, J. M. (2000). The dystrophin complex forms a mechanically strong link between the sarcolemma and costameric actin. *J. Cell Biol.* **150**, 1209-1214.
Sadoulet-Puccio, H. M., Rajala, M. and Kunkel, L. M. (1997). Dystrobrevin and dystrophin: an interaction through coiled-coil motifs. *Proc. Natl. Acad. Sci. USA* **94**, 12413-12418.
Sam, M., Shah, S., Friden, J., Milner, D. J., Capetanaki, Y. and Lieber, R. L. (2000). Desmin knockout muscles generate lower stress and are less vulnerable to injury compared with wild-type muscles. *Am. J. Physiol. Cell Physiol.* **279**, C1116-C1122.
Schröder, R., Mundegar, R. R., Treusch, M., Schlegel, U., Blumcke, I., Owaribe, K. and Magin, T. M. (1997). Altered distribution of plectin/HD1 in dystrophinopathies. *Eur. J. Cell Biol.* **74**, 165-171.
Schröder, R., Warlo, I., Herrmann, H., van der Ven, P. F., Klasen, C., Blümcke, I., Mundegar, R. R., Fürst, D. O., Goebel, H. H. and Magin, T. M. (1999). Immunogold EM reveals a close association of plectin and the desmin cytoskeleton in human skeletal muscle. *Eur. J. Cell Biol.* **78**, 288-295.
Schröder, R., Pacholsky, D., Reimann, J., Matten, J., Wiche, G., Furst, D. O. and van der Ven, P. F. (2002). Primary longitudinal adhesion structures: plectin-containing precursors of costameres in differentiating human skeletal muscle cells. *Histochem. Cell Biol.* **118**, 301-310.
Schweitzer, S. C., Klymkowsky, M. W., Bellin, R. M., Robson, R. M., Capetanaki, Y. and Evans, R. M. (2001). Paranemin and the organization of desmin filament networks. *J. Cell Sci.* **114**, 1079-1089.

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Sevcík, J., Urbániková, L., Kost'an, J., Janda, L. and Wiche, G. (2004). Actin-binding domain of mouse plectin. Crystal structure and binding to vimentin. *Eur. J. Biochem.* **271**, 1873-1884.

Straub, V. and Campbell, K. P. (1997). Muscular dystrophies and the dystrophin-glycoprotein complex. *Curr. Opin. Neurol.* **10**, 168-175.

Titeux, M., Brocheriou, V., Xue, Z., Gao, J., Pellissier, J. F., Guicheney, P., Paulin, D. and Li, Z. (2001). Human synemin gene generates splice variants encoding two distinct intermediate filament proteins. *Eur. J. Biochem.* **268**, 6435-6449.

Tokuyasu, K. T., Dutton, A. H. and Singer, S. J. (1983). Immunoelectron microscopic studies of desmin (skeletin) localization and intermediate filament organization in chicken skeletal muscle. *J. Cell Biol.* **96**, 1727-1735.

van der Neut, R., Krimpenfort, P., Calafat, J., Niessen, C. M. and Sonnenberg, A. (1996). Epithelial detachment due to absence of hemidesmosomes in integrin beta 4 null mice. *Nat. Genet.* **13**, 366-369.

Wilhelmsen, K., Litjens, S. H., Kuikman, I., Tshimbalanga, N., Janssen, H., van den Bout, I., Raymond, K. and Sonnenberg, A. (2005). Nesprin-3, a novel outer nuclear membrane protein, associates with the cytoskeletal linker protein plectin. *J. Cell Biol.* **171**, 799-810.

Witt, S., Zieseniss, A., Fock, U., Jockusch, B. M. and Illenberger, S. (2004). Comparative biochemical analysis suggests that vinculin and metavinculin cooperate in muscular adhesion sites. *J. Biol. Chem.* **279**, 31533-31543.

Yoshida, M., Suzuki, A., Yamamoto, H., Noguchi, S., Mizuno, Y. and Ozawa, E. (1994). Dissociation of the complex of dystrophin and its associated proteins into several unique groups by n-octyl beta-D-glucoside. *Eur. J. Biochem.* **222**, 1055-1061.

Yoshida, M., Hama, H., Ishikawa-Sakurai, M., Imamura, M., Mizuno, Y., Araishi, K., Wakabayashi-Takai, E., Noguchi, S., Sasaoka, T. and Ozawa, E. (2000). Biochemical evidence for association of dystrobrevin with the sarcoglycan-sarcospan complex as a basis for understanding sarcoglycanopathy. *Hum. Mol. Genet.* **9**, 1033-1040.