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(Article begins on next page)

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The Muscle-Specific Laminin Receptor $\alpha7\beta1$ Integrin Negatively Regulates $\alpha5\beta1$ Fibronectin Receptor Function

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$\alpha7\beta1$ is the major integrin complex expressed in differentiated muscle cells where it functions as a laminin receptor. In this work we have expressed the $\alpha7$ integrin subunit in CHO cells to investigate the functional properties of this receptor. After transfection with $\alpha7$ CHO cells acquired the ability to adhere and spread on laminin 1 consistent with the laminin receptor activity of the $\alpha7\beta1$. $\alpha7$ transfectants, however, showed a 70% reduction in the ability to adhere to fibronectin and were unable to assemble a fibronectin matrix. The degree of reduction was inversely related to the level of $\alpha7$ expression. To define the mechanisms underlying this adhesive defect we analyzed surface expression and functional properties of the $\alpha5\beta1$ fibronectin receptor. Although cell surface expression of $\alpha5\beta1$ was reduced by a factor of 20–25% in $\alpha7$ transfectants compared to control untransfected cells, this slight reduction was not sufficient to explain the dramatic reduction in cell adhesion (70%) and matrix assembly (close to 100%). Binding studies showed that the affinity of ^{125}I-fibronectin for its surface receptor was decreased by 50% in $\alpha7$ transfectants, indicating that the $\alpha5\beta1$ integrin is partially inactivated in these cells. Inactivation can be reversed by Mn^{2+}, a cation known to increase integrin affinity for their ligands. In fact, incubation of cells with Mn^{2+} restored fibronectin binding affinity, adhesion to fibronectin, and assembly of fibronectin matrix in $\alpha7$ transfectants. These data indicate that $\alpha7$ expression leads to the functional down regulation of $\alpha5\beta1$ integrin by decreasing ligand binding affinity and surface expression. In conclusion, the data reported establish the existence of a *negative cooperativity* between $\alpha7$ and $\alpha5$ integrins that may be important in determining functional regulation of integrins during myogenic differentiation. © 1999 Academic Press

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INTRODUCTION

Integrins are a large family of cell surface receptors that regulate cell-matrix adhesion and consequently affect several important processes such as cell proliferation and differentiation [1, 2]. Expression and functional properties of integrins have been investigated in several systems undergoing differentiation such as keratinocytes [3], mammary epithelial cells [4], nerve cells [5], and myogenic cells. In the latter system several different α subunits are expressed, including $\alpha1$, $\alpha3$, $\alpha4$, $\alpha5$, $\alpha6$, $\alpha7$, $\alpha9$, and αV. Most of these molecules are expressed in undifferentiated myoblasts but are down regulated during muscle cell differentiation and cannot be detected in adult skeletal and cardiac muscle [6–12]. Among these the $\alpha5\beta1$ fibronectin receptor and the $\alpha7\beta1$ laminin receptor have been investigated more extensively.

The $\alpha5\beta1$ integrin undergoes functional down regulation in differentiating postmitotic chick embryo myocytes. At this stage, $\alpha5\beta1$ is expressed at the cell surface in an inactive form that does not support myocyte–fibronectin interaction [13]. At later stages of differentiation $\alpha5\beta1$ expression is reduced and this molecule is absent *in vivo* in adult muscle cells [14–16]. Down regulation of the $\alpha5\beta1$ fibronectin receptor seems to be important for myogenic differentiation as shown by the fact that forced expression of an active $\alpha5\beta1$ in quail myoblasts inhibits their differentiation, maintaining the proliferative phase [17].

An opposite behavior has been reported for the laminin receptor $\alpha7\beta1$ integrin during myogenesis. $\alpha7$ integrin was originally described as a cell surface marker of striated and cardiac muscle [18] and its expression is up regulated during muscle terminal differentiation [19, 20]. Different isoforms of this molecule are generated by alternative splicing, $\alpha7$B and $\alpha7$A representing the best characterized forms. During myogenesis *in vivo*, $\alpha7$B appears between stage E10 and stage E13 of mouse development and its expression increases constantly up to adult stage [12, 21]. The $\alpha7$A is expressed in differentiated muscle cells and represents a specific marker of adult striated muscle tissue [21, 22]. $\alpha7$

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integrin pairs with the β1 subunit [20, 23] that also undergoes alternative splicing during myogenesis, the β1A isoform being expressed in the earlier phases and β1D in the late phases of differentiation [24, 25]. During mouse myogenesis *in vivo*, at developmental stages between E17 and newborn, α7B heterodimerizes with both the β1A and the β1D isoforms [12]. At this stage of development (E17–newborn) the fibronectin receptor $\alpha5\beta1$ is also present at the surface of differentiating muscle cells, but, in contrast to α7, α5 integrin is down regulated at later stages and it disappears from muscle sarcolemma during early postnatal development [16].

In this study we have transfected α7B into CHO cells expressing the endogenous $\alpha5\beta1$ fibronectin receptor together with either β1A or β1D isoforms. This model mimics the $\alpha7\beta1/\alpha5\beta1$ integrin expression pattern of myogenic cells during differentiation *in vitro* or *in vivo* from stage E13 to newborn. Our data show that α7 is capable of interfering with α5 function and expression and demonstrate the existence of a mechanism of *negative cooperativity* between α integrin subunits belonging to the β1 group.

METHODS

Antibodies. The following antibodies were used: the rabbit polyclonal antibody 242 against the α7B extracellular domain [26]; rabbit polyclonal antisera to human fibronectin and to αV, α3, α5, β1A, and β1D integrin cytoplasmic domains were previously characterized [24, 27–29]. The antiserum against the cytoplasmic domain of α7B was prepared by immunizing rabbits with the synthetic peptide KHPILAADWHPELGPDGHPVPATA corresponding to the last 23 amino acids of mouse α7B; the aminoterminal lysine was added for coupling to the carrier protein keyhole limpet hemocyanin. The monoclonal antibodies PB1 to the $\alpha5\beta1$ CHO integrin and MF20 to sarcomeric myosin were obtained from the Developmental Studies Hybridoma Bank (University of Iowa). Fluorescein-labeled goat anti-rabbit IgG, rhodamine-labeled goat anti-rabbit IgG, and peroxidase-labeled goat anti-rabbit IgG were all from Sigma.

Transfection and integrin analysis by flow cytometry. CHO cells were cultured under standard conditions as previously described [29]. Stable transfectants of CHO cells expressing the human β1A or β1D were obtained as described previously [24]. cDNA coding for the mouse α7B integrin isoform and containing the X2 exon sequence in the extracellular domain [26] was cloned in the eukaryotic expression vector pCDNA3 (Invitrogen). CHO-β1A or CHO-β1D cells, transfected respectively with the human β1A or β1D subunit [24] (10^6 cells/50-cm^2 plate), were cotransfected with 20 μg of the plasmid containing α7B cDNA and 2 μg of the plasmid pECV-hyg coding for hygromycin resistance [30], using the calcium phosphate precipitation method as described in Balzac *et al.* [29]. After 10 days of selection in medium containing 500 μg/ml hygromycin B (Boehringer Mannheim, Germany), cells expressing the transfected α7B protein were isolated by plating on dishes coated with 20 μg/ml laminin 1 (Becton–Dickinson) for 5–10 min at room temperature. Dishes were then rinsed several times with PBS (10 mM phosphate buffer, pH 7.3, 150 mM NaCl) to remove unbound cells. Adherent cells were grown and subjected to repeated cycles of selection on laminin 1 to isolate cell populations expressing high levels of α7B.

The expression of α7B was assessed by flow cytometry; cells were incubated with the polyclonal antibody 242 against the α7B extracellular domain followed by a fluorescein-conjugated secondary antibody as previously described [31].

Analysis of integrin expression by immunoprecipitation and immunofluorescence. To detect integrin α subunit expression, cells were surface biotinylated according to the following protocol. CHO cells grown in monolayer were washed twice with PBS and then labeled with 0.5 mg/ml biotin 3-sulfo-*N*-hydroxisuccinimide ester (Sigma) in Hanks' buffer (1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 5 mM KCl, 138 mM NaCl, 5.6 mM D-glucose, 25 mM Hepes, pH 7.4) for 15 min at 4°C. Labeling was terminated by washing three times with 0.6% BSA in DMEM, followed by three washes with Hanks' buffer.

To analyze integrins in differentiating mouse skeletal myoblasts, C2C12 cells were plated on plastic dishes coated with 0.2% gelatin in Dulbecco's modified Eagle's medium with 10% FCS. At confluence, growth medium was replaced with Dulbecco's modified Eagle's medium with 2% horse serum and cells were kept in culture for an additional 5 days to allow myotube formation. Myotubes were isolated by mild trypsinization of differentiated cells under conditions leaving myoblasts adherent to the dishes (0.005% trypsin and 0.002% EDTA in PBS for 5 min at room temperature), while myoblasts were obtained by a second cycle of standard trypsinization. Myoblast and myotube populations were surface biotinylated in suspension according to the protocol described above.

For integrin immunoprecipitation, surface-biotinylated cells were extracted for 20 min at 4°C with 0.5% Triton X-100 in 20 mM Tris–HCl, pH 7.4, 150 mM NaCl (TBS) with 10 μg/ml leupeptin, 4 μg/ml pepstatin, and 0.1 TIU/ml aprotinin (all from Sigma). After centrifugation at 14,000 rpm for 10 min extracts were incubated with nonimmune rabbit IgG coupled to Sepharose to remove the material that nonspecifically binds to the resin. Integrin complexes were immunoprecipitated by incubating cell extracts with α-specific antibodies and protein A–Sepharose beads (Pharmacia) for 15 h at 4°C with gentle agitation. After extensive washing, protein A–Sepharose bound material was separated on 6% SDS–PAGE under reducing (α7 immunoprecipitates) or nonreducing conditions (α3, α5, and αV immunoprecipitates) and transferred to nitrocellulose using a semidry apparatus (Novablot; Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The membranes were saturated by 1 h incubation at 42°C in TBS containing 5% BSA. Biotinylated proteins were detected by peroxidase-conjugated streptavidin and ECL (all from Amersham, UK).

To visualize $\alpha5\beta1$ integrin expression myoblasts were seeded at low density and after 24 h either fixed or induced to differentiate for 5 days in DMEM with 2% horse serum. After fixation with paraformaldehyde and permeabilization with 0.1% Triton X-100 for 1 min at +4°C cells were stained with 5 μg/ml affinity-purified polyclonal antibodies to the α5 integrin cytoplasmic domain [28] followed by rhodamine-labeled secondary antibodies. Cells were also stained with DAPI to visualize DNA.

To analyze association of integrin α chains with β1 isoforms, unlabeled CHO-β1A and CHO-β1D cells were extracted and immunoprecipitated with antibodies specific for α subunit as described above. After SDS–PAGE (reducing conditions) and transfer to nitrocellulose the coprecipitated β1 subunit isoform was detected by antibodies that specifically react with the β1A or β1D integrin cytoplasmic domains. Peroxidase-conjugated anti-rabbit IgG was used as secondary antibody followed by chemiluminescent substrate ECL.

To detect myosin expression in myoblasts and myotube populations, 40 μg of total protein extracts was separated by SDS–PAGE under reducing conditions and blotted to nitrocellulose membrane. The membrane was then incubated in TBS containing 1% BSA and the MF20 monoclonal antibody against sarcomeric myosin, followed by peroxidase-conjugated anti-mouse IgG and chemiluminescent substrate ECL (Amersham).

Adhesion assays. Tissue culture microtiter plates were coated by overnight incubation at 4°C with the indicated concentration of purified matrix proteins in PBS and postcoated with 1% BSA in PBS for 1 h at 37°C. Fibronectin was purified from human plasma by affinity chromatography on gelatin–Sepharose according to Engvall and Ruoslathi [32]. Vitronectin was purified according to Yatohgo *et*

al. [33], mouse laminin 1 from EHS tumor was obtained from Becton–Dickinson. Cells at confluence were detached by incubation in 5 mM EDTA in PBS for 10 min, washed twice in serum-free DMEM, and plated on the coated tissue culture plates for 40 min at 37°C. To analyze the role of Mn^{2+}, cells were plated in adhesion medium (20 mM Tris, pH 7.4, 135 mM NaCl, 5 mM KCl, 2 mM L-glutamine, 1.8 mM glucose, and 1% BSA) containing 1 mM $MnCl_2$; when indicated, the blocking monoclonal antibody PB1 at 15 μg/ml was added. Cell adhesion was evaluated by colorimetric assay for acid phosphatase activity as described in Defilippi *et al.* [28] and values are expressed as percentage of the cells plated in the well.

Fibronectin matrix assembly assay. Matrix assembly in CHO cells was evaluated by analyzing the incorporation of exogenously added fibronectin. Briefly, 100 nM purified human fibronectin was added for 15 h to confluent CHO cell monolayers grown on glass coverslips in medium containing 1% serum or in adhesion medium (20 mM Tris, pH 7.4, 135 mM NaCl, 5 mM KCl, 2 mM L-glutamine, 1.8 mM glucose, and 1% BSA) in the absence or in the presence of 0.1 mM $MnCl_2$. When indicated, the monoclonal antibody PB1 was added at 15 μg/ml. The resulting fibronectin matrix was then visualized by immunofluorescence by incubating paraformaldehyde-fixed monolayers with 1:500 dilution of the polyclonal antibody to human fibronectin followed by rhodamine-labeled secondary antibodies. Cells were also stained with fluorescein-phalloidin (Sigma) to visualize F-actin.

Matrix assembly in C2C12 cells was evaluated by measuring the ability to organize the endogenous secreted fibronectin. Briefly, C2C12 myoblasts were seeded on 35-mm^2 dishes and grown to confluence for 48 h in DMEM medium with 10% FCS; C2C12 differentiated myotubes were instead obtained by growing in differentiation medium for 5 days as described above and the fibronectin matrix organization was assayed by immunofluorescence analysis.

Fibronectin binding to cells. Binding of ^{125}I-labeled fibronectin to CHO-β1A and CHO-β1Aα7 was performed as described in O'Toole *et al.* [34]. Purified bovine serum fibronectin (Gibco) was dissolved in distilled water at 2 mg/ml. Five hundred micrograms was labeled with 0.5 mCi of ^{125}I by iodogen to a specific activity of 9×10^7 cpm/nmol. Cells (2×10^6) in 200 μl of Tyrode's buffer (150 mM NaCl, 2.5 mM KCl, 2 mM $NaHCO_3$, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mg/ml BSA, and 1 mg/ml dextrose) were incubated with the specified concentrations of ^{125}I-fibronectin for 1 h at 37°C in the absence or in the presence of 1 mM $MnCl_2$. Aliquots of 60 μl were layered on 0.3 ml of 20% sucrose and centrifuged for 3 min at 12,000 rpm. Radioactivity associated with the cell pellet was determined in a gamma counter. Specific binding was obtained by adding 10 times excess unlabeled fibronectin.

RESULTS

Transfection of α7 in CHO Cells

CHO cells expressing the human β1A or β1D integrin subunits were previously characterized [24, 35]. These cells were transfected with the full-length mouse α7B integrin cDNA using standard procedures (see Methods) and cell populations expressing different levels of α7 were selected by multiple cycles of adhesion to laminin 1. CHO-β1A and CHO-β1D adhere very poorly to laminin 1 and acquire the ability to attach to this matrix protein only after α7 transfection. As shown in Figs. 1A and 1B, CHO-β1Aα7 and CHO-β1Dα7 cell populations with a uniform expression of α7 (mean fluorescence intensities are 93 and 87 for CHO-β1Aα7 and CHOβ1Dα7, respectively) were selected after five cycles of adhesion on laminin 1. CHO-β1Aα7 cells se-

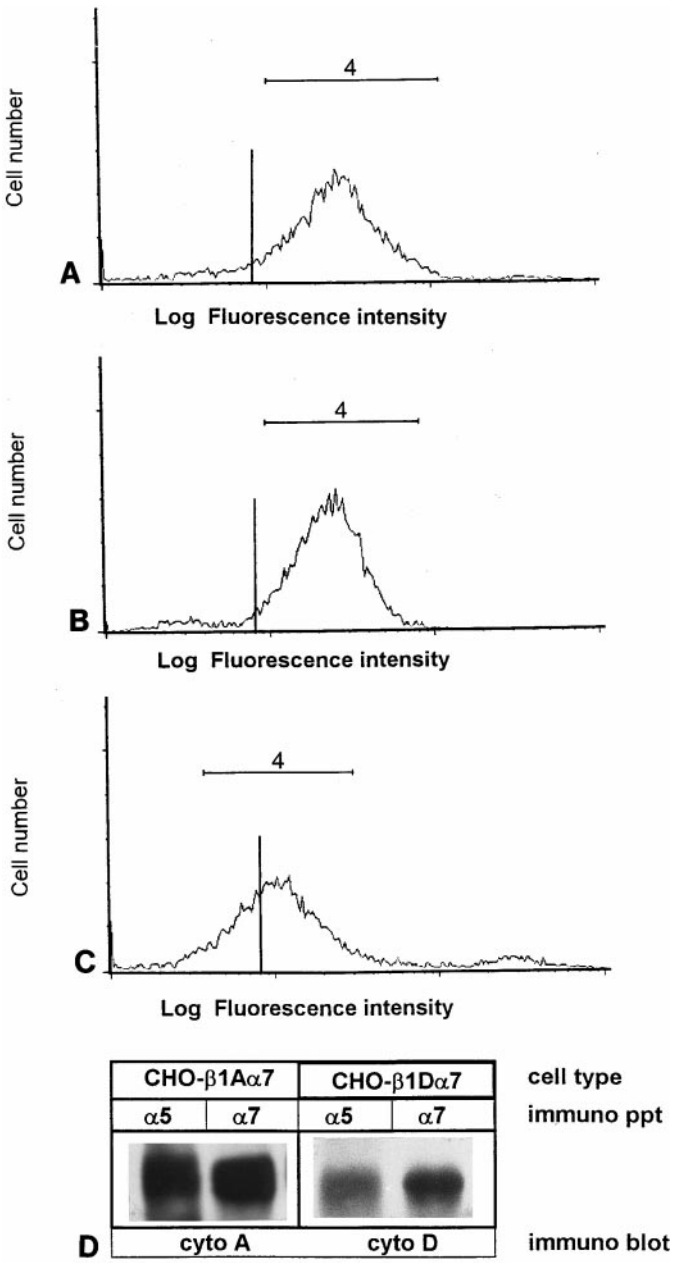
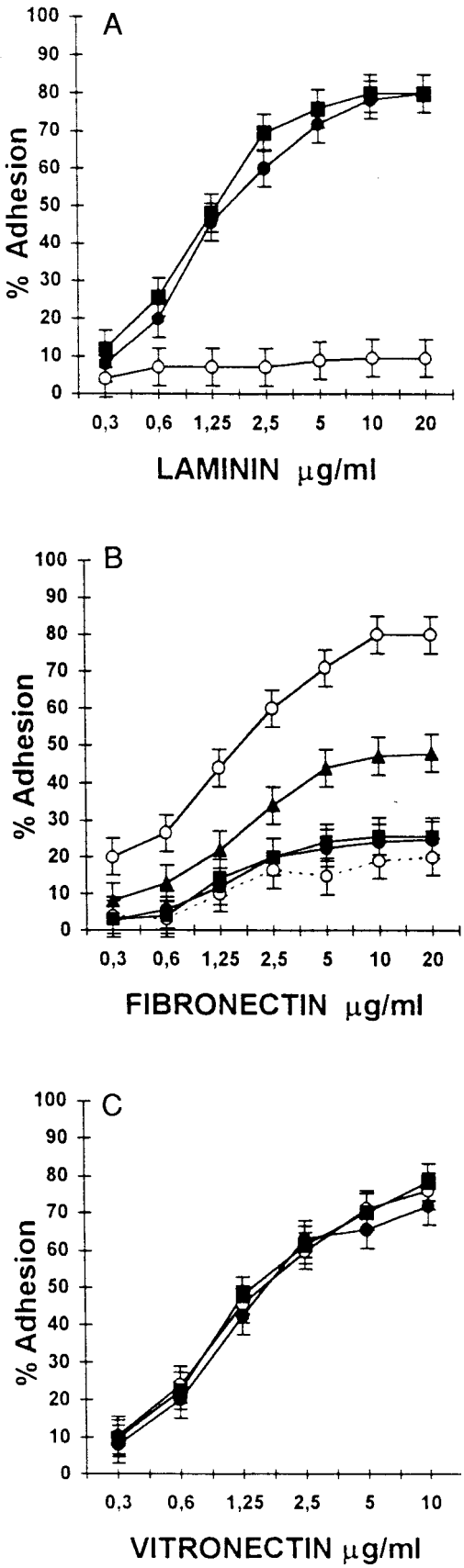


FIG. 1. (A, B, and C) Expression of integrin α7 in CHO-β1A and CHO-β1D cells. CHO cells expressing the human β1A or the human β1D integrin isoforms were transfected with the mouse α7B cDNA. Cells were selected for α7 expression by multiple cycles of adhesion to laminin 1 and analyzed by flow cytometry. CHO-β1Aα7 (A) and CHO-β1Dα7 (B) cell populations after five cycles of selection on laminin 1-coated dishes. CHO-β1Aα7 cells after two cycles of selection (C). The cells were stained with a polyclonal serum against the extracellular domain of α7 and a fluorescein-conjugated goat anti-rabbit secondary antibody. The vertical bar represents the maximum fluorescence intensity in the negative control (CHO cells stained with the anti α7B antibody). (D) Association of integrin α subunits with β1 integrin isoforms. Cells were detergent extracted and integrin complexes were immunoprecipitated with antibodies specific for α5 and α7B integrin subunits. After electrophoresis under reducing conditions and electrotransfer, coprecipitated β1 integrin isoforms were visualized by reacting with antibodies specific to the β1A isoform or to the β1D isoform. Note that endogenous α5 and transfected α7B paired with both β1A and β1D isoforms.



lected by only two cycles of adhesion to laminin 1 (Fig. 1C) express a lower level of $\alpha 7$ (mean fluorescence intensity 65). Immunoprecipitation experiments followed by Western blotting showed that the transfected $\alpha 7$ integrin paired both with the $\beta 1D$ and with the $\beta 1A$ isoform (Fig. 1D). Similar results were obtained for the $\alpha 5$ integrin subunit showing that both $\alpha 7$ and $\alpha 5$ subunits associated to the $\beta 1$ isoforms in a similar fashion.

Adhesive Properties of α7 Transfected Cells

To test adhesive properties of $\alpha 7$-expressing cells, we performed adhesion assays on dishes coated with purified matrix proteins. While untransfected CHO-$\beta 1A$ cells showed undetectable adhesion to laminin 1, $\alpha 7$ transfectants strongly adhered to laminin 1 and the percentages of adherent CHO-$\beta 1A\alpha 7$ and CHO-$\beta 1D\alpha 7$ cells were not significantly different (Fig. 2A).

In contrast, transfectants expressing high levels of $\alpha 7$ showed a 70% reduction in the ability to adhere to fibronectin compared to control cells (Fig. 2B). Reduction in cell adhesion to fibronectin was proportional to the $\alpha 7$ expression level; in fact, only 40% reduction in adhesion to fibronectin was observed in the cell population expressing low level of $\alpha 7$ (Fig. 2B). The reduced adhesion to fibronectin did not reflect a general defect in cell adhesion since all cell types analyzed adhered normally and comparably when plated on vitronectin-coated dishes (Fig. 2C). Moreover, this effect was not a transfection artifact because the reduction of adhesion to fibronectin occurred not in one single clone, but in cell populations from two independent transfections. In CHO cells adhesion to fibronectin is mediated by the endogenous $\alpha 5\beta 1$ integrin as shown by inhibition with the monoclonal antibody PB1 specific for this integrin complex (Fig. 2B). These data suggest a specific inhibitory effect of $\alpha 7$ expression on the fibronectin receptor $\alpha 5\beta 1$.

Organization of Fibronectin Matrix in α7 Transfected CHO Cells

To test if functional properties of $\alpha 5\beta 1$ are altered in $\alpha 7$ transfectants we analyzed fibronectin matrix assembly by immunofluorescence staining.

CHO cells assemble a very poor endogenous fibronec-

FIG. 2. Adhesion of $\alpha 7$-transfected CHO cells on purified matrix proteins. Microtiter wells were coated with increasing concentration of mouse laminin 1 (A), human plasma fibronectin (B), or human plasma vitronectin (C). Cells were suspended by EDTA treatment and plated in serum-free medium for 40 min at 37°C. Cell adhesion was measured as described under Methods and the mean values from triplicate measurements are presented. CHO-$\beta 1A\alpha 7$ (■) and CHO-$\beta 1D\alpha 7$ (●) expressing high levels of $\alpha 7$ (see Figs. 1A and 1B), CHO-$\beta 1A\alpha 7$ (▲) expressing low $\alpha 7$ level (see Fig. 1C). CHO-$\beta 1A$ cells either in the absence (—○—) or in the presence (---○---) of the $\alpha 5\beta 1$-blocking monoclonal antibody PB1 at 15 μg/ml.

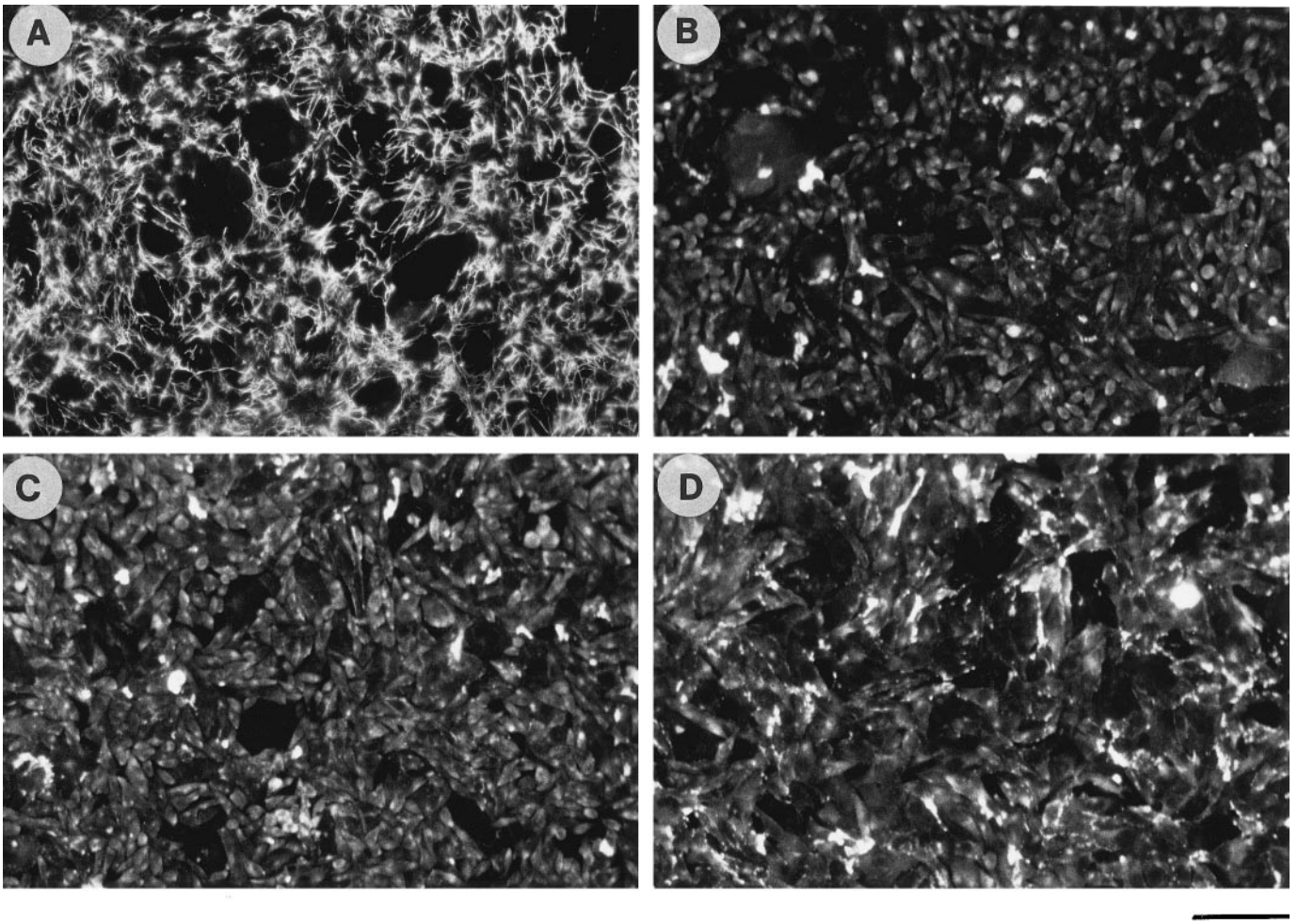


FIG. 3. Fibronectin matrix organization in α7-transfected CHO cells. Confluent monolayers of CHO-β1A (A and B), CHO-β1Aα7 (C), and CHO-β1Dα7 (D) cells were cultured for 15 h with 100 nM exogenous human plasma fibronectin in the absence (A, C, and D) or in the presence of the blocking α5β1 monoclonal antibody PB1 (B). Fibronectin organized on the cell monolayer was visualized by immunofluorescence staining as described under Methods. Note that fibronectin matrix assembly is strongly reduced both in CHO-β1Aα7 cells (C) and in CHO-β1Dα7 cells (D). Bar, 300 μm.

tin matrix, but are able to organize exogenously added fibronectin in fibrils on their apical surface [36]. Figure 3A shows fibronectin matrix assembly in CHO-β1A monolayer cells incubated overnight with 100 nM purified human fibronectin. This process is driven by α5β1 integrin, as shown by inhibition with the α5β1-blocking PB1 monoclonal antibody (Fig. 3B). When CHO-β1Aα7 cells were tested, lack of fibronectin matrix assembly was observed (Fig. 3C) as judged by the fluorescence staining. A similar defect in matrix assembly was observed also in α7β1D-expressing cells (Fig. 3D). The use of higher fibronectin concentrations (up to 300 nM) or longer incubation times (24 h) did not significantly increase matrix assembly in α7 transfectants.

Thus, both adhesion and matrix assembly assays indicate a functional down regulation of the α5β1 fibronectin receptor in cells expressing the α7β1 laminin receptor.

Expression of Integrin α Subunits in α7 Transfectants

To investigate the mechanisms leading to reduced fibronectin adhesion and matrix assembly, we analyzed surface expression of the α5 and of the other α integrin subunits before and after α7 transfection.

Cell-surface-biotinylated proteins were immunoprecipitated from CHO-β1A and CHO-β1Aα7 using polyclonal antibodies against α3, α5, α7B, and αV, which represent the major α integrin subunits in these cells. As shown in Fig. 4A a 25% reduction in surface expression of α5 was observed in α7 transfectants as quantitated by densitometric scanning of gel bands. The surface expression of α5β1 integrin was further analyzed by flow cytometry with PB1 monoclonal antibody (Fig. 4B). The mean fluorescence intensity was reduced by 23% in CHO-β1Aα7 compared to CHO-β1A (mean fluorescence values were 102 and 133, respectively). These differences in α5β1 integrin surface expression

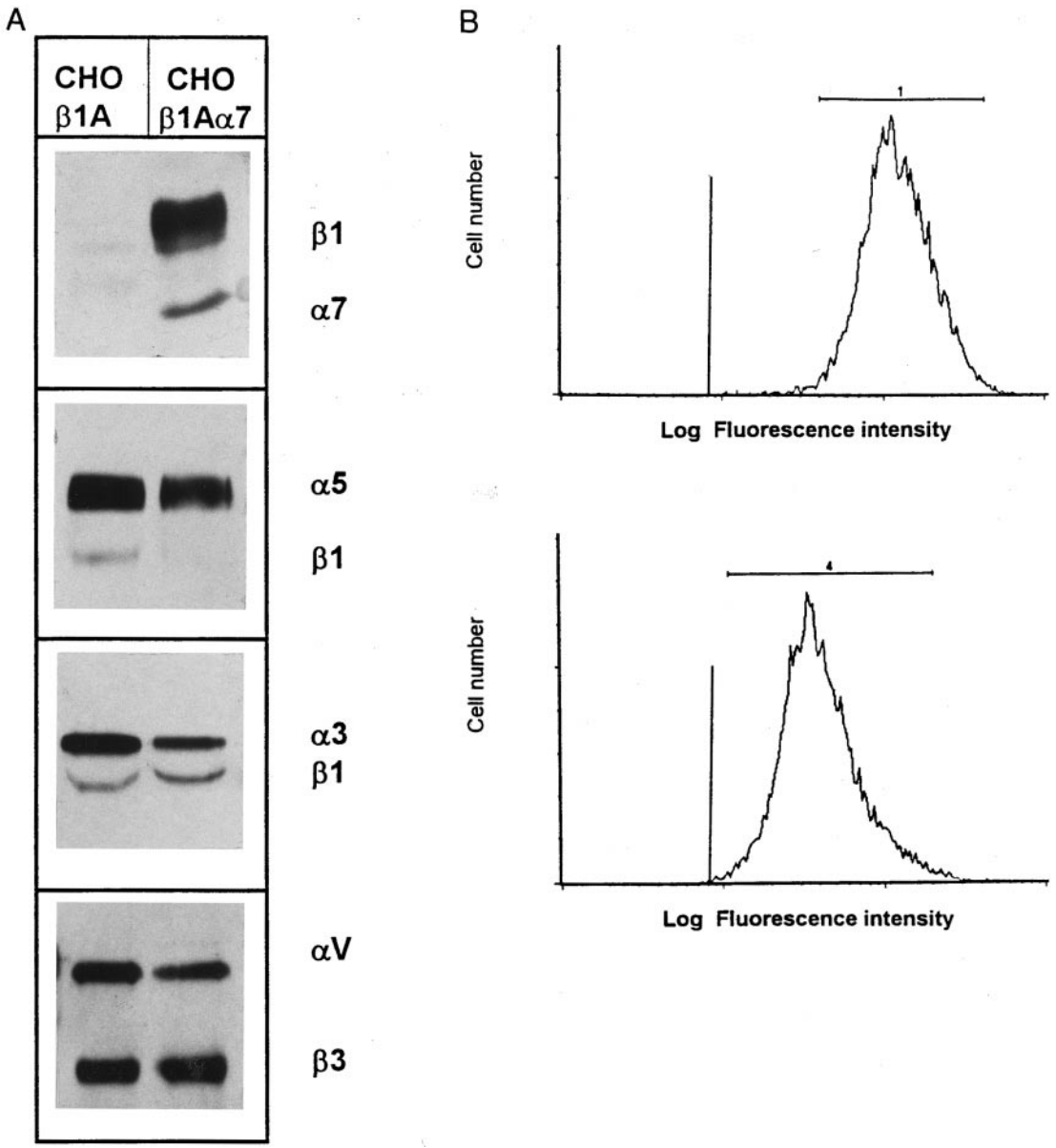


FIG. 4. Reduction in the surface expression of $\alpha 5$ in $\alpha 7$-transfected CHO cells. (A) Surface-biotinylated CHO-$\beta 1$A and CHO-$\beta 1$A$\alpha 7$ were immunoprecipitated with anti-$\alpha 7$B, -$\alpha 5$, -$\alpha 3$, and -αV polyclonal antibodies. To resolve the $\alpha 7$ subunit from comigrating $\beta 1$, $\alpha 7$B immunoprecipitate was separated by SDS–PAGE under reducing conditions and transferred to nitrocellulose membrane. Immunoprecipitates of $\alpha 5$, $\alpha 3$, and αV were separated under nonreducing conditions. Proteins were visualized by incubation with peroxidase-conjugated streptavidin and then detected by ECL. The positions of the α and the coprecipitated β subunits are indicated. (B) Flow cytometry detection of $\alpha 5\beta 1$ in CHO-$\beta 1$A (top) and CHO-$\beta 1$A$\alpha 7$ cells (bottom). Cells were stained with the anti-$\alpha 5\beta 1$ monoclonal antibody PB1 and a rhodamine-conjugated anti-mouse secondary antibody. The vertical bar represents the maximum fluorescence intensity in the negative control (CHO-$\beta 1$A cells stained only with the secondary antibody).

were further confirmed by fibronectin binding studies (see below).

$\alpha 3\beta 1$ subunit was also slightly decreased in surface expression in $\alpha 7$ transfectants, while no significant changes were detected for $\alpha V\beta 3$ integrin (Fig. 4A).

Binding of Fibronectin to α7 Transfected Cells

The decreased $\alpha 5\beta 1$ expression observed in $\alpha 7$ transfectants is relatively small compared to the dramatic reduction in cell adhesion or matrix assembly. In order to test the possibility that fibronectin binding affinity was altered in $\alpha 7$ transfectants, we examined the binding of ^{125}I-fibronectin to CHO-$\beta 1$A and CHO-$\beta 1$A$\alpha 7$ cells kept in suspension. The data shown in Fig. 5 indicate a reduced fibronectin binding in $\alpha 7$-expressing cells. Scatchard analysis allowed the definition of apparent dissociation constants of 2.8×10^{-7} M for CHO-$\beta 1$A cells and of 5.7×10^{-7} M for CHO-$\beta 1$A$\alpha 7$ cells,

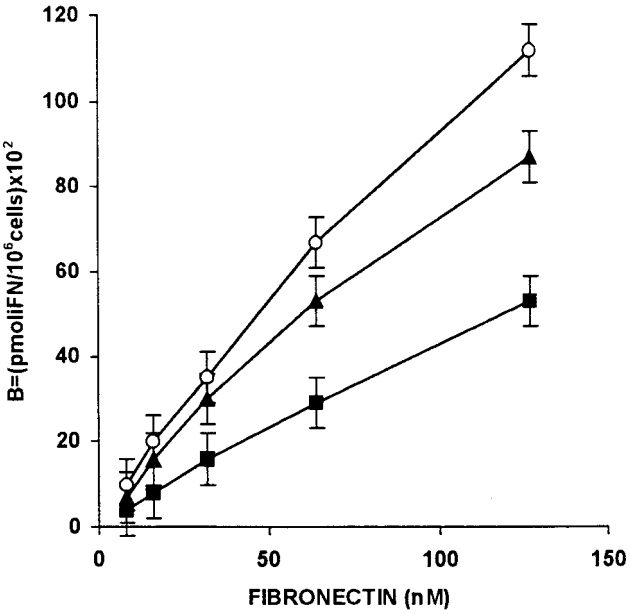


FIG. 5. Binding of ^{125}I-fibronectin to α7-transfected CHO cells. Increasing concentrations of ^{125}I-fibronectin (9×10^7 cpm/nmol) were mixed with 2×10^6 cells in a total volume of 200 μl for 1 h at 37°C. 60-μl aliquots were layered on 0.3 ml of 20% sucrose and centrifuged. Radioactivity associated with the cell pellet was determined in a gamma counter. CHO-β1A (○), CHO-β1Aα7 (■), and CHO-β1Aα7 in the presence of 1 mM Mn^{2+} (▲). Specific binding was obtained by subtracting values obtained in the presence of 10-fold excess cold fibronectin. The mean values from triplicates are reported.

thus indicating that upon α7 transfection, the binding affinity of the fibronectin receptor for its ligand is reduced by 50%. Incubation of the CHO-β1Aα7 cells with Mn^{2+}, a divalent cation known to increase ligand binding affinity of several integrins [37] restored the binding affinity to values comparable to those observed in CHO-β1A cells ($K_d = 3.1 \times 10^{-7}$ M). The *x* intercept in the Scatchard plot indicated that the number of fibronectin binding sites did not change significantly in the presence of Mn^{2+} (1.70×10^5 binding sites for CHOβ1Aα7 and 1.8×10^5 for CHO-β1Aα7+Mn^{2+}), confirming that this cation affects mainly the integrin ligand binding affinity.

Scatchard analysis also confirmed reduced expression of the fibronectin receptor in α7 transfectants. The number of fibronectin binding sites was 2.1×10^5 for CHO-β1A and 1.70×10^5 for CHO-β1Aα7 (19% reduction).

Effect of Mn^{2+} Ions on Adhesion and Matrix Assembly in α7 Transfectants

Based on the results described above we tested whether Mn^{2+} ions can rescue the adhesive phenotype of α7 transfectants. As shown in Fig. 6A, when CHO-β1Aα7 were incubated with 1 mM Mn^{2+}, adhesion to fibronectin-coated dishes was restored to levels close to those observed in untransfected cells. When the α5β1-blocking monoclonal antibody PB1 was added together with Mn^{2+}, CHO-β1Aα7 no longer adhered to fibronectin, showing that Mn^{2+}-stimulated adhesion to fibronectin specifically involves α5β1 integrin.

We then tested whether fibronectin assembly ability of CHO-β1Aα7 can be modified by Mn^{2+}. As shown in Fig. 6B treatment of cell monolayer with 0.1 mM Mn^{2+} was sufficient to restore the ability of α7 transfectants to assemble fibronectin fibrils at the apical surface, and Mn^{2+} effect was blocked by PB1 monoclonal antibody, confirming specific involvement of α5β1 in this process.

These results demonstrated that on the surface of CHO-β1Aα7 cells the integrin α5β1 exists in an inactive state, likely due to a change in the molecular conformation, which can be reverted by Mn^{2+} treatment.

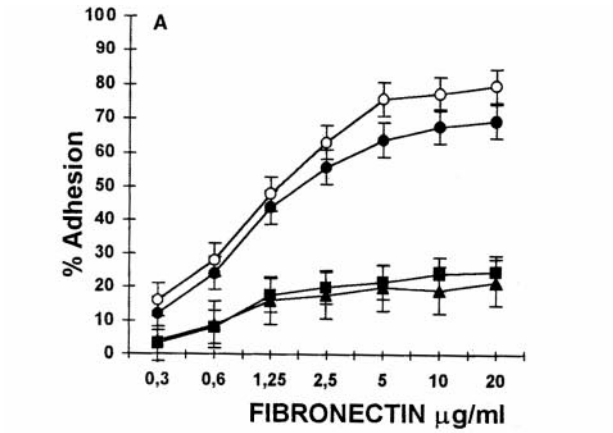
Fibronectin Matrix Assembly and α5β1 Expression in Differentiating C2C12

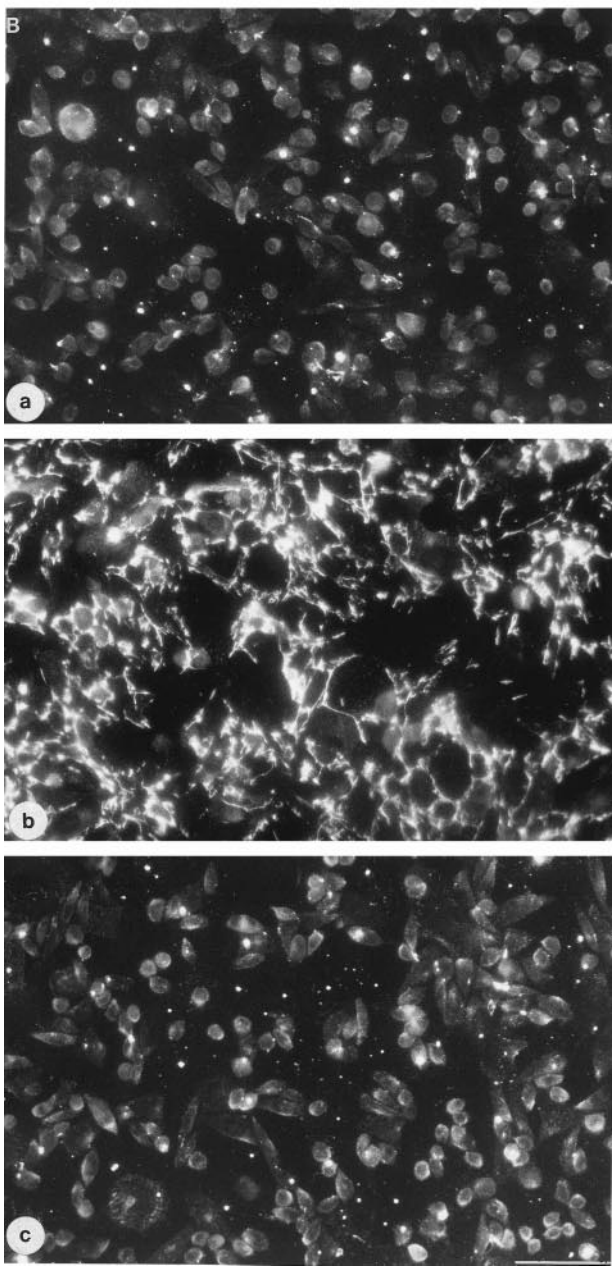
In order to test whether the negative cooperativity observed between α7 and α5 integrins was unique of CHO transfectants we analyzed mouse myogenic cells that normally express these integrin heterodimers. Undifferentiated C2C12 myoblasts *in vitro* synthesize and assemble on their apical surface a dense fibronectin matrix (Fig. 7A, a). Upon serum withdrawal C2C12 cells start to differentiate and after 5 days large multinucleated myotubes are formed (Fig. 7A, d). These myotubes failed to organize fibronectin matrix (Fig. 7A, b) as shown by immunofluorescence staining and rather organized a laminin network (our unpublished results; see also [43]). Lack of fibronectin assembly was not due to lack of synthesis since fibronectin is still produced and assembled by undifferentiated myoblasts present in the culture (Fig. 7A, b).

Analysis of integrin expression by immunoprecipitation of surface-biotinylated differentiating C2C12 indicated that myotubes express levels of α5β1 integrin at their surface not significantly lower than those detected on undifferentiated myoblasts (Fig. 7B). α5β1 integrin expression on differentiated myotubes was also confirmed by immunofluorescence experiments (Fig. 8). In addition α7 expression is up regulated in differentiated cells (Fig. 7B), suggesting that, also during myogenic differentiation, high α7 expression level leads to functional down regulation of α5β1 function.

DISCUSSION

In this study we analyzed CHO cells transfected with the laminin-binding, muscle-specific α7B integrin. The results reported here reveal the existence of a *negative cooperativity* between the muscle-specific α7β1 laminin receptor and the α5β1 fibronectin receptor. In fact, the expression of α7β1 in CHO cells causes a functional





down regulation of the endogenous $\alpha5\beta1$ fibronectin receptor resulting in a strong decrease of cell adhesion to fibronectin and of the ability to assemble a fibronectin matrix. The decrease in cell adhesion to fibronectin was inversely related to the level of $\alpha7$ surface expression. Moreover, inhibition of fibronectin matrix assembly was nearly complete as judged by immunofluorescence staining intensity. Since antibody inhibition studies indicated that adhesion to fibronectin and matrix assembly are supported by the endogenous $\alpha5\beta1$ integrin, we investigate the expression of this molecule by immunoprecipitation, flow cytometry, and ligand binding studies. These analyses consistently showed that $\alpha5\beta1$ expression was reduced by 20–25% in $\alpha7$-expressing CHO cells. A reduction of $\alpha5$ surface expression after overexpression of $\alpha7$B in human epithelial kidney 293 cells was observed also by Echtermeyer *et al.* (submitted for publication).

The limited extent of reduction in $\alpha5\beta1$ surface expression (20–25%) can hardly explain the strong reduction of cell adhesion and matrix assembly (70–100%) occurring in $\alpha7$-transfected CHO. Measurements of fibronectin binding to the cell surface indicated that upon $\alpha7$ transfection fibronectin binds with an affinity that is 50% lower compared to untransfected cells, indicating that $\alpha5\beta1$, although expressed at the cell surface, is partially inactivated by the presence of the $\alpha7\beta1$ integrin heterodimer. The reduction of fibronectin binding affinity can be almost completely recovered by Mn^{2+} treatment. This cation is known to increase ligand binding affinity of several integrin complexes by changing the conformation of the integrin complex ectodomain [38]. The ability of Mn^{2+} to restore fibronectin binding affinity clearly indicates that the $\alpha5\beta1$ is functionally inactivated in a reversible manner and suggests that conformational changes occurring in the receptor are responsible for the reduced binding affinity.

These data suggest that the functional down regulation of the $\alpha5\beta1$ integrin occurring in $\alpha7$ CHO transfectants can be explained by the concomitant reduction

FIG. 6. Mn^{2+} treatment restores adhesion (A) and matrix assembly (B) of CHO-$\beta1A\alpha7$ transfectants. (A) Cells were suspended by EDTA treatment and plated in serum-free or adhesion medium (see Methods) for 40 min at 37°C. CHO-$\beta1A$ (○), CHO-$\beta1A\alpha7$ (■), CHO-$\beta1A\alpha7$ cells in presence of Mn^{2+} (●), CHO-$\beta1A\alpha7$ cells in presence of Mn^{2+} and of monoclonal antibody PB1 (▲). The reduced adhesion of CHO-$\beta1A\alpha7$ cells was restored to levels observed in untransfected cells by adding Mn^{2+} during adhesion. Blocking the $\alpha5\beta1$ integrin with monoclonal antibody PB1 abolished adhesion of Mn^{2+}-treated cells. (B) Confluent monolayers of CHO-$\beta1A\alpha7$ cells were cultured for 15 h with 100 nM exogenous human plasma fibronectin either in the absence (a) or in the presence of (b) 0.1 mM $MnCl_2$ or (c) 0.1 mM $MnCl_2$ and the blocking monoclonal antibody PB1. Fibronectin matrix was detected by immunofluorescence. Note that Mn^{2+} treatment restores fibronectin matrix assembly in CHO-$\beta1A\alpha7$ cells (b) in a $\alpha5\beta1$ integrin-dependent manner (c). Bar, 150 μm.

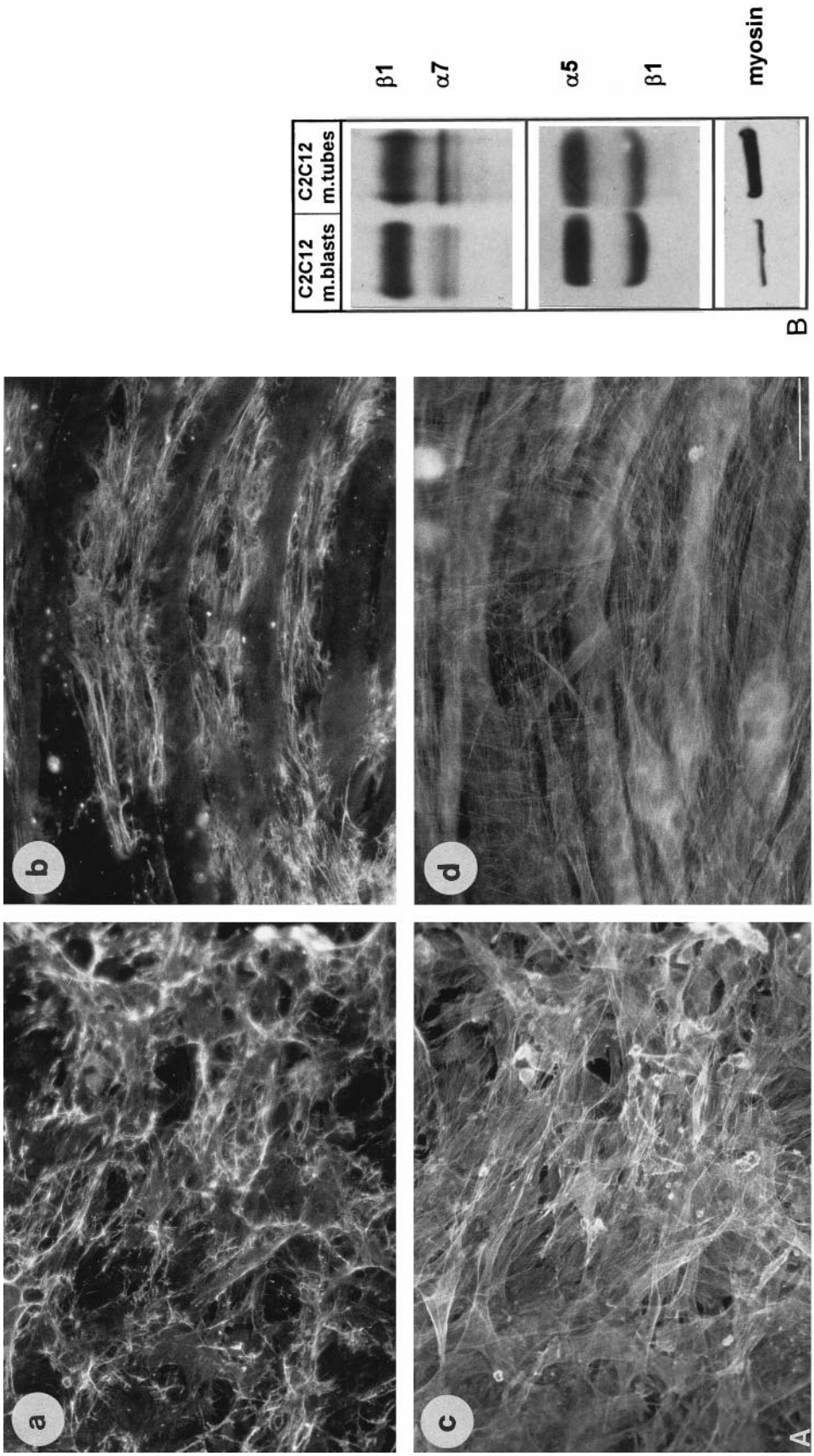


FIG. 7. Fibronectin matrix deposition by C2C12 myoblasts and myotubes. The organization of fibronectin was analyzed in myoblasts grown to confluence (a, c) or in myotubes differentiated for 5 days (b, d). Cells were stained for fibronectin (a, b) and simultaneously for f-actin (c, d). Note that when differentiated myotubes and undifferentiated myoblasts are present in the same culture, fibronectin is assembled on myoblasts but not on multinucleated myotubes surface (b). Bar, 50 μm. (B) Surface expression of α7B and α5 integrin subunits in C2C12 cells. Myoblast and myotube populations were isolated from differentiated C2C12 cultures as described under Methods and surface biotinylated. α7B and α5 integrin complexes were immunoprecipitated and visualized by peroxidase-conjugated streptavidin and ECL. The positions of the α and the coprecipitated β subunits are shown. The bottom shows a Western blot of total cell extracts with mAb MF20 against sarcomeric myosin to demonstrate the induction of differentiation (see Methods).

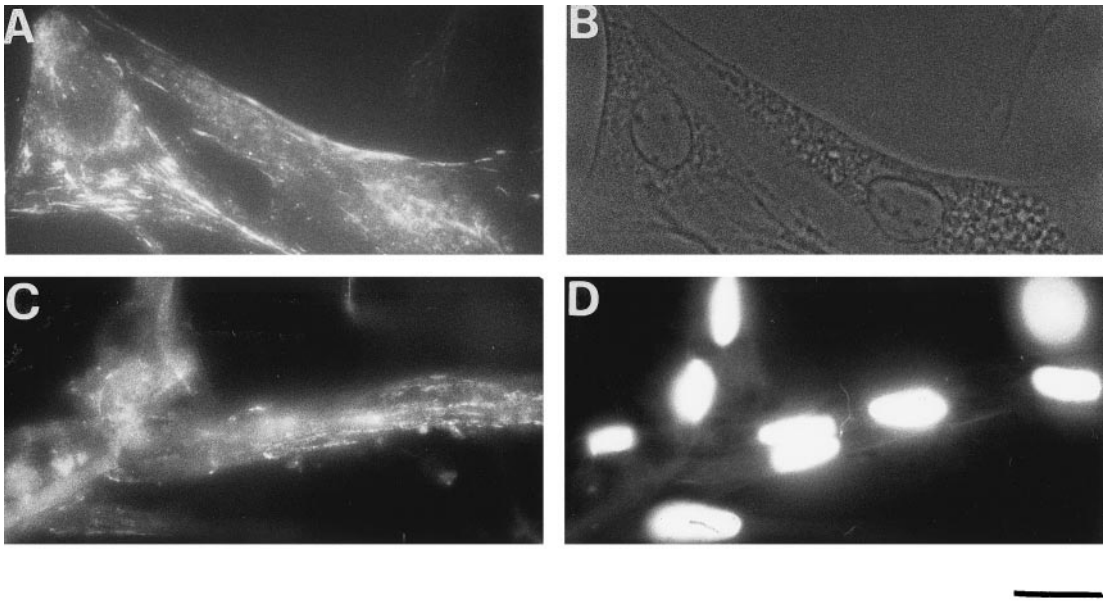


FIG. 8. Expression of $\alpha5\beta1$ integrin at the cell surface of C2C12 myoblasts and myotubes. Cells were seeded at low density (A and B) and incubated with differentiation medium for 5 days (C and D). After fixation and permeabilization $\alpha5$ integrin was visualized with polyclonal antibodies to the cytoplasmic domain (A and C). Cells in A were photographed under phase contrast (B). Cells in B were simultaneously stained for DNA with DAPI to visualize nuclei (D). Bar, 15 μm.

of surface expression and binding affinity. The reduction in binding affinity plays an important role in the functional down regulation since Mn^{2+} treatment of $\alpha7$ transfectants restores cell adhesion to fibronectin and matrix assembly to levels close to those of $\alpha7$-negative cells.

The understanding of the mechanisms by which $\alpha7$ expression leads to reduced $\alpha5\beta1$ expression and binding affinity deserves further work. A possible explanation for the reduced $\alpha5$ surface expression can be the competitive recruitment of $\beta1$ subunit by the endogenous $\alpha5$ and the transfected $\alpha7$ integrins. This possibility is suggested by the fact that reduced expression occurred for α subunits associated with $\beta1$, but not for $\alpha V\beta3$ heterodimers (see Fig. 4). More complex mechanisms are likely to be responsible for the reduced ligand binding. This function is known to be regulated both by intracellular signals [2] and by association with other membrane proteins like the recently described CD98 antigen [39]. Thus it is possible that $\alpha7$ affects one of such pathways. Interestingly the $\alpha7\beta1A$ and $\alpha7\beta1D$ heterodimers were found to have similar effects on the $\alpha5\beta1$ complex inactivation, suggesting that this effect is specifically mediated by the $\alpha7$ subunit.

Previous work demonstrated that a given integrin may negatively regulate another integrin complex. For example, expression of $\alpha V\beta3$ in K562 cells inhibits the phagocytic function of the endogenous $\alpha5\beta1$ [40]. Similarly, transfected $\alpha IIb\beta3$ inhibits the function of cotransfected $\alpha2\beta1$ or endogenous $\alpha5\beta1$ in CHO cells [41] and activation of LFA-1 on T cell surface decreases adhesion mediated by $\alpha4\beta1$ [42]. In all these examples functional interference occurs between integrin belonging to different classes and in particular $\beta3$ or $\beta2$ integrins affect $\beta1$ class heterodimers. The $\alpha7$ effect reported in the present study differs from those discussed above since in our case the interference occurs between heterodimers belonging to the same $\beta1$ class. While in the previous cases the β subunits are thought to be involved in the generation of an inhibitory signals, in our case the $\alpha7$ subunit is responsible for this effect. We, thus, believe that the current example represents a novel mechanism of negative cooperativity between different heterodimers sharing the same β subunit.

The possible physiological significance of the negative interference of $\alpha7\beta1$ on $\alpha5\beta1$ integrin is suggested by the functional behavior of these integrins during myogenesis. It has been shown that mononucleated myoblasts assemble a dense fibronectin matrix, but this property is lost in multinucleated myotubes [43, 44]. *In vitro* differentiation of myogenic cells, however, does not involve loss of $\alpha5\beta1$ surface expression as shown by immunoprecipitation experiments [13, 45, 46]. By analyzing C2C12 myogenic cells we have confirmed loss of fibronectin matrix assembly in myotubes without appreciable loss of $\alpha5\beta1$ integrin expression (see Figs. 7 and 8). These data suggest that the fibronectin receptor is functionally inactivated in differentiated muscle cells. This interpretation is also supported by the work of Boettiger *et al.* [13], who showed that chicken postmitotic myocytes have reduced capacity to adhere to fibronectin compared to undifferenti-

ated myoblasts. This lack of function can be rescued by treating myocytes with an α5-activating monoclonal antibody, indicating that the $\alpha5\beta1$ integrin is exposed at the myocyte surface in an inactive conformation.

Concomitantly with decreased $\alpha5\beta1$ integrin function a strong increase in $\alpha7$ integrin subunit expression occurs during myogenesis [12, 19, 20, 22] (Fig. 7 this work). Our results in CHO cells suggest that $\alpha7$ integrin can be responsible for the functional down regulation of the fibronectin receptor during myogenesis.

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