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Interaction of the disintegrin and cysteine-rich domains of ADAM12 with integrin α7β1

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Abstract

We describe a novel interaction between the disintegrin and cysteine-rich (DC) domains of ADAM12 and the integrin α7β1. Integrin α7β1 extracted from human embryonic kidney 293 cells transfected with α7 cDNA was retained on an affinity column containing immobilized DC domain of ADAM12. 293 cells stably transfected with α7 cDNA adhered to DC-coated wells, and this adhesion was partially inhibited by 6A11 integrin α7 function-blocking antibody. The X1 and the X2 extracellular splice variants of integrin α7 supported equally well adhesion to the DC protein. Integrin α7β1-mediated cell adhesion to DC had different requirements for Mn^{2+} than adhesion to laminin. Furthermore, integrin α7β1-mediated cell adhesion to laminin, but not to DC, resulted in efficient cell spreading and phosphorylation of focal adhesion kinase (FAK) at Tyr397. We also show that adhesion of L6 myoblasts to DC is mediated in part by the endogenous integrin α7β1 expressed in these cells. Since integrin α7 plays an important role in muscle cell growth, stability, and survival, and since ADAM12 has been implicated in muscle development and regeneration, we postulate that the interaction between ADAM12 and integrin α7β1 may be relevant to muscle development, function, and disease. We also conclude that laminin and the DC domain of ADAM12 represent two functional ligands for integrin α7β1, and adhesion to each of these two ligands via integrin α7β1 triggers different cellular responses.

Keywords: Myoblast; Muscle; Laminin; Cell–cell interaction

Introduction

ADAM proteins constitute a family of transmembrane or secreted glycoproteins that play important roles in cell surface proteolysis, cell–cell or cell–matrix interactions, cell migration, and differentiation [1–3]. The extracellular portion of a typical ADAM protein contains an N-terminal secretion signal, prodomain, metalloprotease, disintegrin, cysteine-rich, and epidermal growth factor-like domains. The prodomain, metalloprotease, disintegrin, and cysteine-rich domains of ADAMs are related to the P-III class of soluble snake venom metalloproteases (SVMPs) [4]. By analogy with the interactions mediated by SVMPs, the disintegrins and/or the cysteine-rich domains of ADAMs have been proposed to bind to integrins and to mediate cell–cell or cell–matrix interactions [5]. The disintegrin domains of ADAM2 (fertilin β) and ADAM9 have been shown to bind to integrin $\alpha_6\beta_1$ [6–9], the disintegrin domain of ADAM15 interacts with integrins $\alpha_v\beta_3$ [10,11] and $\alpha_5\beta_1$ [11], the disintegrin domain of ADAM23 associates with integrin $\alpha_v\beta_3$ [12], and the disintegrin domain of ADAM28 is a ligand for integrin $\alpha_4\beta_1$ [13]. The disintegrin and cysteine-rich (DC) domains of ADAM13, in addition to interacting with β1 integrins, bind to fibronectin [14], an extracellular matrix protein that associates with several integrins containing β1 chains.

ADAM12 is highly expressed in developing and regenerating skeletal muscle [15–19]. It is an active metalloprotease that cleaves insulin-like growth factor binding protein-3 and -5 [20,21], and heparin binding-epidermal growth factor (HB-EGF) [22]. ADAM12 cytoplasmic domain inter-

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acts with protein tyrosine kinase Src [23], phosphatidylinositol 3-kinase [24], and cytoskeletal proteins α-actinin-2 [18] and α-actinin-1 [25]. Transgenic expression of ADAM12 in differentiated myofibers of skeletal muscle using muscle creatine kinase promoter results in a pronounced accumulation of adipocytes in skeletal muscle [26]. Overexpression of ADAM12 in myofibers of the dystrophin-deficient *mdx* mice alleviates the skeletal muscle pathology [27].

The disintegrin and cysteine-rich domains of ADAM12, similar to the corresponding domains in other ADAM proteins, appear to interact with cell adhesion receptors. The cysteine-rich domain of ADAM12 binds to syndecan-4, a member of the heparan sulfate proteoglycan family [28–30], and the disintegrin domain of ADAM12 interacts with integrin α9β1 [31,32]. To identify integrin $\alpha_9\beta_1$ as a binding partner for the disintegrin domain of ADAM12, a series of CHO cells stably transfected with individual integrin α chains (α2, α3, α4, α5, α6, or α9) was tested for adhesion to the recombinant disintegrin domain of ADAM12 [31]. The list of integrin α chains transfected into CHO cells did not include integrin α7. Integrin α7, which forms a dimer with the β1 chain, is the major laminin-binding integrin in skeletal muscle and it plays an important role in muscle development, disease, and regeneration [33]. Since ADAM12 has been implicated in muscle development and regeneration as well [18,19,34], we asked whether integrin α7β1 can represent a binding partner for ADAM12. The α7 chain can exist as one of the three cytoplasmic tail splice variants (A, B, or C) and two extracellular domain splice variants (X1 and X2) [33]. In this report, we demonstrate that the disintegrin and cysteine-rich domains of ADAM12 bind to the α7β1 dimer (containing either the X1B or X2B variant of α7) in vitro and in intact 293 human embryonic kidney cells and L6 myoblasts. However, we also provide evidence that integrin α7β1-mediated adhesion to disintegrin and cysteine-rich domains, unlike adhesion to laminin, does not efficiently induce cell spreading and phosphorylation of focal adhesion kinase (FAK) at Tyr397. These results point to important differences between integrin α7β1-mediated signaling initiated by two different ligands: laminin and ADAM12.

Materials and methods

Cells

Rat L6 myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in the presence of 5% CO_2 at 37°C. 293EBNA cells [human embryonic kidney 293 cells stably expressing the Epstein Barr Virus (EBV) EBNA-1 gene] were cultured in DMEM supplemented with 250 μg/ml G418. 293-X1 and 293-X2 cells (293EBNA cells stably transfected with mouse integrin α7X1B or α7X2B isoforms, respectively, in pCEP4, an episomal mammalian expression vector containing the EBV replication origin, Ref. [35]) were cultured in DMEM/medium F12 (1:1) supplemented with 5% FBS, 250 μg/ml G418, and 300 μg/ml hygromycin.

Affinity chromatography using recombinant disintegrin and cysteine-rich domains of ADAM12

Recombinant DC-6xHis protein (amino acids 425–657, CGNGYV … VFGVHK, from mouse ADAM12, containing a C-terminal 6xHis tag) was produced in *Spodoptera frugiperda* Sf21 cell line using baculovirus expression system, and was purified from cell-associated material, as described [37]. DC-6xHis was immobilized on a fresh Ni-NTA affinity column (1.5 mg/ml resin, 0.1 ml bed volume). In parallel, a control column containing Ni-NTA resin and no DC-6xHis was subjected to the same treatment. 293-X1 or 293-X2 cells were extracted with 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 50 mM octylglucopyranoside, 1 mM $MnCl_2$, 1 mM AEBSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin; cell extracts were supplemented with 10 mM imidazole, divided into two equal parts, loaded onto the DC-6xHis column and the control column, and incubated for 1 h at 4°C. Columns were then washed with 10 ml of 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 1% Triton X-100 (washing buffer), then with washing buffer supplemented with 0.1 M imidazole, and then eluted with 0.3 ml of washing buffer containing 0.5 M imidazole.

Cell adhesion assays

Wells of 96-microtiter plates were coated with indicated concentrations of EHS laminin (Invitrogen) or DC-6xHis in $NaHCO_3$, pH 9.0, overnight at 4°C, and then blocked with 2.5% (w/v) BSA in DPBS for 1 h at 37°C. Adherent cells were detached in DPBS containing 1 mM EDTA and 0.05% (w/v) trypsin, washed with DPBS containing 1 mg/ml of trypsin inhibitor, suspended at 10^6 cells/ml in Tyrode's buffer (5 mM HEPES–KOH, pH 7.4, 12 mM $NaHCO_3$, 150 mM NaCl, 2.6 mM KCl, 5 mM glucose, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$,) containing 1% BSA. Cells were then pre-incubated for 15 min with or without the following function-blocking antibodies: anti-α7 (clone 6A11, Ref. [35]; 10 μg/ml), anti-β1 (clone Ha2/5, Pharmingen; 20 μg/ml), anti-β1 (clone P4C10), anti-α1 (clone FB12), anti-α2 (clone P1E6), anti-α3 (clone P1B5), anti-α5 (clone P1D6), anti-α6 (clone NKI-GoH3), or anti-α9 (clone Y9A2) (obtained from Chemicon, used at 20 μg/ml). Cells were then incubated in the wells of a 96-well plate (10^5 cells/well) for 1 h at 37°C. Unbound cells were removed by washing the wells three times with DMEM. Adherent cells were fixed for 15 min at room temperature with 3% glutaraldehyde in DPBS and stained for 5 min with 0.04% crystal

violet in 20% methanol. After washing the wells and adding 1% SDS, absorbance was measured with a Vmax microplate reader (Molecular Devices) at 550 nm; the linear range of the plate reader was 0–3.5 O.D. Each assay point was derived from two to four wells in at least two independent experiments.

Immunoblotting

To analyze FAK phosphorylation, cells were serum starved for 24 h; to analyze integrin α7 binding to the DC protein, cells were used without starvation. Cellular proteins were extracted with 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM AEBSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 5 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4 and 10 mM $Na_4P_2O_7$; 2 ml/100-mm plate. Samples were centrifuged for 30 min at 21,000 × *g*, supernatants were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked in DPBS containing 3% (w/v) dry milk (for α7B and FAK immunoblotting) or 3% (w/v) BSA [for FAK (pY397) immunoblotting] and 0.3% (v/v) Tween-20, then incubated with a primary antibody in blocking buffer, followed by incubation with a horseradish peroxidase-labeled secondary antibody and development with a chemiluminescence detection method (West Pico, Pierce, Rockford, IL). The following dilutions of primary antibodies were used: rabbit anti-integrin α7 antibody recognizing the C-terminus of the α7B isoform [36], 1:2000; anti-FAK (Santa Cruz, sc-558), 1:4000; anti-FAK (pY397) (BioSource), 1: 5000.

Results

Integrin α7β1 interacts with the DC domain of ADAM12 in vitro

To examine whether integrin α7β1 can bind to the disintegrin and cysteine-rich (DC) domains of ADAM12 in vitro, protein extracts from cells expressing the α7β1 integrin were passed through Ni-NTA columns containing immobilized DC protein, and the eluate was analyzed by SDS-PAGE and Western blotting using an antibody specific for the C-terminus of integrin α7. As shown in Fig. 1, mouse integrin α7X1 and α7X2 chains stably expressed in 293 cells (293-X1 and 293-X2, respectively) were retained on the DC-containing columns but not on control Ni-NTA columns without DC. Immunoblotting with an antibody specific for integrin β1 further confirmed the presence of the β1 integrin chain in the eluates from DC but not from control columns (result not shown). This result indicates the α7β1 dimer containing either the X1 or X2 splice variant of the integrin α7 interacts with the DC protein in vitro.

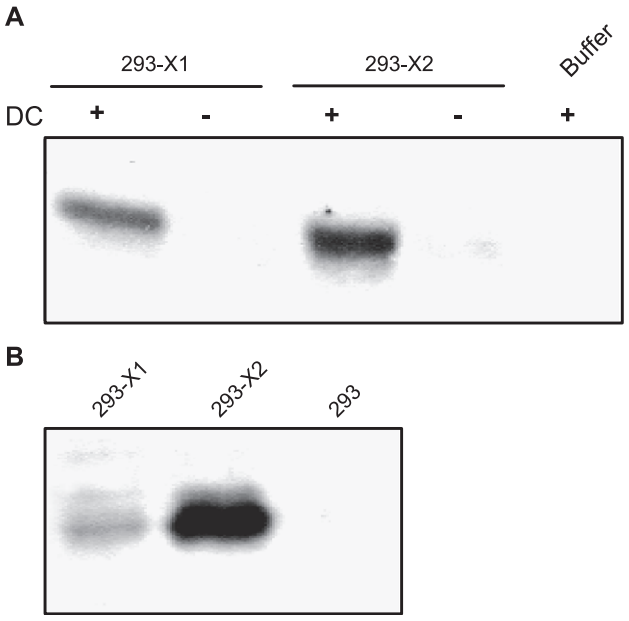

Fig. 1. Interaction of integrin α7β1 with the disintegrin-like and cysteine-rich (DC) domains of ADAM12 in vitro. (A) Total cell extracts from 293 cells stably transfected with mouse integrin α7X1B or α7X2B (293-X1 and 293-X2, respectively) were loaded on Ni-NTA columns containing immobilized DC-6xHis protein or on control Ni-NTA columns without DC-6xHis. Another DC-6xHis-containing column was incubated with cell extraction buffer only. Columns were washed and eluted with 0.5 M imidazole; samples of the eluates were subjected to SDS-PAGE under reducing conditions, followed by Western blotting with antibody raised against a C-terminal peptide from integrin α7B. The protein band of approximately 35 kDa detected by the antibody and corresponding to the C-terminal "light chain" of integrin α7B is shown. (B) Expression of integrin α7B in total cell lysates of 293-X1, 293-X2, but not in control mock-transfected 293 cells, was confirmed by Western blotting with anti-integrin α7B antibody.

Integrin α7β1 mediates cell adhesion to the DC domain of ADAM12 in the presence of Mn^{2+}

We next examined whether recombinant DC domain of ADAM12 can support cell adhesion via integrin α7β1. In the presence of 1 mM Mn^{2+}, 1 mM Ca^{2+}, and 0.5 mM Mg^{2+}, both integrin α7-expressing cells (293-X1 and 293-X2) and mock-transfected 293 cells adhered well to DC-coated plates (Fig. 2A). Pre-incubation of 293-X1 and 293-X2 cells with integrin α7-blocking antibody 6A11 inhibited cell adhesion by approximately 50% (Fig. 2A). The same antibody did not have any effect on the adhesion of mock-transfected 293 cells to DC (Fig. 2A). This suggests that adhesion of 293-X1 and 293-X2 cells to DC under these conditions was mediated, at least in part, by integrin α7β1. In contrast, in the presence of 1 mM Ca^{2+}, 0.5 mM Mg^{2+}, and in the absence of Mn^{2+}, all three cell lines (293-X1, 293-X2, and 293) adhered very poorly to DC-coated plates (Fig. 2B).

In the presence of Mn^{2+}, the attachment of 293 cells to DC was partially inhibited by integrin β1 blocking antibodies. The extent of this inhibition was similar to the

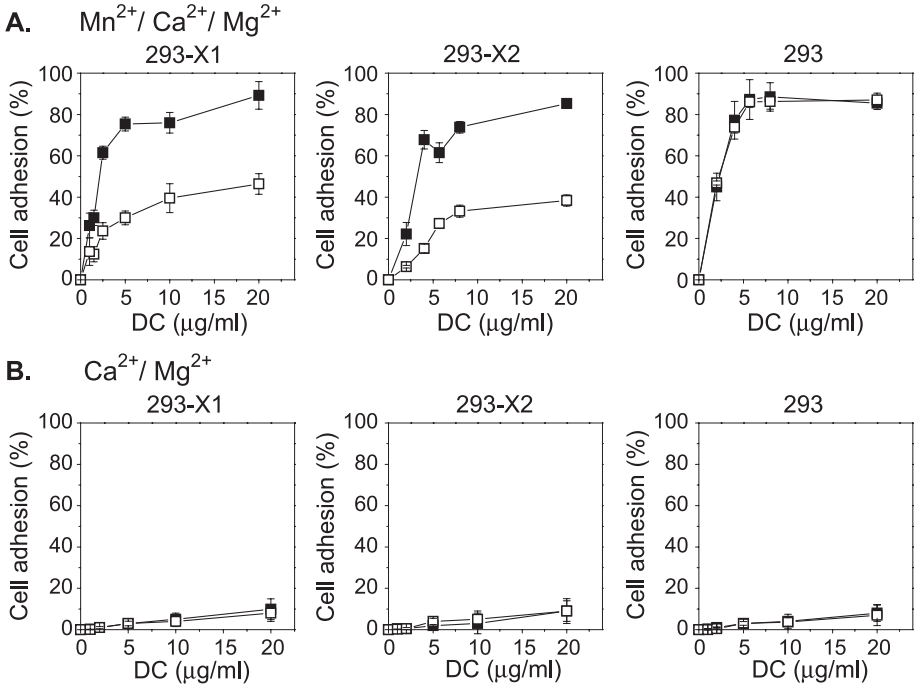

Fig. 2. Integrin α7β1 supports adhesion of 293-X1 and 293-X2 cells to the DC domain of ADAM12 in the presence of Mn^{2+}. Wells of 96-well plates were coated with DC protein at indicated concentrations. 293 cells stably transfected with mouse integrin α7X1B, α7X2B, or vector only (293-X1, 293-X2, and 293, respectively) were incubated for 15 min in Tyrode's buffer containing 1 mM Mn^{2+}, 1 mM Ca^{2+}, and 0.5 mM Mg^{2+} (A) or 1 mM Ca^{2+}, and 0.5 mM Mg^{2+} and no Mn^{2+} (B), without (closed symbols) or with 10 μg/ml of 6A11 integrin α7 function-blocking antibody (open symbols). Cells were then added to the wells (10^5 cells/well) and incubated for 1 h at 37°C. After removing unbound cells, bound cells were fixed and stained with 0.04% crystal violet, as described in Materials and methods. Results are shown as the average from three measurements, ±SE.

inhibition observed for 293-X1 and 293-X2 cells (Fig. 3). Thus, integrin α7β1 is not the only integrin capable of binding to the DC domain of ADAM12, and other β1 integrins present in 293 cells may mediate the adhesion to DC as well. Previous studies demonstrated that 293 cells express multiple integrin α chains that can dimerize with the β1 chain [35,38]. We tried to determine which of these integrins might have interacted with DC. We reasoned that the best candidates were the integrins that were down-regulated after overexpression of integrin α7, namely α1, α2, α3, α5, and α6 [35]. Therefore, we tested the effect of function-blocking antibodies against these integrins on the adhesion of 293 cells to DC. In addition, since the disintegrin domain of ADAM12 was shown to bind to integrin α9β1 [31,32] and since 293 cells express the α9 chain [38], the effect of an anti-integrin α9 antibody was also examined. None of the tested antibodies, however, added either individually or in combination, inhibited 293 cell adhesion to DC (data not shown). This suggests that either a different integrin α subunit was involved in adhesion to DC or that the potency of one or more of the antibodies used in our studies was diminished in the presence of Mn^{2+}.

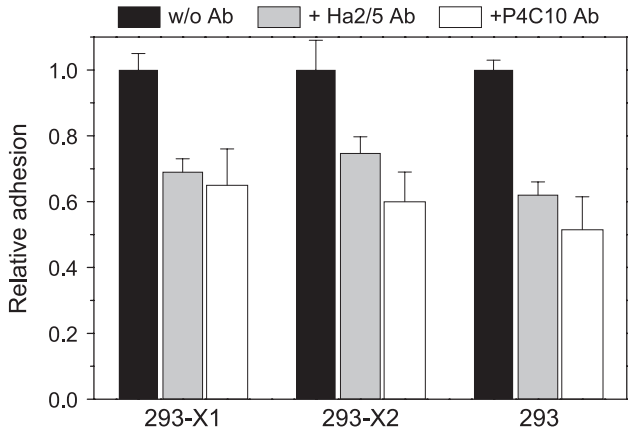

Fig. 3. The effect of integrin β1 function-blocking antibodies on adhesion of 293-X1, 293-X2, and 293 cells to DC. 293-X1, 293-X2, or 293 cells in Tyrode's buffer/1% BSA/1 mM Mn^{2+} were incubated for 15 min without (black bars) or with integrin β1 function-blocking antibody Ha2/5 (20 μg/ml; gray bars) or P4C10 (20 μg/ml; white bars) and then transferred to wells coated with 10 μg/ml of DC. The amounts of cells bound to the wells were determined as in Fig. 2.

Integrin α7β1-mediated cell adhesion to laminin has different requirements for Mn^{2+} than adhesion to DC

Integrin α7β1 is a laminin-binding protein [33,39,40] and it efficiently mediates adhesion to laminin-coated plates [35,36,41–43]. As integrin α7β1 appears to bind to the DC domain of ADAM12 as well, we were interested in comparing integrin α7β1-mediated cell adhe-

sion to DC and to laminin. As shown in Fig. 2, the interaction of integrin α7β1 with DC was critically dependent on Mn^{2+}. In contrast, adhesion of 293-X1 and 293-X2 cells to laminin was very efficient in the presence of 0.5 mM Mg^{2+} and 1 mM Ca^{2+}, and it was only weakly further stimulated by Mn^{2+} (Figs. 4A, B). Moreover, adhesion of 293-X1 and 293-X2, but not control 293 cells, to laminin in the presence of 0.5 mM Mg^{2+} and 1 mM Ca^{2+} was efficiently blocked by 6A11 antibody (Fig. 4B). Adhesion of 293-X1 and 293-X2 cells incubated in the presence of 6A11 antibody was lower than adhesion of 293 cells, which is consistent with decreased levels of several other laminin-binding integrins (mainly integrin α3β1 and α6β1) in 293-X1 and 293-X2 cells as a result of overexpression of integrin α7 [35]. Thus, in the absence of Mn^{2+}, integrin α7β1 is the major integrin in 293-X1 and 293-X2 cells that mediates adhesion to laminin.

In contrast, the 6A11 antibody had a much weaker effect on the adhesion of 293-X1 and 293-X2 cells to laminin when 1 mM Mn^{2+} was included in the incubation medium (Fig. 4A). This suggests that other laminin-binding integrins that are present in 293-X1 and 293-X2 cells contribute significantly to cell adhesion to laminin in the presence of 1 mM Mn^{2+}. Collectively, the results of the experiments shown in Figs. 2 and 4 indicate that integrin α7β1 dimers expressed in 293-X1 and 293-X2 have different requirements for Mn^{2+} to mediate cell adhesion to DC or to laminin.

293-X1 and 293-X2 cells spread well on laminin and poorly on DC

We examined the ability of integrin α7β1 to mediate cell spreading on laminin and on DC. 293-X1, 293-X2, and 293 cells plated on laminin-coated plates in the presence of 0.5 mM Mg^{2+} and 1 mM Ca^{2+} were well spread and assumed a flattened morphology (Fig. 5). Since in the absence of Mn^{2+}, adhesion of 293-X1 and 293-X2 cells to laminin is mediated predominantly via integrin α7β1 (Fig. 4B); this result indicates that integrin α7β1 binding to laminin-coated plates induces 293-X1 and 293-X2 cell spreading. In contrast, in the presence of 0.5 mM Mg^{2+}, 1 mM Ca^{2+}, and 1 mM Mn^{2+}, 293-X1 and 293-X2 cells plated on DC were mostly rounded and had few filopodia-like thin protrusions (Fig. 5). Since 293-X1 and 293-X2 cells in the presence of Mn^{2+} adhere to DC in large part via integrin α7β1, this result suggested that integrin α7β1 binding to laminin and to DC may trigger different signaling pathways in these cells. The extent of spreading of control 293 cells plated on laminin or DC was similar and most cells appeared spread and flattened.

Integrin α7β1-mediated adhesion to laminin leads to higher Tyr397 phosphorylation of FAK than adhesion to DC

We next asked whether integrin α7β1-mediated cell adhesion to DC- and to laminin-coated plates results in the activation of the same intracellular signaling pathways.

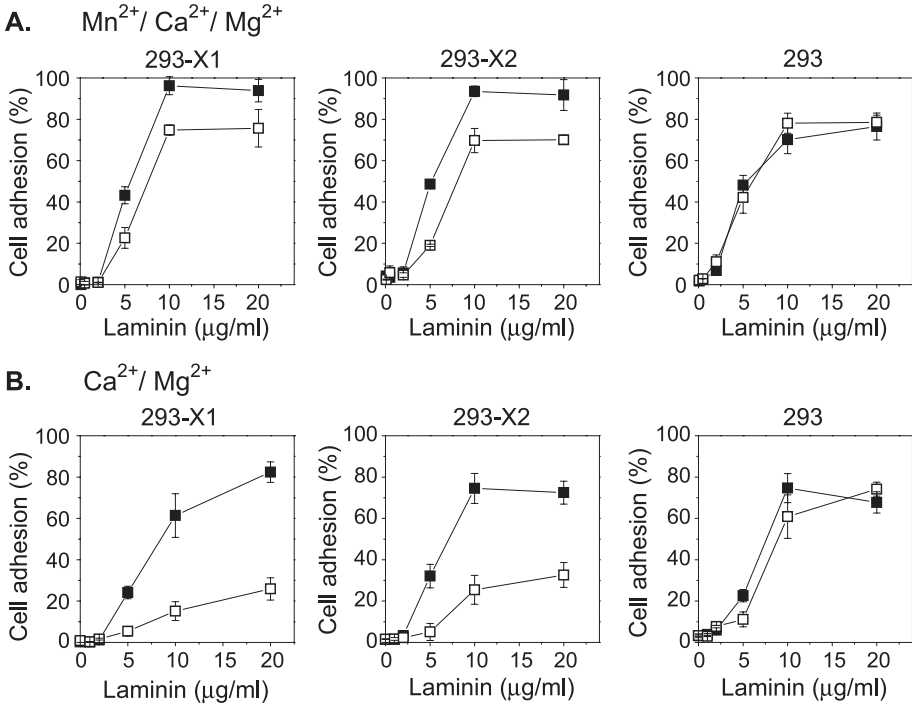

Fig. 4. Integrin α7β1-mediated adhesion of 293-X1 and 293-X2 cells to laminin has a different requirements for Mn^{2+} than adhesion to DC. Wells of 96-well plates were coated with laminin at indicated concentrations. Adhesion of 293-X1, 293-X2, and 293 cells in the presence of 1 mM Mn^{2+} (A) or in the absence of Mn^{2+} (B), without (closed symbols) or with 10 μg/ml of 6A11 integrin α7 function-blocking antibody (open symbols), was determined as in Fig. 2. Results are shown as the average from three measurements, ±SE.

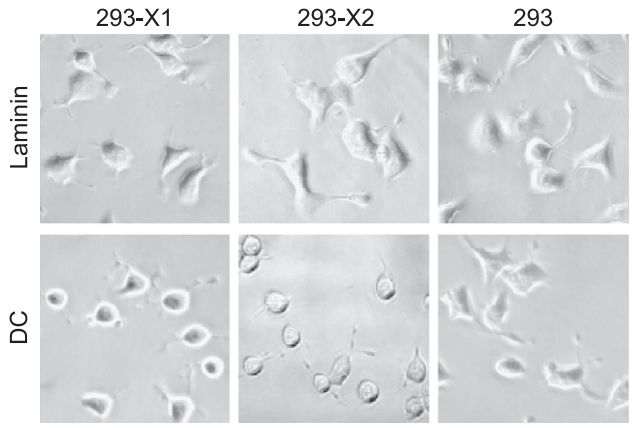

Fig. 5. Integrin α7β1-mediated adhesion to DC induces less cell spreading than adhesion to laminin. Wells of 96-well plates were coated with laminin or DC at 20 μg/ml. 293-X1, 293-X2, or 293 cells suspended in Tyrode's buffer containing 0.5 mM Mg^{2+}, 1 mM Ca^{2+} (for adhesion to laminin) or 0.5 mM Mg^{2+}, 1 mM Ca^{2+}, and 1 mM Mn^{2+} (for adhesion to DC) were added to the wells (10^4 cells/well). After 1 h, cells were examined by phase contrast microscopy.

Autophosphorylation at Tyr397 and subsequent activation of focal adhesion kinase (FAK) are the early events that are observed in many cells after the engagement of integrins with their immobilized ligands [43,44]. To examine the level of FAK phosphorylation after cell adhesion to DC, 293-X1, 293-X2, and 293 cells were serum-starved for 24 h, detached from plates, and then allowed to attach to DC-coated wells in the presence of 1 mM Mn^{2+}. After 1 h incubation, the levels of FAK (pY397) and total FAK protein were analyzed by Western blotting. As shown in Figs. 6A and B, the level of FAK (pY397) in 293-X1 and in 293-X2 cells plated on DC protein was at least three times lower than in control 293 cells, whereas the amount of total FAK protein was the same. Since in the presence of 1 mM Mn^{2+} the adhesion of 293-X1 and 293-X2, but not 293 cells, to DC is mediated in large part by integrin α7β1 (see also Fig. 2A), this result suggests that the interaction between α7β1 and DC provides a poor stimulus for FAK phosphorylation.

In contrast, the interaction between integrin α7β1 and laminin does lead to efficient phosphorylation of FAK at Tyr397. In 0.5 mM Mg^{2+} and 1 mM Ca^{2+}, the adhesion of 293-X1 and 293-X2 cells to laminin is mediated mostly by integrin α7β1 (see Fig. 4B), whereas 293 cells adhere to laminin most likely via integrin α3β1 and α6β1 (Ref. [35]). The levels of FAK (pY397) in 293-X1, 293-X2, and 293 cells attached to laminin in the absence of Mn^{2+} were very similar (Fig. 6), indicating that integrin α7β1 engagement (in 293-X1 and 293-X2 cells) was as efficient as the engagement of other laminin-binding integrins (in 293 cells) in inducing FAK phosphorylation. Collectively, these results suggest that although laminin and the DC domain of ADAM12 represent two ligands for integrin α7β1, adhesion to each of these two ligands via integrin α7β1 triggers different cellular responses. Specifically, α7β1-mediated adhesion to laminin leads to higher Tyr397 phosphorylation of FAK (Fig. 6), which is accompanied by more efficient cell spreading (Fig. 5).

The endogenous integrin α7β1 expressed in myoblasts contributes to cell adhesion to DC

We asked whether skeletal myoblasts expressing the endogenous α7 chain utilize the α7β1 integrin dimer during adhesion to the DC domain of ADAM12. As shown in Fig. 7A, L6 myoblasts incubated in the presence of 1 mM Mn^{2+}, 1 mM Ca^{2+}, and 0.5 mM Mg^{2+} adhered well to DC-coated plates. Similarly to 293-X1 and 293-X2 cells, the adhesion of L6 myoblasts to DC was critically dependent on the presence of Mn^{2+}. Pre-incubation with 6A11 integrin α7 function-blocking antibody inhibited cell adhesion at lower DC-coating concentrations (<2 μg/ml), but did not have any effect at higher DC concentrations (Fig. 7A), suggesting that other integrins may also play a role in L6 cell adhesion to

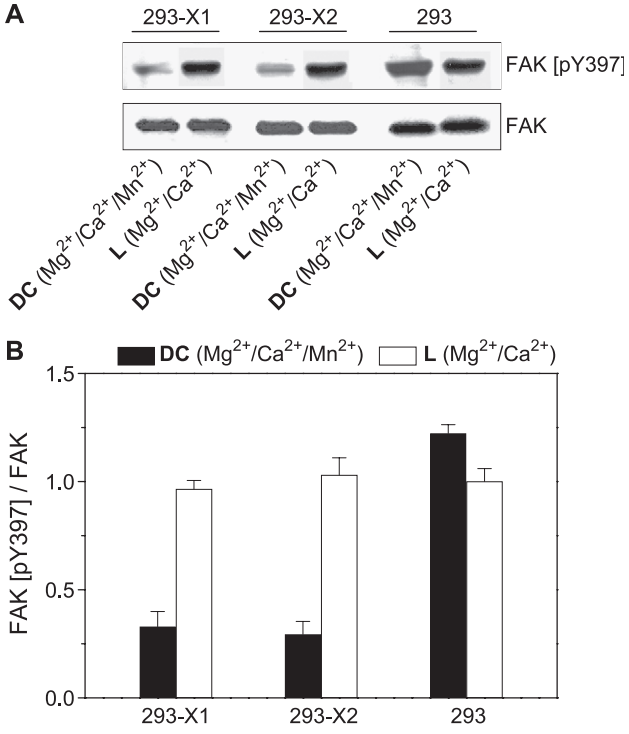

Fig. 6. FAK phosphorylation at Tyr397 in cells adhering to DC and to laminin. (A) Wells of 96-well plates were coated with DC protein or EHS laminin (L) at 20 μg/ml. 293-X1, 293-X2, or 293 cells were suspended in Tyrode's buffer containing 0.5 mM Mg^{2+}, 1 mM Ca^{2+}, with or without 1 mM Mn^{2+}, as indicated. Cells were then added to the wells (10^5 cells/well) and incubated for 1 h at 37°C. After removing unbound cells, bound cells were treated with lysis buffer containing protease and phosphatase inhibitors, centrifuged, and supernatants were analyzed by Western blotting using a phospho-specific antibody recognizing phosphorylated Tyr397 in FAK (top) or total FAK (bottom). (B) The intensities of the FAK (pY397) and total FAK signals shown in panel A were quantified by densitometry. The experiment was repeated three times, representative results from one experiment are shown in A, the average FAK (pY397)/FAK signals (in arbitrary units, ±SE) are shown in B.

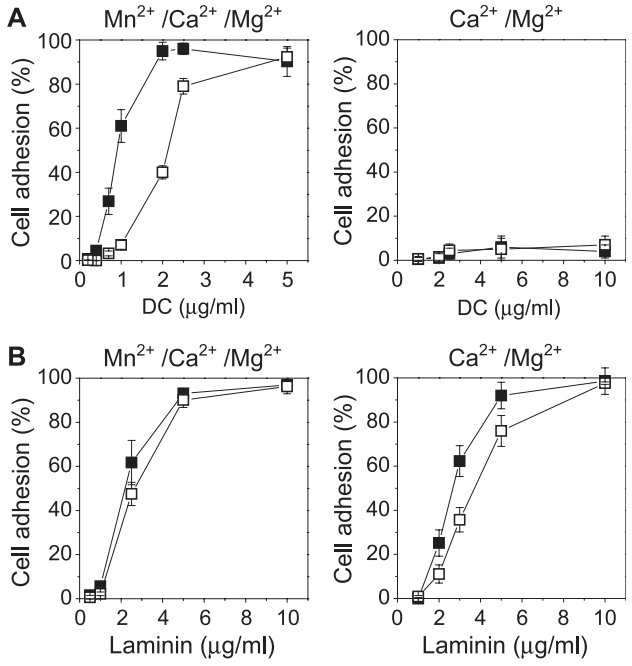

Fig. 7. Adhesion of L6 myoblasts to DC is partially mediated by integrin α7β1 and it requires the presence of Mn^{2+}. Wells of 96-well plates were coated with DC protein (A) or laminin (B) at indicated concentrations. 293-X1, 293-X2, and 293 cells were incubated for 15 min in Tyrode's buffer containing 1 mM Mn^{2+}, 1 mM Ca^{2+}, and 0.5 mM Mg^{2+} (left panels) or 1 mM Ca^{2+}, and 0.5 mM Mg^{2+} and no Mn^{2+} (right panels), without (closed symbols) or with 10 μg/ml of 6A11 integrin α7 function-blocking antibody (open symbols). Cells were then added to the wells (10^5 cells/well) and incubated for 1 h at 37 °C. The amount of bound cells was determined as in Fig. 2. Results are shown as the average from three measurements, ±SE.

DC. Interestingly, the inhibitory effect of the antibody was more pronounced when L6 cells were plated on DC than on laminin (Fig. 7B). We conclude that the endogenous integrin α7β1 expressed in L6 myoblasts does contribute to cell adhesion to DC, and its contribution is most evident at low concentrations of DC protein.

Discussion

In this report, we describe a novel interaction between the disintegrin and cysteine-rich domains of ADAM12 and integrin α7β1. Using affinity columns containing immobilized DC domain of ADAM12, we showed that integrin α7β1 bound to the DC protein in vitro. 293 cells stably transfected with α7 cDNA, as well as L6 myoblasts expressing the endogenous α7 integrin, adhered to DC-coated wells, and this adhesion was partially inhibited by 6A11 integrin α7 function-blocking antibody.

Integrin α7 chain can exist as one of the three cytoplasmic (A, B, or C) and two extracellular (X1 or X2) splice variants [33]. Our cell adhesion and column binding assays utilized 293 cells expressing the X1B or X2B forms of the α7 chain, which are the major variants expressed in proliferating myoblasts [45,46]. Although the X1 and X2 splice variants confer different specificities for laminin in cell adhesion [36,42], cell migration [35,36], acetylcholine receptor clustering [47], or solid phase binding assays [43], these two variants supported equally well adhesion to the DC protein.

Whereas laminin and the DC domain of ADAM12 represent two functional ligands for integrin α7β1, our results suggest that there are important differences between α7β1 binding to these two ligands. While α7β1-mediated adhesion to laminin did not require activation of the integrin by Mn^{2+}, Mn^{2+} was necessary to detect adhesion to the DC protein. In addition, cellular responses initiated upon integrin α7β1 binding to laminin and to DC seem to be different as well, as α7β1-mediated adhesion to laminin results in better cell spreading and more potent Tyr397 phosphorylation than adhesion to DC. Interestingly, our results are similar to a previous report in which integrin α6β1-mediated adhesion of fibrosarcoma cells to the recombinant extracellular domain of ADAM9 led to a rounded cell morphology and higher cell motility than adhesion to laminin [9].

The different requirement for Mn^{2+} of α7β1 binding to laminin and to DC is consistent with the recently proposed model of integrin activation [48–50]. According to this model, in the presence of Mn^{2+}, there is an equilibrium of the bent integrin conformer (with a low affinity for ligand), an extended conformer with closed headpiece (with an intermediate affinity for ligand), and an extended conformer with open headpiece (with a high affinity for ligand). In the absence of Mn^{2+}, the major conformational state of integrin is the bent conformer, with a very minor population of the extended conformers. Addition of a ligand shifts the equilibrium toward the extended conformer with an open headpiece [48]. Because the equilibria between conformational changes and ligand binding are thermodynamically linked, a ligand (such as laminin) that binds strongly to integrin α7β1 may shift the equilibrium towards the extended conformer with an open headpiece even in the absence of Mn^{2+}. In the case of a ligand with weaker binding (such as DC), Mn^{2+} is required to destabilize the bent conformation and to induce the extended conformer with the open headpiece that can interact with the ligand. Our results suggest, therefore, that DC binding to integrin α7β1 is significantly weaker than the binding of laminin. It should be noted, however, that the DC protein corresponds to a domain of ADAM12 and its integrin binding properties may be different from the properties of the intact transmembrane ADAM12.

Integrin α7β1 is not the only protein capable of binding to the DC domain of ADAM12, as adhesion of 293-X1 and 293-X2 cells to DC was only partially inhibited by anti-α7 antibodies and parental 293 cells lacking integrin α7 chain also adhered well to DC-coated plates. Indeed, it has been shown recently that multiple receptors present on the same cell can participate in cell binding to recombinant ADAM disintegrin domains [8]. In fact, two other proteins were reported to bind to the extracellular domain of ADAM12

previously: syndecan-4, a member of heparan sulfate family [28–30], and integrin α9β1 [31,32]. Syndecan-4 binds to the cysteine-rich fragment of ADAM12 located between amino acids 564 and 708 [28], a region that has a limited overlap (93 amino acids) with the DC construct used in our studies (amino acids 425–657). Syndecans, however, are not activated by Mn^{2+} and they seem unlikely to mediate cell adhesion observed in our studies. Integrin α9β1 binds to the RX_6DLPEF recognition motif that is present in the disintegrin domains of many ADAMs, including ADAM12 [32]. Thus, the α9β1 integrin is a receptor for many ADAM disintegrins. As both α9β1 and ADAMs are widely expressed, α9β1-ADAM interactions are predicted to be of a broad biological importance [32]. Integrin α7β1, on the other hand, is expressed predominantly in skeletal and cardiac muscle and therefore it might be involved in tissue-specific interactions with ADAM12. Interestingly, it was recently observed that transgenic overexpression of ADAM12 in mice using the muscle creatine kinase promoter leads to increased expression of integrin α7 in skeletal muscle [51]. Clearly, an interaction between the DC domain of ADAM12 and integrin α7β1 is one of the possible mechanisms underlying this effect.

Integrin α7 plays an important role in muscle development and disease [33]. Spatiotemporal pattern of expression of individual splice variants of integrin α7 is developmentally regulated during myogenesis [52–54]. Mice lacking the integrin α7 gene develop a specific form of muscular dystrophy that affects myotendinous junction [55], and mutations in the integrin α7 gene in humans cause congenital myopathies [56]. Moreover, an altered expression of integrin α7 has been observed in Duchenne muscular dystrophy and in *mdx* mice that lack dystrophin [57]. Enhanced expression of integrin α7 reduced muscular dystrophy and stabilized muscle regeneration in *mdx/utr−/−* mice that lacked both dystrophin and utrophin [58]. Since ADAM12 has been implicated in muscle development and/or regeneration as well [18,19,34,51], we postulate that the interaction between ADAM12 and integrin α7β1 reported here may contribute to integrin α7β1 functions in muscle cell growth, stability, and/or survival.

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