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Expression of a Dystrophin–Sarcoglycan Complex in Serum-Deprived BC_3H1 Cells and Involvement of α-Sarcoglycan in Substrate Attachment

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Dystrophin and α- and γ-sarcoglycans were newly expressed in BC_3H1 cells during differentiation induced by serum withdrawal. These proteins formed a tight complex with other dystophin-associated proteins (DAPs), as detected by immunoprecipitation with anti-dystrophin antibody. Integrins β_1 and β_3, vinculin, and focal adhesion kinase were also detected in the same immunoprecipitate. In a cell adhesion assay, differentiated BC_3H1 cells attached more efficiently to type I collagen-coated dishes than nondifferentiated cells and loss of α-sarcoglycan induced by antisense ODN in differentiated cells resulted in significant inhibition of cell adhesion. Thus dystrophin and DAPs, at least partly, form a complex with the focal adhesion proteins in differentiated BC_3H1 cells and α-sarcoglycan seems to modulate the function of the focal adhesion complex in these cells.

In skeletal muscle, dystrophin, whose defect causes Duchenne and Becker dystrophies, is localized at the cytoplasmic surface of the sarcolemma, forming a tight complex with dystrophin-associated proteins (DAPs) (1, 2). The latter consists at least of a peripheral membrane protein triplet (syntrophins), the dystroglycan complex (α- and β-dystroglycans), the sarcoglycan complex (α-, β-, and γ-sarcoglycans), and a 25-kDa transmembrane protein. Since dystrophin binds to actin and α-dystroglycan binds to laminin, an extracellular matrix protein, the dystrophin-DAP complex links the actin cytoskeleton to the extracellular matrix. This linkage is considered to be essential for the structural and functional integrity of the sarcolemma in striated muscle. However, little is known about the mechanism by which defect in this linkage leads to muscle cell necrosis (1, 2).

The sarcoglycan complex is specifically expressed in the striated muscle, whereas dystroglycans are expressed in nonmuscle cells as well as in myocytes (1-4). Recently, autosomally inherited muscular dystrophies of variable severity and phenotype have been reported to occur in patients carrying defects in the sarcoglycan genes (3-5). Thus sarcoglycans are as important as dystrophin for the viability of striated muscle cells. At present, however, there is little information regarding the interaction of sarcoglycans with other components of the dystrophin-DAP complex or with other cellular proteins. Like other components of the dystrophin-DAP complex, the functional role of sarcoglycans is not known.

BC_3H1 cells, a nonfusing muscle cell line derived from a mouse brain tumor (6), have received considerable attention as a model for studying the control of the expression of muscle-specific proteins. In response to reduced serum concentration, these cells stop proliferation and start to express skeletal and smooth muscle proteins including creatine phosphokinase, nicotinic acetylcholine receptor, ryanodine receptor, skeletal and smooth muscle actins, and high-molecular-weight caldesmon (7-10). In this study, we attempted to establish the utility of this myogenic cell line as a model for investigating the function of the dystrophin-DAP complex in the striated muscle. We found that differentiated BC_3H1 cells expressed the dys-

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trophin-sacoglycan complex. By using an antisense DNA strategy, we present evidence that α-sarcoglycan is involved in attaching differentiated BC_3H1 cells to the substrate.

MATERIALS AND METHODS

Cell culture. BC_3H1 cells were grown on 100-mm culture dishes in DMEM supplemented with 20% heat inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. To induce differentiation, cells were switched to the same medium containing 0.5% FCS.

Immunoprecipitation and immunoblotting. BC_3H1 cells (1.5×10^6 cells) were washed three times with phosphate-buffered saline and lysed with IP buffer (50 mM Tris/HCl, pH 7.4 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 0.5 % NP-40, 0.1 % Na-deoxycholate, 1 mM PMSF, 1 mM Na-orthovanadate, 10 mM NaF, 10 mM Na-pyrophosphate) for 5 min on ice. The lysates were then clarified by centrifugation and precleared for 1 hr with 50 μl of protein A-sepharose beads. After centrifugation, the supernatants were incubated for 2 hr with an antibody and then for 1 hr with 100 μl of protein A-sepharose beads. Immune complex was extensively washed with ice-cold IP buffer and finally boiled in 30 μl of SDS/PAGE sample buffer for 5 min. SDS-PAGE and Immunoblot analysis were performed as described previously (11).

Antibodies. Anti-α-sarcoglycan monoclonal antibody (mAb) IVD3-1 (UBI, Lake Placid NY), anti-dystrophin mAb VIA4-2 A3 (UBI), anti-utrophin mAb NCL-DRP1 (Novocastra), anti-β-dystroglycan mAb NCL-43DAG (Novocastra), anti-laminin mAb (Sigma), anti-syntrophin mAb mab SYN1351, and anti-γ-sarcoglycan polyclonal antibody (pAb) were used. mab SYN1351 (12) was a gift from Dr. Stanley C. Froehner. Anti-γ-sarcoglycan IgG pAb was raised by immunizing outbred white leghorn (Babcock B300) with a glutathione S-transferase (GST) fusion protein containing the C-terminal fragment (aa 64 - 291) of rabbit skeletal muscle γ-sarcoglycan (4). The GST fusion protein construct was generated by subcloning the PCR-amplified segment of the γ-sarcoglycan cDNA into pGEX 4T-3 vector (Pharmacia). The fusion protein was purified from transformed E. coli DH5α according to the manufacturer's protocol.

Cell adhesion assay. Differentiated BC_3H1 cells (1×10^5 cells) were dispersed by treating them with 0.25 % trypsin/1 mM EDTA, and washed 3 times with serum-free DMEM. Cells were then plated onto dishes coated with

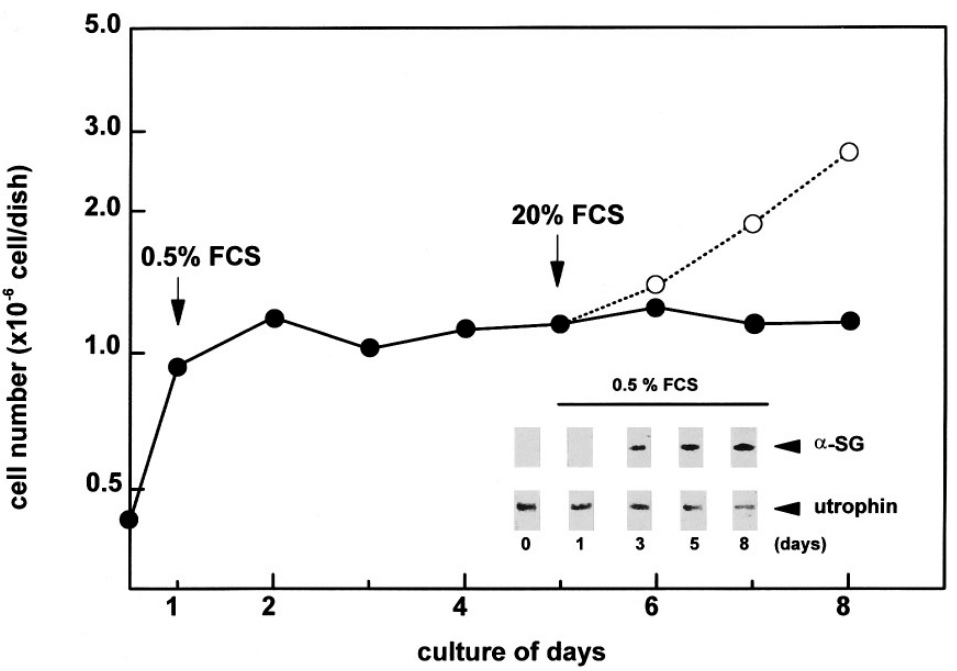

FIG. 1. Effects of serum on growth of BC_3H1 cells and expression of α-sarcoglycan and utrophin. BC_3H1 cells were plated on 100-mm dishes at 1.5×10^5 cells/dish in DMEM containing 20% FCS. On day 1, cultures were transferred to medium containing 0.5% FCS. On day 5, half of the cultures were transferred to 20% FCS (○), while the other half were maintained in 0.5% FCS (●). The inset shows time-dependent changes in expression levels of α-sarcoglycan and utrophin after the transfer of cells to 0.5% FCS.

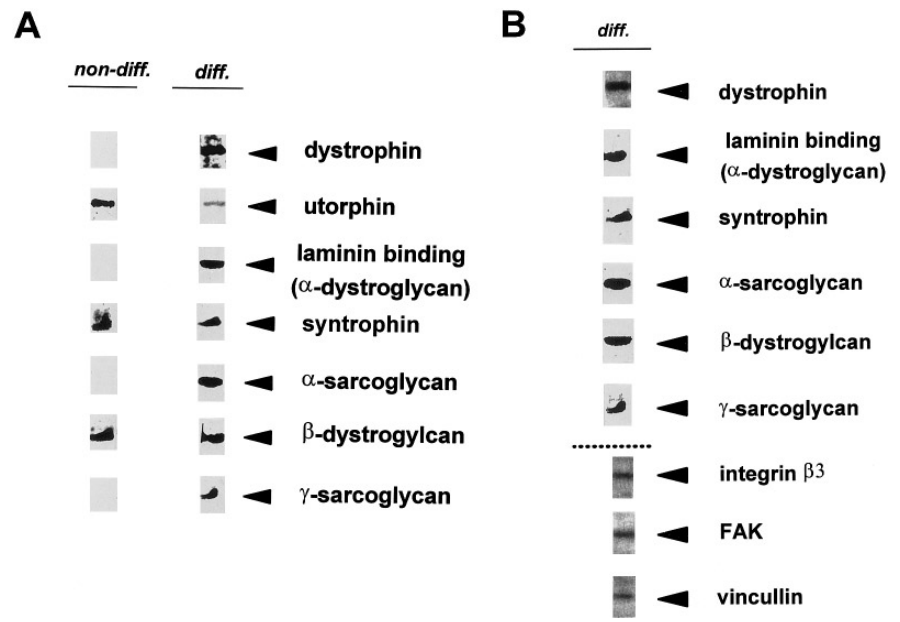

FIG. 2. Induction of the dystrophin-DAP complex in differentiated BC_3H1 cells. Lysates were prepared from BC_3H1 cells (1×10^7 cells) that had been maintained for 5 days in 20% or 0.5% FCS. (*A*) Lysates were subjected to immunoblot analysis with antibodies against indicated proteins. (*B*) Lysates were subjected to immunoprecipitation with anti-dystrophin antibody and then the immunoprecipitated material was analyzed by immunoblotting as in (*A*).

type I collagen and incubated with serum-free DMEM at 37°C in 5 % CO_2 for the indicated periods. Attached cells were counted using trypan blue.

Antisense oligodeoxyribonuclotide (ODN) treatment. Following 16-mer phosphoro-thioate-modified ODNs were synthesized (Sawady Technology Inc.): antisense ODN, 5′-GTGGTCTGCTGGGCCT-3′ (nt 64 - 79 of rabbit α-sarcoglycan cDNA (13)); control ODN, the same base composition as the antisense ODN except that the 11th and the 13th G were replaced by As. After cultivation in 0.5% FCS for 7 days, cells were treated with 300 nM antisense or control ODN in the presence of 10 μg/ml lipofectin (Gibco BRL) but in the absence of FCS for 6 hr. Cells were then washed three times with DMEM and left in DMEM containing 0.5% FCS and 300 nM ODN for additional 48 hr. The latter procedure was repeated to expose cells to ODN for a total of 5 days. Cells were then screened for α-sarcoglycan expression by immunoblotting.

RESULTS AND DISCUSSION

BC_3H1 cells grew logarithmically with a doubling time of approximately 20 hr when cultured in medium containing 20% FCS. Transfer of subconfluent cultures to medium containing 0.5 % FCS resulted in arrest of cell division (Fig. 1). On the third day of culture in 0.5 % FCS, we observed induction of α-sarcoglycan in these cells, whose expression level increased with longer time in low serum (Fig. 1. inset). In contrast, a dystrophin-related protein (utrophin), a high-molecular-weight protein that has extensive primary sequence similarity to dystrophin, was expressed in proliferating BC_3H1 cells and its expression decreased after the transfer of cultures to low serum (Fig. 1. inset).

We examined the expression of dystrophin and DAPs in proliferating as well as in differentiated (cultured for 5 days in 0.5% FCS) BC_3H1 cells by immunoblot analysis (Fig. 2A). Proteins reactive with antibodies against dystrophin, β-dystroglycan, syntrophins, and α- and γ-sarcoglycans were detected in differentiated BC_3H1 cells, whereas only proteins recognized by antibodies to syntrophin and β-dystroglycan were present in proliferating BC_3H1 cells. α-Dystroglycan and utrophin were detected by a laminin overlay assay and anti-utrophin antibody

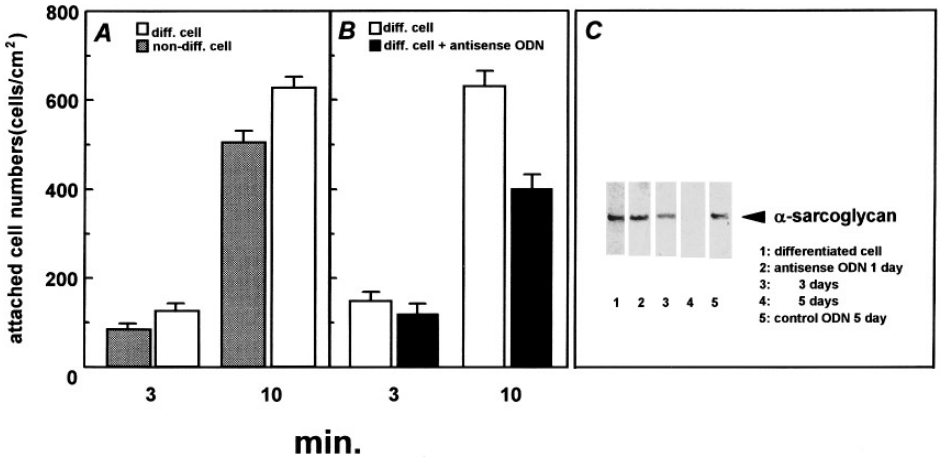

FIG. 3. Cell adhesion assay. (*A*) BC_3H1 cells maintained for 5 days in 20% or 0.5% FCS were detached from dishes by treating with trypsin/EDTA. Cells (10^4 cells each) were then replated onto new collagen-coated dishes in serum-free DMEM for indicated times and attached cells were counted. (*B*) BC_3H1 cells maintained in 0.5% FCS for 5 days were treated with control ODN or antisense ODN directed against α-sarcoglycan for 5 days. Cell adhesion assay was carried out as in (*A*). In (*A*) and (*B*), each column represents the mean ± S.E.M. of data obtained from 4 independent experiments. (*C*) BC_3H1 cells maintained in 0.5% FCS were treated with control or antisense ODN and a time-dependent change in α-sarcoglycan content was estimated by immunoblot analysis.

in both proliferating (data not shown) and differentiated cells (Fig. 2A). These proteins were detected only in the Triton X-100-insoluble fractions from proliferating and differentiated BC_3H1 cells (data not shown).

Fig. 2B shows typical results from immunoprecipitation experiments with anti-dystrophin antibody. All these proteins except for utrophin were present in the immunoprecipitated material from the lysate of differentiated BC_3H1 cells. Intriguingly, we also detected proteins reactive with antibodies against integrin $\beta 3$ subunit, vinculin, and focal adhesion kinase (FAK) in the same immunoprecipitate. Integrin $\beta 1$ was also detected (data not shown). On the other hand, anti-dystrophin antibody did not precipitate any of these proteins from proliferating BC_3H1 cells (data not shown). When similar experiments were repeated using anti-utrophin antibody, α- and β-dystroglycans, and syntrophins, but not α- and γ-sarcoglycans were coimmunoprecipitated with utrophin from lysates of both proliferating and differentiated cells (data not shown).

We compared adhesion activities of proliferating and differentiated BC_3H1 cells. We found that differentiated BC_3H1 cells attached more efficiently to type I collagen-coated dishes than proliferating cells (Fig. 3A). Furthermore, adhesion of differentiated BC_3H1 cells to collagen were inhibited by about 40% when cells had been treated with antisense ODN directed against α-sarcoglycan for 5 days and then were assayed for adhesion activity for 10 (Fig. 3B) or 30 min (data not shown). After the treatment with α-sarcoglycan antisense ODN for 5 days, α-sarcoglycan disappeared completely from BC_3H1 cells (Fig. 3C), although expression levels of dystrophin and other DAPs were not affected by the same treatment (data not shown). Integrins β_1 and β_3 by immunoblot analysis also decreased to less than 20% and to about 50% of the control, respectively. In contrast, control ODN did not affect the expression of these proteins including α-sarcoglycan (Fig. 3C and data not shown).

The expression of α- and γ-sarcoglycans are known to be specific in the striated muscle (3, 4, 13). The results described here strongly suggest that sarcoglycans are expressed forming a complex with dystrophin together with other DAPs in differentiated BC_3H1 cells. In contrast, dystrophin or α- and γ-sarcoglycans as well as short dystrophin-gene products are not detected

in proliferating BC_3H1 cells. In the latter, utrophin forms a complex with α- and β-dystroglycans and syntrophins. In differentiated BC_3H1 cells, the utrophin complex still exists, but in a much reduced amount (Fig. 1A and data not shown). We found that integrins, vinculin, and FAK, which are the proteins of focal adhesions, are coimmunoprecipitated with anti-dystrophin antibody, suggesting the colocalization of these proteins with the dystrophin-DAP complex in differentiated BC_3H1 cells. This finding is consistent with the previous report by Kramarcy and Sealock (14) that by immunostaining, dystrophin and a 48-kDa protein, which is recognized by anti-syntrophin antibody, are localized to talin-positive sites in cultured Xenopus muscle. Thus the dystrophin-DAP complex, at least in part, seems to form an integral part of the focal adhesion complex in these cultured cells. Since differentiated BC_3H1 cells expressing sarcoglycans attached more efficiently to collagen-coated dishes than proliferating cells (Fig. 3A) and since loss of α-sarcoglycan due to treatment with α-sarcoglycan antisense ODN significantly resulted in the inhibition of cell adhesion, α-sarcoglycan appears able to modulate the function of the focal adhesion complex.

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REFERENCES

1. Tinsley, J. M., Blake, D. J., Zuellig, R. A., and Davies, K. E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8307–8313.
2. Campbell, K. P. (1995) *Cell* **80,** 675–679.
3. Roberds, S. L., Leturcq, F., Allamand, V., Piccolo, F., Jeanpierre, M., Anderson, R. D., Lim, L. E., Lee, J. C., Tomé, F. M. S., Romero, N. B., Fardeau, M., Beckmann, J. S., Kaplan, J-C., and Campbell, K. P. (1994) *Cell* **78,** 625–633.
4. Noguchi, S., McNally, E. M., Othmane, K. B., Hagiwara, Y., Mizuno, Y., Yoshida, M., Yamamoto, H., Bönnemann, C. G., Gussoni, E., Denton, P. H., Kyriakides, T., Middleton, L., Hentati, F., Hamida, M. B., Nonaka, I., Vance, J. M., Kunkel, L. M., and Ozawa, E. (1995) *Science* **270,** 819–822.
5. Lim, L. E., Duclos, F., Broux, O., Bourg, N., Sunada, Y., Allamand, V., Meyer, J., Richard, I., Moomaw, C., Slaughter, C., Tome, F. M. S., Fardeau, M., Jackson, C. E., Beckmann, J. S., and Campbell, K. P. (1995) *Nature Genetics* **11,** 257–265.
6. Schubert, D., Harris, J., Devine, C. E., and Heinemann, S. (1974) *J. Cell Biol.* **61,** 398–413.
7. Munson, R., Caldwell, Jr., K. L., and Glaser, L. (1982) *J. Cell Biol.* **92,** 350–356.
8. Ueki, N., Sobue, K., Kannda, K., Hada, T., and Higashino, K. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 9049–9053.
9. Strauch, A. R., and Reeser, J. C. (1989) *J. Biol. Chem.* **264,** 8345–8355.
10. Marks, A. R., Taubman, M. B., Saito, A., Dai, Y., and Fleischer, S. (1991) *J. Cell Biol.* **114,** 303–312.
11. Tawada-Iwata, Y., Imagawa, T., Yoshida, A., Takahashi, M., Nakamura, H., and Shigekawa, M. (1993) *Am. J. Physiol.* **264,** H1447–H1453.
12. Froehner, S. C., Murnane, A. A., Tobler, M., Peng, H. B., Sealock, R. (1987) *J. Cell Biol.* **104,** 1633–1646.
13. Roberds, S. L., Anderson, R. D., Ibraghimov-Beskrovnaya, O., and Campbell, K. P. (1993) *J. Biol. Chem.* **268,** 23739–23742.
14. Kramarcy, N. R., and Sealock, R. (1990) *FEBS Lett.* **274,** 171–174.