Bidirectional Signaling between Sarcoglycans and the Integrin Adhesion System in Cultured L6 Myocytes*

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The rat L6 skeletal muscle cell line was used to study expression of the dystrophin-containing glycoprotein complex and its interaction with the integrin system involved in the cell-matrix adhesion reaction. A complex of dystrophin and its associated proteins was fully expressed in L6 myotubes, from which anti-dystrophin or anti-α-sarcoglycan co-precipitated integrin α5β1 and other focal adhesion-associated proteins vinculin, talin, paxillin, and focal adhesion kinase. Immunostaining and confocal microscopy revealed that dystrophin, α-sarcoglycan, integrin α5β1, and vinculin exhibited overlapping distribution in the sarcoplemma, especially at focal adhesion-like, spotty structures. Adhesion of cells to fibronectin- or collagen type I-coated dishes resulted in induction of tyrosine phosphorylation of α- and γ-sarcoglycans but not β-sarcoglycan. The same proteins were also tyrosine-phosphorylated when L6 cells in suspension were exposed to Arg-Gly-Asp-Ser peptide. All of these tyrosine phosphorylations were inhibited by herbycin A. On the other hand, treatment of L6 myotubes with α- and γ-sarcoglycan antisense oligodeoxynucleotides resulted in complete disappearance of α- and γ-sarcoglycans and in significant reduction of levels of the associated focal adhesion proteins, which caused about 50% reduction of cell adhesion. These results indicate the existence of bidirectional communication between the dystrophin-containing complex and the integrin adhesion system in cultured L6 myocytes.

Several classes of cell adhesion receptors, including integrins, dystroglycan, cadherins, and members of the immunoglobulin family, are coexpressed by skeletal muscle and presumably play critical roles during skeletal muscle differentiation and development (see Ref. 1 for review). Integrins form a large family of heterodimeric transmembrane proteins with different α and β subunits (2). The ligands of the integrins can be components of the extracellular matrix or other integral membrane proteins, such as VCAM-1. Previous studies suggest that different integrins may play specific roles in different developmental and cellular phenomena. The β1 integrin subfamily, with its different α subunits, is a major group of integrins expressed in muscle cells and has been shown to be required for myoblast differentiation and myotube formation (1, 3). A large number of different integrin α subunits are expressed in developing muscle cells, and some of them localize in unique functional cellular regions and seem to play distinct roles in regulation of myogenesis, although their precise functions remain to be clarified (1, 4, 5).

Myogenic cells associate with extracellular matrix, which is advantageous for cell terminal differentiation. The extracellular matrix-integrin interaction occurs at various cytoskeletal-sarcomemmal linkages, such as focal adhesion-like structures, myotendinous and neuromuscular junctions, and the costameres (1, 4, 6). At the focal adhesions present in cultured cells, integrins cluster and associate with many cytoskeletal proteins, such as talin, vinculin, paxillin, and α-actinin, as well as FAK1 and other protein kinases (7–9). The focal adhesion complex is the major site of actin filament attachment. Recent evidence indicates that assembly of the focal complex requires both the engagement of integrins with extracellular matrix and the integrin activation by intracellular signaling, although the physiological mechanism of the latter is largely unknown (9). Integrin occupancy with ligands and clustering have been shown to trigger tyrosine phosphorylation of several intracellular proteins, including FAK, paxillin, tensin, and mitogen-activated protein kinase (2, 7, 9). Recent evidence suggests that tyrosine phosphorylation of FAK and other focal adhesion proteins is not required for focal adhesion formation but is important for the other integrin-induced signalings, such as control of cell growth (10). However, assembly of the focal adhesion complex can be inhibited by tyrosine kinase inhibitors (8, 11).

Dystroglycan, which exists as a noncovalently linked complex of α- and β-subunits, is another important cell adhesion receptor that links the extracellular matrix with the actin cytoskeleton (12–14). In skeletal muscle cells, it forms a tight complex with dystrophin together with other dystrophin-associated proteins (DAPs) including α-, β-, γ- and δ-sarcoglycans, the 25-kDa protein, and syntrophins (13, 14, 16–20). Dystroglycan is expressed widely in nonmuscle cells, as well as in myocytes, whereas sarcoglycans are expressed predominantly in striated muscle cells (12, 15–20). The integrity of the dystrophin-DAP complex seems to be essential for the viability of muscle cells, because disruption of the complex due to a defect in dystrophin or any one of the sarcoglycans has been reported to cause various forms of inherited muscular dystrophy (13, 14, 16–18, 21). Sarcoglycans thus seem to be functionally and pathologically as important as dystrophin. At present, however, like all other components of the dystrophin-DAP complex, little is known about the functional and structural roles of sarcoglycans, including their interaction with other cellular proteins.

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1 The abbreviations used are: FAK, focal adhesion kinase; DAP, dystrophin-associated protein; ODN, oligodeoxynucleotide; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin.
In our recent preliminary report (22), we have presented evidence that the dystrophin-DAP complex is associated with the focal adhesion assembly proteins, such as the integrin β1, subunit, vinculin, and FAK in serum-deprived, differentiated BC3H1 cells, a nonfusing muscle cell line. We have also shown that loss of α-sarcoglycan induced by the antisense ODN treatment results in a significant inhibition of adhesion to the substrate by these cells. In this communication, we report that muscle-specific α- or γ-sarcoglycans were tyrosine-phosphorylated when differentiated rat skeletal L6 cells were adhered to the extracellular matrix. On the other hand, treatment of these cells with antisense ODN directed against α- or γ-sarcoglycan significantly decreased the associated focal adhesion proteins and concomitantly inhibited cell adhesion. The results indicate the existence of bidirectional communication between the dystrophin-DAP complex and the integrin adhesion system in these cultured cells.

EXPERIMENTAL PROCEDURES

Antibodies—Monoclonal mouse antibodies against dystrophin (VLA4-2 A3, Upstate Technology Inc.), α-sarcoglycan (VVD-2, Upstate Technology Inc.), β-dystroglycan (Novocastra), γ-sarcoglycan (anti-laminin monoclonal antibody, Sigma), integrin β1 (MAR4, Pharmingen), vinculin (VIN-11–5, Sigma), and phosphotyrosine (PY20, Upstate Technology Inc.) were used. Anti-FAK, anti-paxillin, anti-ezrin, and anti-cortactin monoclonal antibodies were obtained from Transduction Laboratories. Anti-syntrophin monoclonal antibody SYN1351 was a gift from Dr. Stanley C. Froehner (23). Polyclonal antibodies against integrin αβ1 (VLA-5), and αβ2, integrin subunit (VNRI39) were purchased from Chemicon and Life Technologies, Inc., respectively. Anti-actin polyclonal antibody, which recognizes all actin isoforms, was a gift from Dr. Keigi Fujiwara (24). A polyclonal rabbit antibody against the cytoplasmic domain of α-sarcoglycan fused to glutathione S-transferase was prepared as described previously (25). Preparation of polyclonal chicken antibodies against glial F65-Thr-275 fusion proteins containing amino acids 92–215 of rabbit skeletal muscle β-sarcoglycan and amino acids 64–291 of rabbit skeletal muscle γ-sarcoglycan was also described (26). These chicken antibodies, which were subsequently affinity purified using glutathione S-transferase and glutathione S-transferase-sarcoglycan fusion proteins as described previously (27), recognized single proteins of the expected molecular weights for respective sarcoglycans in the immunoblot assay of L6 cell lysates (see Fig. 3B).

Cell Culture and Cell Adhesion Assay—L6 rat skeletal myoblasts (ATCC) were grown on 100-mm tissue culture dishes (Falcon) in DMEM supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin and 100 μg/ml streptomycin. After 4–5 days, ~80% of cells were fused to form myotubes. We used these cells for all experiments in this study. For cell adhesion assay, these cells were detached from dishes by trypsinization, washed once with DMEM containing 2% FCS and twice with DMEM containing 0.5% BSA, and subsequently preincubated with DMEM containing 0.5% BSA for 15 min at 37 °C on a rotator. Cells (104 cells) were then plated onto dishes coated with fibronectin (4 μg/cm2), or poly-L-lysine (3 μg/cm2) and incubated at 37 °C for 3 h. After being washed three times with PBS containing 0.1% Tween 20, cells were incubated with either rhodamine-labeled donkey anti-rabbit IgG (Chemicon) (1:1000) or fluorescein-labeled donkey anti-mouse IgG (Cappel) (1:500) for 1–3 h at 4 °C and then washed three times with PBS containing 0.1% Tween 20. For double staining with a combination of polyclonal (rabbit) and monoclonal (mouse) antibodies, fixed and permeabilized cells were incubated with a blocking solution (Block ACE, Dainippon Pharmaceuticals) containing 3% BSA for 1 h at room temperature and then washed three times with PBS containing 0.1% Tween 20. Cells were treated with one of the following primary antibodies for 4–6 h at 4 °C: anti-α-sarcoglycan (di-lution, 1:500), anti-dystrophin (1:100), anti-integrin β1 (1:200), anti-integrin αβ1 (1:500). After being washed three times with PBS containing 0.1% Tween 20, cells were incubated with fluorescein- or rhodamine-labeled donkey anti-rabbit IgG (Chemicon) (1:1000) or fluorescein-labeled donkey anti-mouse IgG (Cappel) (1:500) for 1–3 h at 4 °C and then washed three times with PBS containing 0.1% Tween 20. For double staining with a combination of polyclonal (rabbit) and monoclonal (mouse) antibodies, fixed and permeabilized cells were incubated with a mixture of two primary antibodies and then with a mixture of the fluorescein-labeled anti-mouse and rhodamine-labeled anti-rabbit IgGs.

RESULTS

Expression and Localization of Dystrophin-DAP Complex in L6 Myotubes—The rat skeletal muscle cell line has widely been used as a model for studying myogenesis. Upon reaching subconfluency in 10% serum, L6 myoblasts spontaneously fuse with each other to form multinucleated myotubes and express skeletal muscle proteins (30, 31). Using specific antibodies, we examined the expression and distribution of the dystrophin-DAP complex in L6 cells that had been cultured on fibronectin-coated dishes for 4–5 days.

The material immunoprecipitated from adherent L6 cells with anti-dystrophin contained proteins recognized by antibodies against dystrophin, β-dystroglycan, syntrophin, and α-, β-, and γ-sarcoglycans (Fig. 1A). α-Dystroglycan was detected by laminin overlay assay. In the same immunoprecipitate, we detected the proteins reactive with antibodies raised against focal adhesion- and growth factor-associated proteins such as the integrins αβ1, αβ2, vinculin, talin, and FAK (Fig. 1A; see also Fig. 7B). We also detected a protein reactive with anti-paxillin (data not shown). Conversely, we found that the material immunoprecipitated with anti-integrin β1 subunit contained proteins reactive with antibodies against the components of the dystrophin-DAP complex (Fig. 1B). The fractions of focal adhesion proteins recov-
were lysed in immunoprecipitation buffer. A small amount of actin was detected in the precipitates obtained using the usual immunoprecipitation buffer (see Fig. 1A) without a resultant change in the relative abundance of each protein (data not shown). Based on all of these results, it seems likely that the dystrophin antibody did not co-precipitate very large cytoskeletal complexes, suggesting the presence of specific interaction of the dystrophin-DAP complex with focal adhesion-associated proteins.

Antibodies against other cytoskeletal proteins, such as 
integrin subunits, ezrin, and cortactin, did not precipitate the components of the dystrophin-DAP complex from adherent L6 cells (Fig. 1A and data not shown). Conversely, 
integrin subunits, ezrin, and cortactin were not detected in the dystrophin immunoprecipitates (Fig. 1, A and C). Actin was not detected in the 
integrin immunoprecipitates (Fig. 1A). On the other hand, a small amount of actin was detected in the dystrophin immunoprecipitates from adherent and nonadherent cells (Fig. 1A). This actin appears to be one that is bound to dystrophin, because dystrophin is known to be an actin-binding protein (13, 14, 32). Under the conditions used, >80% of actin was not solubilized into cell lysates, and about 90% of the solubilized actin was present in the postimmunoprecipitation supernatant obtained with anti-dystrophin (data not shown). To obtain additional controls for these immunoprecipitation data, we carried out immunoprecipitation with anti-dystrophin using the immunoprecipitation buffer containing 0.2% SDS but omitting Triton X-100 and sodium deoxycholate, instead of our usual buffer containing 0.1% SDS, 1% Triton X-100, and 1% sodium deoxycholate. When adherent cells were dissolved in this solution and immunoprecipitation and washing were performed in the same solution, recovery of proteins with protein A-Sepharose beads was reduced to about half, but anti-dystrophin or anti- 
integrin subunit was able to co-precipitate all of the proteins that were detected in the corresponding immunoprecipitates obtained using the usual immunoprecipitation buffer (see Fig. 1A) at a resultantly change in the relative abundance of each protein (data not shown). Based on all of these results, it seems likely that the dystrophin antibody did not co-precipitate very large cytoskeletal complexes, suggesting the presence of specific interaction of the dystrophin-DAP complex with focal adhesion-associated proteins.

We investigated localization of proteins recognized by antibodies against dystrophin, a-sarcoglycan, integrin  

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β1 or anti-dystrophin stained only the peripheral sarcolemma (Fig. 2, A and B), whereas in a cell focused at the basal portion, spotty staining of cell area was obtained with anti-dystrophin (Fig. 2B, arrowhead). We double-stained cells with rabbit anti-a-sarcoglycan (red) and mouse monoclonal anti-vinculin (green). In a cell focused at the basal portion, signals from both antibodies colocalized at many spotty structures (Fig. 2D, yellow), although they clearly did not overlap at some other similar structures. These spotty structures stained with anti-vinculin may be similar to focal adhesions in fibroblasts (7). When the middle portion of a cell was focused, much less overlap of signals was observed (Fig. 2C). Interestingly, in cells plated on poly-L-lysine-coated dishes, the signal from anti-a-sarcoglycan was much reduced in its intensity in the basal cell area, and correspondingly the overlap of signals from anti-a-sarcoglycan and anti-vinculin was much reduced (Fig. 2E). Of note, in these cells, the basal cell area contacting the substrate was also significantly decreased.

Cell Adhesion-induced Tyrosine Phosphorylation of Sarcoglycans—We investigated tyrosine phosphorylation of proteins in L6 cells in response to cell adhesion. L6 cells plated on fibronectin-coated dishes for 30 min exhibited a high level of tyrosine phosphorylation of proteins, with M r (in thousands) 120–130, 100, 70–85, 50, and 30–45 (Fig. 3A, lane 3). We identified one of the phosphoproteins in the M r 120,000–130,000 range to be FAK (see below), which is consistent with similar findings obtained with fibroblastic cells under comparable conditions (11, 33). We further examined whether the proteins precipitated from adherent L6 cells with anti-dystrophin or anti- 

α-sarcoglycan were tyrosine-phosphorylated. In each immunoprecipitate, proteins of M r 50,000 and 35,000 were most prominently tyrosine-phosphorylated, suggesting that they are a- and γ-sarcoglycans (Fig. 3A, lanes 4–6). These proteins, however, were not tyrosine-phosphorylated in nonadherent L6 cells (Fig. 3A, lane 2, see also Figs. 4A and 5A). Tyrosine phosphorylation of 50- and 35-kDa proteins was not detected in dystrophin immunoprecipitates from adherent and nonadherent cells (Fig. 1A). This actin appears to be one that is bound to dystrophin, because dystrophin is known to be an actin-binding protein (13, 14, 32). Under the conditions used, >80% of actin was not solubilized into cell lysates, and about 90% of the solubilized actin was present in the postimmunoprecipitation supernatant obtained with anti-dystrophin (data not shown). To obtain additional controls for these immunoprecipitation data, we carried out immunoprecipitation with anti-dystrophin using the immunoprecipitation buffer containing 0.2% SDS but omitting Triton X-100 and sodium deoxycholate, instead of our usual buffer containing 0.1% SDS, 1% Triton X-100, and 1% sodium deoxycholate. When adherent cells were dissolved in this solution and immunoprecipitation and washing were performed in the same solution, recovery of proteins with protein A-Sepharose beads was reduced to about half, but anti-dystrophin or anti-
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Fig. 1. Co-precipitation of dystrophin, DAPs, and some focal adhesion-associated proteins from L6 cell lysates. L6 cells were cultured for 4 days on fibronectin-coated plates in DMEM containing 10% FCS. Adherent cells or cells detached by trypsination followed by incubation with DMEM containing 0.5% BSA for 30 min (1×10^7 cells) were lysed in immunoprecipitation buffer. A, lysates were subjected to immunoprecipitation with anti-dystrophin or anti- 
integrin subunit, and then precipitated materials were analyzed by immunoblotting with antibodies against the indicated proteins. B, lysates from adherent cells were subjected to immunoprecipitation with anti-integrin β1 subunit, and the precipitates were analyzed by immunoblotting with antibody against dystrophin (lane 1), β-dystroglycan (lane 3), syntrophin (lane 4), a-sarcoglycan (lane 5), β-sarcoglycan (lane 6), or γ-sarcoglycan (lane 7). -Dystroglycan was detected by laminin overlay assay (lane 2). C, lysates from adherent cells were subjected to immunoprecipitation with anti-dystrophin, and the precipitates (ppt) and postprecipitation supernatants (sup) were analyzed by immunoblotting with antibodies against the indicated proteins. In these and all subsequent figures, 100 µg of samples was loaded to each lane in SDS-polyacrylamide gel electrophoresis.
in the material precipitated with anti-αV integrin subunit (Fig. 3A, lane 7).

To substantiate our suggestion that α- and γ-sarcoglycans are tyrosine-phosphorylated in adherent L6 cells, we examined the effects of treatment with antisense ODNs directed against sarcoglycans on tyrosine phosphorylation of the 50- and 35-kDa proteins. Prior treatment of adherent L6 cells with α-, β-, or γ-sarcoglycan antisense ODN for 5 days resulted in complete disappearance of the 50-, 42-, and 35-kDa proteins from the dystrophin immunoprecipitates (Fig. 3B, lanes 2, 4, and 6). Under the same conditions, the 50- and 35-kDa bands detected with anti-phosphotyrosine also disappeared (Fig. 3B, lanes 8 and 10).

FIG. 2. Confocal laser scanning images showing localization of dystrophin, α-sarcoglycan, integrin α5β1, and vinculin. L6 cells plated on fibronectin-coated dishes for 60 min were stained with anti-integrin α5β1 (A) or anti-dystrophin (B) or double-stained with anti-α-sarcoglycan (red) and anti-vinculin (green) (C and D). L6 cells plated on poly-L-lysine-coated dishes for 60 min were double-stained with anti-α-sarcoglycan (red) and anti-vinculin (green) (E). Focus was adjusted to the middle (A, B, and C) or the basal (D and E) portions of cells. Arrowhead in B and C shows a cell focused at its basal portion.

FIG. 3. Cell adhesion-induced tyrosine phosphorylation of proteins in L6 cells treated without (A) or with (B) sarcoglycan antisense ODNs. A, lysates prepared from L6 cells kept in suspension (lanes 1 and 2) or from cells plated on fibronectin-coated dishes (lanes 3–7) were subjected to immunoblotting with anti-phosphotyrosine (lanes 1 and 3) or to immunoprecipitation with anti-dystrophin followed by immunoblotting with either anti-phosphotyrosine (lanes 2 and 5) or anti-α-sarcoglycan (lane 4). The lysates from adherent L6 cells were also subjected to immunoprecipitation with anti-α-sarcoglycan (lane 6) or anti-αV integrin subunit (lane 7) followed by immunoblotting with anti-phosphotyrosine. B, L6 cells had been treated for 5 days with 300 nM each of control ODN (lanes 1, 3, 5, and 7) or antisense ODNs directed against α-sarcoglycan (lanes 2 and 8), β-sarcoglycan (lanes 4 and 9), or γ-sarcoglycan (lanes 6 and 10). Lysates from these cells were subjected to immunoprecipitation with anti-dystrophin followed by immunoblotting with anti-α-sarcoglycan (lanes 1 and 2), anti-β-sarcoglycan (lanes 3 and 4), anti-γ-sarcoglycan (lanes 5 and 6) or anti-phosphotyrosine (lanes 7–10).

FIG. 4. Time courses of cell adhesion-induced tyrosine phosphorylation of sarcoglycans and FAK (A) and of attachment of L6 cells to dishes coated with different materials (B). A, L6 cells in suspension (1 × 10^5 cells) were replated onto fibronectin-coated dishes for the times indicated. Lysates from cells harvested at indicated time points were subjected to immunoprecipitation with anti-α-sarcoglycan (α-sarcoglycan and γ-sarcoglycan) or anti-FAK (FAK) followed by immunoblotting with the indicated antibodies. B, cells in suspension (10^4 cells) were replated onto dishes coated with poly-L-lysine (open circle), fibronectin (filled circle) or collagen type I (open triangle), and attached cells were counted at the indicated time points. Data are the mean ± S.E. of four independent experiments.
and 10). In contrast, random ODN or β-sarcoglycan antisense ODN did not affect tyrosine phosphorylation of the 50- and 35-kDa proteins (Fig. 3B, lanes 7 and 9). Furthermore, sarcoglycan antisense ODNs did not affect the relative abundance of components of the dystrophin-DAP complex other than the target sarcoglycan in the dystrophin immunoprecipitates (data not shown), indicating that they did not disrupt the preformed dystrophin-DAP complex.

Fig. 4A shows the time courses of tyrosine phosphorylation of α- and γ-sarcoglycans as well as of the protein recognized by anti-FAK in L6 cells plated onto fibronectin-coated dishes. The time courses of cell attachment to dishes coated with other materials are also shown (Fig. 4B). Before cell attachment, phosphotyrosine staining was not detected in α- or γ-sarcoglycan in the α-sarcoglycan immunoprecipitate or in the protein recognized by anti-FAK in the FAK precipitate (Fig. 4A, time 0). After cell plating, tyrosine phosphorylation of the protein recognized by anti-FAK was already significant at 2 min, reaching a high level at 5 min or longer (Fig. 4A). In contrast, phosphotyrosine staining of α- and γ-sarcoglycans was delayed, reaching approximately 20 and 10% of the maximum at 5 min, respectively, although it reached high levels at 15 min. Under equivalent conditions, about 75% of L6 cells adhered to fibronectin-coated dishes at 15 min (Fig. 4B). In contrast, cell attachment to collagen type I- and poly-L-lysine-coated dishes was significantly delayed (Fig. 4B).

Fig. 5A shows that α- and γ-sarcoglycans were tyrosine-phosphorylated to a similar extent in cells plated for 30 min on fibronectin- or collagen-coated dishes. However, tyrosine phosphorylation of these proteins was negligible in cells plated on poly-L-lysine-coated dishes. In the α-sarcoglycan immunoprecipitate from cells plated on poly-L-lysine, the protein recognized by anti-FAK and its tyrosine phosphorylation were significantly decreased compared with those from cells plated on fibronectin (Fig. 5B, lanes 2 and 3). Recovery of α-sarcoglycan and tyrosine phosphorylation of the protein recognized by anti-FAK in the FAK immunoprecipitate from the same cells were also significantly decreased compared with those from cells plated on fibronectin (Fig. 5B, lanes 6 and 7). On the other hand, the protein recognized by anti-FAK was not detected in the α-sarcoglycan immunoprecipitate from nonadherent cells (Fig. 5B, lane 1).

We examined whether RGDS peptide could mimic the effect of cell attachment on tyrosine phosphorylation of sarcoglycans. RGDS peptide is known to be recognized by a variety of integrins (2, 34). Tyrosine phosphorylation of α- and γ-sarcoglycans, as well as of the protein recognized by anti-FAK, was markedly enhanced when L6 cells in suspension were exposed to 1 μM RGDS peptide (Fig. 6). However, tyrosine phosphorylation of β-sarcoglycan was not detectable (data not shown). Prior treatment of L6 cells with 50 μM herbimycin A, a Src tyrosine kinase inhibitor, resulted in suppression of the RGDS peptide- or cell adhesion-induced tyrosine phosphorylation of α- and γ-sarcoglycans and the protein recognized with anti-FAK (Fig. 6), suggesting that the sarcoglycan phosphorylation was catalyzed by a herbimycin A-sensitive tyrosine kinase. By immunoblot, we detected α-sarcoglycan and the protein recognized with anti-FAK in the material precipitated by anti-α-sarcoglycan or anti-FAK from the RGDS peptide-treated cells (data not shown).

**Effect of Sarcoglycan Antisense ODNs on Adhesion of L6 Cells**—In the experiment shown in Fig. 7A, we compared adhesion activity of L6 cells treated with each of sarcoglycan antisense ODNs. We found that adhesion to fibronectin-coated dishes was inhibited by about 50% in cells pretreated with α- or...
unit of the dystrophin precipitates was not changed significantly by pretreatment with either of these antisense ODNs.

**DISCUSSION**

In this study, we have provided several lines of evidence suggesting that the dystrophin-DAP complex in cultured rat L6 myotubes has specific bidirectional interactions with the \( \alpha_\varepsilon \beta_1 \) integrin adhesion system: 1) anti-dystrophin or anti-\( \alpha_1 \)-sarcoglycan co-precipitated integrin \( \alpha_\varepsilon \beta_1 \) and other focal adhesion-associated proteins (vinculin, talin, paxillin, and FAK) together with the components of the dystrophin-DAP complex (Figs. 1A and 5B). Anti-dystrophin did not co-precipitate other cytoskeletal proteins, such as \( \alpha_\varepsilon \) integrin subunit, ezrin, cortactin, and actin (Fig. 1A and C). Conversely, anti-\( \beta_1 \) integrin subunit or anti-FAK co-precipitated the components of the dystrophin-DAP complex, although antibodies against \( \alpha_\varepsilon \) integrin subunit, ezrin, and cortactin did not precipitate them (Figs. 1B and 5B and “Results”). 2) Immunofluorescence study using confocal microscopy revealed that \( \alpha_1 \)-sarcoglycan and vinculin exhibited overlapping distribution in the sarcolemma, especially at the spotty structures in the basal cell area, when L6 cells were plated on fibronectin-coated dishes (Fig. 2). 3) Adhesion of L6 cells to fibronectin or collagen type I resulted in induction of tyrosine phosphorylation of \( \alpha_\varepsilon \) and \( \gamma \)-sarcoglycans (Figs. 3–5). Tyrosine phosphorylation of these proteins was similarly induced when L6 cells in suspension were exposed to RGDS peptide that interacts with integrins (2, 34) (Fig. 6). 4) Finally, treatment of L6 cells with \( \alpha_\varepsilon \) and \( \gamma \)-sarcoglycan antisense ODNs resulted in complete disappearance of \( \alpha_\varepsilon \) and \( \gamma \)-sarcoglycans and significant reduction of the associated focal adhesion proteins (Figs. 3B and 7B), which caused about 50% reduction of cell adhesion (Fig. 7A).

The integrin system involved in the bidirectional interaction with the dystrophin-DAP complex in L6 cells seems to be mainly one that is engaged in the cell-matrix adhesive reaction because adhesion-induced physical association of the dystrophin-DAP complex with focal adhesion-associated proteins is required for the tyrosine phosphorylation of \( \alpha_\varepsilon \) and \( \gamma \)-sarcoglycans. In L6 cells in suspension, \( \alpha_\varepsilon \) and \( \gamma \)-sarcoglycans and FAK were not tyrosine-phosphorylated, and anti-dystrophin or anti-\( \alpha_1 \)-sarcoglycan did not co-precipitate FAK, vinculin, and talin from these cells, although they co-precipitated some integrin \( \alpha_\varepsilon \beta_1 \) (Figs. 1 and 5). However, when cells in suspension had been treated with RGDS peptide, \( \alpha_\varepsilon \) and \( \gamma \)-sarcoglycans and FAK were co-precipitated, and their tyrosine phosphorylation occurred as in cells plated on fibronectin (Fig. 6 and “Results”). On the other hand, tyrosine phosphorylation of \( \alpha_\varepsilon \) and \( \gamma \)-sarcoglycans was not detectable in cells plated on poly-l-lysine (Fig. 5A, lane 2), although a small amount of phosphorylated FAK was found to be present in the \( \alpha_\varepsilon \)-sarcoglycan immunoprecipitate (Fig. 5B, lane 2). Confocal double-labeling study revealed a marked reduction of the overlap of signals from anti-\( \alpha_1 \)-sarcoglycan and anti-vinculin at the basal portion of a cell plated on poly-l-lysine (Fig. 2E). Thus, adhesion-induced recruitment of relevant proteins to the focal adhesion-like, spotty structures seems to be required for the phosphorylation of sarcoglycans. Of note, the amount of integrin \( \alpha_\varepsilon \beta_1 \), vinculin, talin, or FAK co-precipitated with anti-dystrophin was about 15% of the amount of each that was present in the total cell lysate (see “Results”), suggesting that relatively small fractions of these proteins are tightly associated with the dystrophin-DAP complex. This could be due to existence in L6 cells of much larger amounts of integrin \( \alpha_\varepsilon \beta_1 \) and other focal adhesion proteins compared with the dystrophin-DAP complex. Another possibility could be that colocalization of these focal adhesion proteins with the dystrophin complex occurs only at the limited cell area. By immunofluorescence staining, dystrophin, \( \alpha_1 \)-sarcoglycan antisense ODNs on adhesion activity of L6 cells and expression of focal adhesion-associated proteins. A, L6 cells were pretreated for 5 days with 300 nm each of random ODN (open columns) or sarcoglycan antisense ODNs (filled columns) and then replated onto fibronectin-coated dishes at a density of \( 10^6 \) cells in serum-free DMEM for the indicated periods of time. Cell adhesion assay were performed as described under “Experimental Procedures.” Each column represents the mean ± S.E. obtained from four independent experiments. B, lyses from cells pretreated with random ODN (AS-ODN) or antisense ODN directed against \( \alpha_1 \)-sarcoglycan antisense ODN (\( \alpha \)-SG AS-ODN), \( \beta_1 \)-sarcoglycan antisense ODN (\( \beta \)-SG AS-ODN), or \( \gamma \)-sarcoglycan antisense ODN (\( \gamma \)-SG AS-ODN) were subjected to immunoprecipitation with antibody for anti-dystrophin followed by immunoblotting with specific antibodies against the indicated proteins.
can, \( \alpha \beta_2 \), and vinculin were present at the sarcolemma in both the basal and nonbasal portions of the cell (Fig. 2). Signals of \( \alpha \)-sarcoglycan and vinculin were partially colocalized in both of these membrane areas with much intense overlap at the basal portion of the cell (Fig. 2C). At present, the quantitative aspect of the interaction between these proteins is unclear.

In fibroblasts and other cells, formation of the focal adhesion contact induces autophosphorylation and subsequent activation of FAK, a member of a family of structurally distinct tyrosine kinases (9, 35). Once phosphorylated on its tyrosine residue (Tyrr\(^{pS} \)), FAK binds to SH2 domain of pp60\(^{Src} \), recruiting the latter to focal adhesion contacts (36). In this study, we found that recombinant pp60\(^{Src} \) is able to tyrosine phosphorylate \( \alpha \)- and \( \gamma \)-sarcoglycans in vitro. 2 Thus, phosphorylation of sarcoglycans in response to cell adhesion in L6 cells is likely to be catalyzed by either FAK or an FAK-stimulated tyrosine kinase, such as a member of the Src family of kinases.

Sarcoglycans are intrinsic membrane proteins of \( M_2 \), 35,000–50,000, forming a tight subcomplex, which is associated with the dystroglycan subcomplex, dystrophin, and other proteins to form the dystrophin-DAP complex (see “Introduction”). Sarcoglycans possess large extracellular domains and small cytoplasmic tails containing no obvious catalytic domains (17–20, 37). We found that cell adhesion induces tyrosine phosphorylation of \( \alpha \)- and \( \gamma \)-sarcoglycans but not \( \beta \)-sarcoglycan and that phosphorylated \( \alpha \)-sarcoglycan co-precipitates with phosphorylated \( \gamma \)-sarcoglycan and vice versa. 3 Thus, phosphorylation of sarcoglycans in response to cell adhesion in L6 cells is likely to be catalyzed by either FAK or an FAK-stimulated tyrosine kinase, such as a member of the Src family of kinases.

The antisense ODN directed against \( \alpha \)- or \( \gamma \)-sarcoglycan for 5 days inhibited adhesion of L6 cells to fibronectin by about 50% (Fig. 7A). After each antisense ODN treatment, \( \alpha \)- or \( \gamma \)-sarcoglycan disappeared completely from these cells (Fig. 7B), although expression levels, as well as the ability of dystrophin and other DAPs to form a tight complex, were not affected by the treatment (see “Results”). Under these conditions, integrin \( \beta_1 \), subunit, vinculin, talin, and FAK co-precipitated with anti-dystrophin were decreased by 60–70%, although levels of these proteins in the total cell lysates were not reduced greatly (Fig. 7B and “Results”). In contrast, antisense ODN directed against \( \beta \)-sarcoglycan exerted minimal effects on cell adhesion, as well as on the association of the dystrophin-DAP complex with focal adhesion proteins. Thus, \( \alpha \)- and \( \gamma \)-sarcoglycans may be involved directly in L6 cell adhesion by interacting with focal adhesion-associated proteins. Of note, in skeletal muscle of patients with various forms of limb-girdle muscular dystrophy, mutations in each of the sarcoglycans result in the concomitant absence of the other sarcoglycans from the sarcolemma (16–18, 21). Such findings are in marked contrast to the observed absence of the effect of loss of the sarcoglycan protein on the stability of the dystrophin-DAP complex in L6 cells. This may reflect difference in molecules that contact or surround sarcoglycans in the dystrophin-DAP complex in cultured L6 cells versus adult skeletal muscle.

Localization of dystrophin in integrin-rich cell areas has been reported previously. By immunofluorescence staining, Kramarey and Sealock 38 observed that dystrophin and a 48-kDa protein, which is recognized by anti-syntrophin, are colocalized to talin-positive focal adhesion-like structures in cultured Xenopus muscle cells. In addition, Lakonishok et al. 39 observed transient overlapping localization of \( \alpha \beta_2 \) integrin and dystrophin in a punctate lattice-like structure on the surface of the cultured chick skeletal myotube during muscle development. We have also reported that some focal adhesion-associated proteins are co-precipitated with the dystrophin-DAP complex from detergent-solubilized nonfusing muscle cell line BC3H1 cells that have been cultured under differentiation conditions (22). On the other hand, in platelets in which dystrophin is not expressed, a dystrophin-related protein (utrophin) is a component of the membrane skeleton, and upon binding of adhesive extracellular ligands to integrin \( \alpha g \beta_2 \), utrophin has been shown to redistribute, along with other membrane skeleton proteins including talin and vinculin, to the low-speed detergent-insoluble fraction from the high-speed detergent-insoluble fraction (40).

The bidirectional interaction between the dystrophin-DAP complex and the integrin-cytoskeletal system in cultured muscle cells suggests that it could potentially play an important role in the regulation of the function of each adhesion system. Integrins are the best studied adhesion receptor, and much is known about the mechanism by which they mediate the bidirectional transfer of information across the plasma membrane (2, 7, 9). In contrast, little is known about the signaling role of the dystrophin-DAP complex, for which dystroglycan is a transmembrane adhesion receptor. As briefly discussed under “Introduction,” sarcoglycans seem to be particularly important for the function of the dystrophin-DAP complex in striated muscle cells. However, there has been no information available for the interaction of sarcoglycans with other cellular proteins. It is intriguing to ask whether there is a similar bidirectional interaction involving sarcoglycans between the dystrophin-DAP complex and the integrin-containing structures in the adult skeletal muscle. Previous immunofluorescence studies of striated muscle have suggested that dystroglycan, dystrophin, integrin \( \beta_2 \) subunit, vinculin, talin, and spectrin localize to the submembrane two-dimensional lattice structures of cosmeratos that mediate lateral attachment of the contractile apparatus to the sarcolemma (6, 41–43).

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