Metalloprotease-disintegrin ADAM 12 interacts with α-actinin-1

Yi CAO, Qing KANG and Anna ZOLKIEWSKA[1]

Department of Biochemistry, Kansas State University, 104 Willard Hall, Manhattan, KS 66506, U.S.A.

ADAM 12, a member of the ADAM family of proteins (containing A Disintegrin And Metalloprotease domain), has been implicated in differentiation and fusion of myoblasts. While the extracellular domain of ADAM 12 contains an active metalloprotease and a region involved in cell adhesion, the function of the cytoplasmic tail of ADAM 12 has been less clear. Here we show that the cytoplasmic domain of ADAM 12 interacts *in vitro* and *in vivo* with α-actinin-1, an actin-binding and cross-linking protein. Green fluorescent protein fused to ADAM 12 cytoplasmic domain co-localizes with α-actinin-1-containing actin stress fibres in C2C12 cells. The interaction between ADAM 12 and α-actinin-1 is direct and involves the 58-amino acid C-terminal fragment of ADAM 12 and the 27 kDa N-terminal domain of α-actinin-1. Consistently, expression of the 27 kDa fragment of α-actinin-1 in C2C12 cells using a mitochondrial targeting system results in recruitment of the co-expressed ADAM 12 cytoplasmic domain to the mitochondrial surface. Moreover, α-actinin-1 co-purifies with a transmembrane, His_6-tagged form of ADAM 12 expressed in C2C12 myoblasts, indicating that the transmembrane ADAM 12 forms a complex with α-actinin-1 *in vivo*. These results indicate that the actin cytoskeleton may play a critical role in ADAM 12-mediated cell–cell adhesion or cell signalling during myoblast differentiation and fusion.

Key words: cell adhesion, cell–cell fusion, cytoskeleton, differentiation, myoblasts.

INTRODUCTION

ADAMs (proteins containing A Disintegrin And Metalloprotease domain) are cell-surface receptors that have been implicated in cell adhesion, communication and fusion [1–3]. ADAMs are composed of a pro-domain, a metalloprotease domain, a disintegrin-like domain, a cysteine-rich region and, in most cases, an epidermal growth factor-like domain, a single transmembrane domain and a cytoplasmic tail. ADAM 12 is primarily expressed in skeletal muscle where it has been postulated to promote differentiation and fusion of myoblasts [4–6]. Indeed, overexpression of a fragment of ADAM 12 extending from the disintegrin to the cytoplasmic domain leads to accelerated fusion of C2C12 myoblasts, whereas transfection of the cells with the same construct in antisense orientation results in inhibition of fusion [4]. ADAM 12 is also found in placenta [6], bone [7], macrophage-derived giant cells [8] and osteoclasts [8], and its expression is dramatically upregulated in many tumour cell lines [9].

We have demonstrated previously [10] that the disintegrin-like domain of ADAM 12, together with the cysteine-rich region, supports cell adhesion. Moreover, recombinant cysteine-rich domain alone was found to bind to cell-surface proteoglycans [9], consistent with the postulated involvement of ADAM 12 in cell–cell adhesion and communication. In addition, the metalloprotease domain in the extracellular part of ADAM 12 has been shown to possess a Zn^{2+}-dependent proteolytic activity [11]. The function of the cytoplasmic portion of ADAM 12 is less clear. Recently [12], it has been reported that this domain interacts with a skeletal-muscle-specific protein α-actinin-2, but the exact effect of this interaction on ADAM 12 is unknown.

α-Actinin is an actin-binding and cross-linking protein that is expressed in virtually all cells. In humans, four different genes encoding α-actinin have been identified [13]. The *ACTN1* gene gives rise to α-actinin-1, which can exist as a non-muscle-specific or a smooth muscle isoform as a result of alternative mRNA splicing. *ACTN2* and *ACTN3* encode two skeletal muscle isoforms (α-actinin-2 and -3 respectively), and *ACTN4* encodes a novel non-muscle isoform, α-actinin-4. The non-muscle-specific isoform of α-actinin-1 is involved in bundling actin filaments into stress fibres and connecting them to the cell membrane. Skeletal and smooth muscle α-actinins play key roles in organizing actin filaments at the Z discs in skeletal muscle and dense bodies in smooth muscle respectively [13]. In cultured skeletal muscle myoblasts, α-actinin-1 is expressed both before and after cell differentiation and fusion, whereas α-actinin-2 and -3 appear at the onset of cell fusion [14,15].

The present study is a part of an ongoing effort to identify novel proteins that interact with ADAM 12. We expressed the cytoplasmic domain of ADAM 12 fused to green fluorescent protein (GFP) and examined its localization in C2C12 myoblasts. Since GFP-ADAM 12 cytoplasmic domain fusion protein co-localized with actin stress fibres and, to some extent, with focal adhesions, we investigated whether ADAM 12 could interact with α-actinin-1, the main α-actinin form present in myoblasts before their differentiation and fusion. We found that the cytoplasmic tail of ADAM 12, when expressed either as an autonomous soluble domain, or as a part of a transmembrane form of ADAM 12, directly interacted with α-actinin-1 *in vitro* and *in vivo*. These observations suggest a potential role for the actin cytoskeleton in ADAM 12-mediated cell–cell adhesion or cell signalling during myoblast differentiation and fusion.

EXPERIMENTAL

Antibodies

Anti-ADAM 12 antibody was raised against the peptide Met-Asn-Arg-Ala-Pro-His-Phe-Asn-Thr-Pro-Lys-Asp-Arg-His-Ser-Leu-Lys-Cys from mouse ADAM 12 cytoplasmic domain (amino acids 774–791) and affinity purified (Bethyl Laboratories, Montgomery, TX, U.S.A.). Mouse anti-vinculin monoclonal

Abbreviations used: ADAM, A Disintegrin And Metalloproteinase; GFP, green fluorescent protein; GST, glutathione S-transferase; AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride; DPBS, Dulbecco's PBS.

[1] To whom correspondence should be addressed (e-mail zolkiea@ksu.edu).

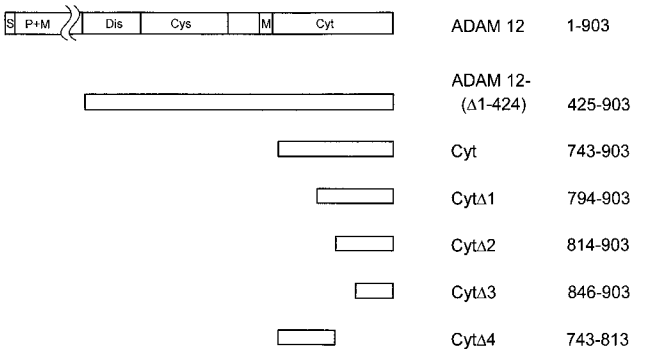

Figure 1 Schematic representation of the constructs used to study the interaction between ADAM 12 and α-actinin-1

Mouse ADAM 12 (residues 1–903) contains a signal peptide (S), a pro- and a metalloprotease domain (P + M), a disintegrin-like domain (Dis), a cysteine-rich domain (Cys), a transmembrane domain (M) and a cytoplasmic tail (Cyt). ADAM 12 fragments, comprising the amino acids shown on the right, were fused to GST, GFP or a His_6-tag.

antibody and rabbit anti-α-actinin polyclonal antibody raised against chicken gizzard α-actinin were obtained from Sigma, mouse anti-GFP monoclonal antibody (clone 3E6) was from Molecular Probes (Eugene, OR, U.S.A.), mouse Penta-His monoclonal antibody, recognizing five consecutive histidine residues, was from Qiagen, mouse anti-c-Myc monoclonal antibody was from Invitrogen (Carlsbad, CA, U.S.A.), and goat anti-glutathione S-transferase (GST) polyclonal antibody was from Amersham Pharmacia Biotech.

Generation of ADAM 12 and α-actinin-1 expression constructs

cDNAs encoding different regions of mouse ADAM 12 cytoplasmic domain (Figure 1) were amplified by PCR using mouse skeletal muscle cDNA (ClonTech, Palo Alto, CA, U.S.A.) as template, *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA, U.S.A.) and appropriate sets of primers. The Cyt fragment of ADAM 12 (residues 743–903) was cloned into pEGFP-C1 vector (ClonTech) between *Bgl*II and *Eco*RI sites to express GFP-Cyt fusion protein in C2C12 cells, with GFP present at the N-terminus of the recombinant ADAM 12 fragment. Fragments CytΔ1 (residues 794–903), CytΔ2 (residues 814–903), CytΔ3 (residues 846–903) and CytΔ4 (residues 743–813) were cloned into pGEX-2T vector (Amersham Pharmacia Biotech) between *Bam*HI and *Eco*RI sites for expression of GST-fusion proteins in *Escherichia coli*. In the GFP- and all the GST-expression constructs, the 3′-end of the ADAM 12 coding region was immediately followed by a stop codon so that the resulting GFP- and GST-fusion proteins did not contain any foreign amino acids at the C-termini. To express the full size ADAM 12, the entire coding region of ADAM 12 (residues 1–903) was cloned into the *Cla*I/*Not*I sites of the pIRESpuro vector (ClonTech). In addition, a cDNA fragment encoding a transmembrane form of ADAM 12 extending from the disintegrin to the cytoplasmic domain [ADAM 12 (Δ1–424), residues 425–903] was cloned into the pIRESpuro vector between *Cla*I and *Not*I sites. This construct contained the secretion signal of the mouse Igκ chain derived from pSecTag2A vector (Invitrogen), as described in [10], with the C-terminus of ADAM 12 immediately followed by a His_6-tag. The N-terminal actin-binding domain of mouse α-actinin-1 comprising amino acids 1–250 was amplified using the primers 5′-ATGGACCATTATGATTCCCA-3′ (derived from a mouse expressed sequence tag, accession number AW107046) and 5′-TGAAAAGGCATGGTAGAAGC-3′ (derived from a mouse expressed sequence tag, accession number AA033333), and cloned between the *Xho*I/*Not*I sites in pCMV/Myc/mito vector (Invitrogen). Recombinant α-actinin-1 fragment in transfected C2C12 cells was detected by Western blotting using anti-Myc monoclonal antibody (Invitrogen) or anti-α-actinin polyclonal antibody (Sigma). Localization of the recombinant α-actinin-1 fragment to mitochondria was confirmed by co-staining of the transfected cells with anti-Myc antibody and MitoTracker Red CMXRos, a mitochondria-specific dye (Molecular Probes). The identity of all the constructs was verified by sequencing the entire lengths of the inserts (performed at the DNA Sequencing Facility, Iowa State University, Ames, IA, U.S.A.).

Expression of GST fusion proteins

All GST fusion proteins were expressed in *E. coli* as soluble proteins and were purified on GSH–Sepharose columns (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The apparent molecular masses of the major forms of purified GST-CytΔ1, GST-CytΔ2, GST-CytΔ3 and GST-CytΔ4 proteins were 40 kDa, 38 kDa, 35 kDa and 35.5 kDa respectively. The predicted molecular masses of the four proteins were 38.2 kDa, 36.0 kDa, 32.7 kDa and 34.3 kDa respectively.

Protein binding under native conditions

Purified GST-CytΔ1 or GST alone (100 μg of each protein) were immobilized on GSH–Sepharose (100 μl bed volume), followed by washing the beads with 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1% (v/v) Triton X-100 and 3% (w/v) BSA. Chicken gizzard α-actinin (Sigma) was dialysed against 50 mM Tris/HCl (pH 8.0) and 150 mM NaCl. A 50 μg portion of α-actinin (0.1 mg/ml) was incubated with GST-CytΔ1 or GST columns for 15 min at 4 °C. The columns were washed with 10 ml of Dulbecco's PBS (DPBS) and eluted with 200 μl of the SDS gel-loading buffer. The eluates were subjected to SDS/PAGE and Western blotting with anti-α-actinin antibody. To study the binding of endogenous α-actinin to GST-CytΔ1, non-differentiated C2C12 cells were incubated with lysis buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), 5 μg/ml aprotinin, 5 μg/ml leupeptin and 1 μg/ml pepstatin A; 2 ml of the buffer/100 mm plate), the lysate was centrifuged for 20 min at 21000 ***g***, and the supernatant (10 ml) was applied to 100 μl GST-CytΔ1 or GST columns. The columns were washed with 10 ml of lysis buffer and eluted with 200 μl of the SDS gel-loading buffer, followed by Western blotting of the eluates with anti-α-actinin antibody.

Blot overlays

GST fusion proteins were resolved by SDS/PAGE and transferred to a nitrocellulose membrane. The membrane was incubated in blocking buffer [50 mM Tris/HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2% BSA and 0.1% β-mercaptoethanol] for 5 h at 4 °C, and then with chicken gizzard α-actinin (30–100 μg/ml) in blocking buffer for 16 h at 4 °C. The membrane was washed twice with 50 mM Tris/HCl (pH 8.0), 50 mM NaCl, 1% Triton X-100 and 1% I-Block (Tropix, Bedford, MA, U.S.A.), followed by incubation with an anti-α-actinin antibody, then incubation with horseradish peroxidase-labelled anti-rabbit IgG antibody, and visualization with a chemiluminescent-detection method. In the experiment shown in Figure 4(C), the NaCl concentration in the buffer during incubation with α-actinin was raised from 50 to 150 mM.

Blot overlays with GST fusion proteins

Chicken gizzard α-actinin was electrophoresed and transferred to a nitrocellulose membrane. The membrane was incubated with 50 mM Tris/HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 2 % BSA and 0.1 % β-mercaptoethanol for 5 h at 4 °C, and then with GST-CytΔ1 or GST alone (100 μg/ml each) in 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1 % Triton X-100 and 2 % BSA for 16 h at 4 °C. The membrane was washed twice with 150 mM Tris/HCl (pH 8.0), 50 mM NaCl, 1 % Triton X-100, 1 % I-Block, followed by incubation with anti-GST antibody (1:2000 dilution in the same buffer), incubation with horseradish peroxidase-conjugated anti-goat IgG antibody, and visualization with a chemiluminescent-detection method. Proteolytic fragments of α-actinin were obtained by incubation of chicken gizzard α-actinin with thermolysin (protease/substrate, 1:20; Sigma) in 50 mM Tris/HCl (pH 8.0) and 150 mM NaCl for 2 h at 37 °C.

Cell culture and transfections

C2C12 cells were incubated in growth medium (Dulbecco's modified Eagle's medium with 10 % fetal bovine serum) in the presence of 5 % CO_2 under humidified atmosphere. After reaching confluence, cells were transferred to differentiation medium (Dulbecco's modified Eagle's medium with 2 % horse serum). Cells in growth medium (5×10^5 cells/100 mm plate or 6×10^4 cells/well in a 6-well plate containing a 22 mm glass coverslip) were transfected with expression vectors using LIPOFECTAMINE™ Plus reagent (Life Technologies) according to the manufacturer's instructions. Expression of the recombinant proteins was analysed 40 h after transfection.

Fluorescence microscopy

Cells on coverslips were fixed for 15 min at room temperature with 3.7 % paraformaldehyde in DPBS. Coverslips were rinsed with DPBS, and cells were permeabilized by incubation with 0.1 % Triton X-100 in DPBS at room temperature for 5 min. For filamentous actin staining, cells were incubated with rhodamine phalloidin (0.1 μg/ml; Sigma) in DPBS for 30 min. For immunocytochemistry, cells were incubated with the anti-ADAM 12 antibody (1:500 dilution), anti-vinculin (1:200 dilution), or anti-c-Myc antibody (1:200 dilution), and then with rhodamine- or FITC-conjugated anti-rabbit or anti-mouse IgG (1:200 dilution). The coverslips were rinsed with DPBS, mounted on slides with 20 μl of 10 % (w/v) Mowiol 4-88 (Calbiochem) in 25 % glycerol, and viewed on a Zeiss laser scanning confocal microscope (model LSM 410), equipped with an Axiovert 100 inverted microscope, an Argon/Krypton 488/568/647 laser, a KP 600 line-selection filter, an FT 488/568 dichroic beam-splitter, an LP 590 emission filter for viewing red fluorochromes, a BP 515–540 emission filter for viewing GFP, and the software package LSM 3.993. Images were processed using Adobe Photoshop 5.5 software.

Chromatography on α-actinin column

Chicken gizzard α-actinin (0.25 mg) was dialysed against buffer containing 0.1 M $NaHCO_3$ (pH 8.3), and 0.3 M NaCl and then coupled to CNBr-activated Sepharose (0.1 ml bed volume; Amersham Pharmacia Biotech) according to the manufacturer's instructions. A control column was prepared that underwent the same treatment as the α-actinin-containing column. COS-7 cells transiently transfected with the full length ADAM 12, ADAM 12 (Δ1–424), or vector only were lysed with buffer containing DPBS, 1 % Triton X-100, 1 mM AEBSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 1 μg/ml pepstatin A (2 ml of the lysis buffer/100-mm plate). The lysate was centrifuged for 20 min at 21000 ***g*** and the supernatant (4 ml) was applied to the α-actinin or to the control column. After washing the columns with 10 ml of lysis buffer and elution with 600 μl of SDS gel-loading buffer, proteins were analysed by SDS/PAGE and Western blotting with anti-ADAM 12 antibody.

Detection of ADAM 12 and α-actinin complex *in vivo*

C2C12 cells on five 100 mm plates were transfected with pIRESpuro vector encoding ADAM 12 (Δ1–424) with a C-terminal His_6-tag. At 40 h after transfection, cells were lysed with buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 1 % Triton X-100, 1 mM AEBSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 1 μg/ml pepstatin A (2 ml/plate). The lysate was centrifuged for 20 min at 21000 ***g*** and the supernatant was applied to an Ni^{2+}-nitrilotriacetate column (100 μl bed volume; Qiagen). The lysate of cells transfected with vector only was applied to an identical column and used as a control. After 30 min incubation at 4 °C, the columns were washed with 10 ml of wash buffer [50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 1 % Triton X-100 and 10 mM imidazole] and then eluted with 300 μl of elution buffer [50 mM NaH_2PO_4 (pH 7.0), 300 mM NaCl, 1 % Triton X-100 and 500 mM imidazole]. The eluted proteins were analysed by SDS/PAGE and Western blotting with Penta-His, anti-ADAM 12, or anti-α-actinin antibodies

Immunoblotting

Proteins were resolved by SDS/PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with blocking buffer [DPBS containing 1 % (w/v) I-Block and 0.3 % (v/v) of Tween 20], then with a primary antibody in blocking buffer, followed by washing with DPBS, incubation with a horseradish peroxidase-labelled secondary antibody in blocking buffer, followed by washing with DPBS and visualization with a chemiluminescent detection method (SuperSignal West Pico; Pierce, Rockford, IL, U.S.A.). The following concentrations or dilutions of primary antibodies were used: anti-ADAM 12 antibody, 0.3 μg/ml; anti-GFP antibody, 0.7 μg/ml; Penta-His antibody, 0.1 μg/ml; anti-α-actinin antibody, dilution 1:30000.

RESULTS

Co-localization of GFP-ADAM 12 cytoplasmic domain fusion protein with actin stress fibres

In a search for cellular proteins that interact with ADAM 12 in skeletal muscle cells, we decided to examine the cellular distribution of a recombinant soluble form of ADAM 12 cytoplasmic domain. A region of mouse ADAM 12 comprising amino acids 743–903 in the cytoplasmic domain of ADAM 12 (Figure 1) was fused to GFP and expressed in C2C12 cells (GFP-Cyt; Figure 2). Immunocytochemistry and direct fluorescence microscopy of transfected C2C12 cells revealed that, while GFP was distributed diffusely in the cytosol and was somewhat concentrated in the nucleus (Figure 3H), GFP-Cyt co-localized to a large extent with actin stress fibres (Figures 3D–3F) and partially co-localized with vinculin (Figures 3A–3C), a marker protein for focal adhesions.

Direct interaction between ADAM 12 cytoplasmic domain and α-actinin-1

Recently, the cytoplasmic domain of mouse ADAM 12 has been found to interact with α-actinin-2, a skeletal muscle-specific form of α-actinin. Since the main form of α-actinin that is expressed in

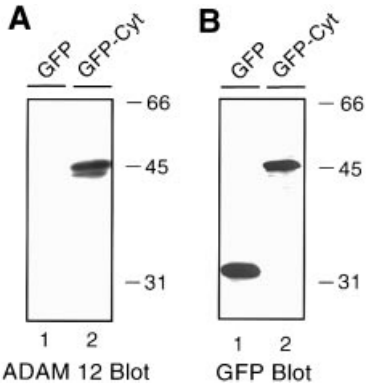

Figure 2 Expression of GFP-ADAM 12 cytoplasmic domain fusion protein in C2C12 cells

C2C12 cells were transfected with vectors encoding GFP or GFP fused to the Cyt fragment of ADAM 12. Expression of the recombinant proteins was analysed 2 days later by subjecting the extracts from GFP- (lane 1) or GFP-Cyt-transfected cells (lane 2) to SDS/PAGE and Western blotting with anti-ADAM 12 antibody (**A**) or anti-GFP antibody (**B**).

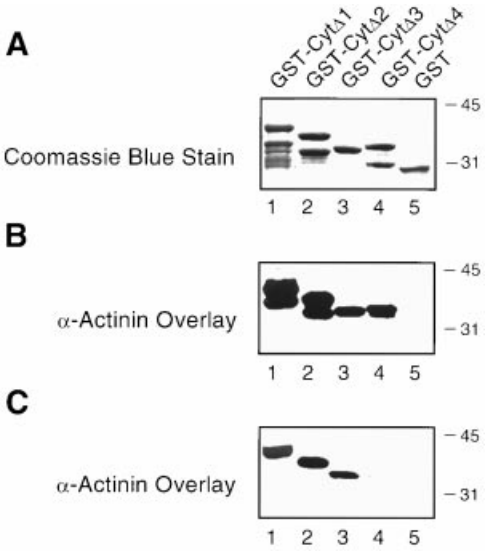

Figure 4 Direct interaction between the cytoplasmic domain of ADAM 12 and α-actinin-1 detected in a blot overlay assay

GST fusion proteins harbouring CytΔ1 (lane 1), CytΔ2 (lane 2), CytΔ3 (lane 3) or CytΔ4 (lane 4) fragments of the cytoplasmic domain of ADAM 12 and GST alone (lane 5) were subjected to SDS/PAGE and stained with Coomassie Blue (**A**) or transferred on to a nitrocellulose membrane and incubated with chicken gizzard α-actinin (50 μg/ml) in the presence of 50 mM NaCl (**B**) or 150 mM NaCl (**C**), followed by detection with anti-α-actinin antibody.

non-differentiated C2C12 cells is α-actinin-1, whereas α-actinin-2 is not detected in C2C12 cells prior to differentiation [14,15], and since α-actinin-1 is a component of actin stress fibres and focal adhesions, i.e. the sites where the GFP-Cyt fusion protein

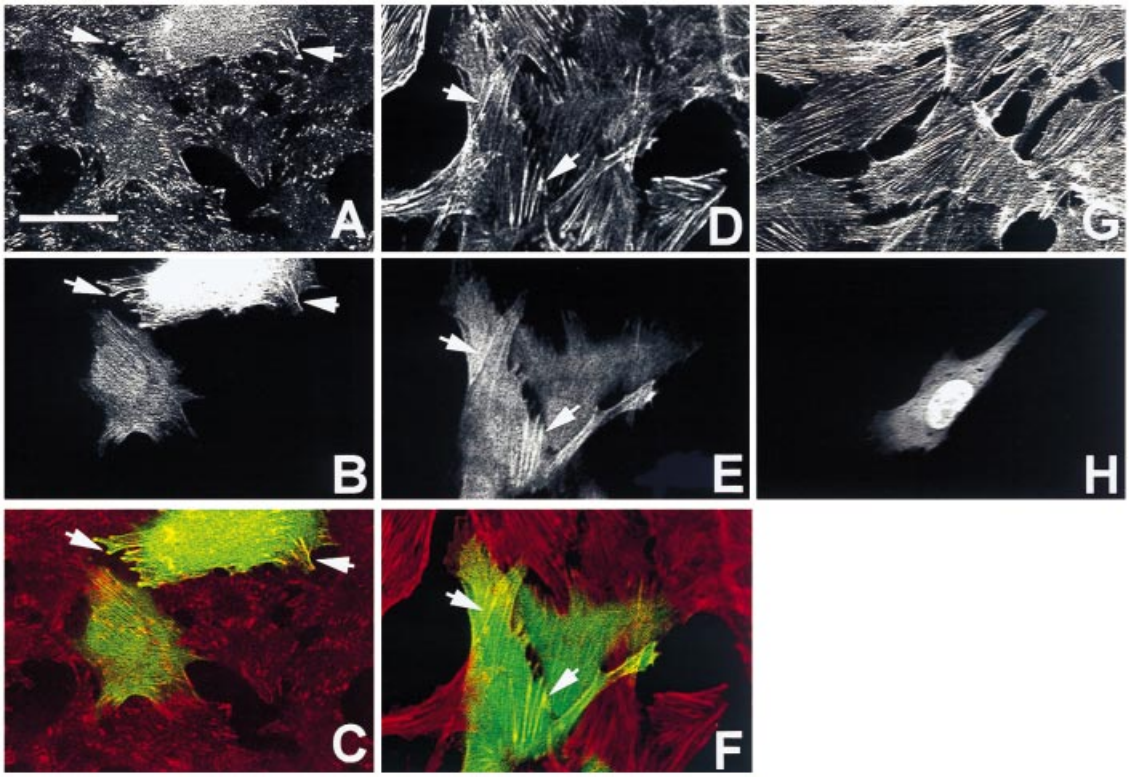

Figure 3 Subcellular localization of GFP fused to the cytoplasmic domain of ADAM 12

C2C12 cells were transfected with vectors encoding GFP fused to the Cyt fragment of ADAM 12, GFP-Cyt (**A**–**F**), or GFP alone (**G**, **H**). At 2 days after transfection, cells were fixed and immunostained with mouse anti-vinculin primary antibody and rhodamine-conjugated anti-mouse IgG secondary antibody to visualize focal adhesions (**A**), stained with rhodamine phalloidin to visualize actin stress fibres (**D**, **G**), or analysed by direct fluorescence microscopy to visualize GFP-Cyt (**B**, **E**) or GFP (**H**). (**C**) An overlay of (**A**, **B**); (**F**) an overlay of panels (**D**, **E**). Arrowheads indicate the co-localization of the GFP-Cyt protein with focal adhesions or actin stress fibres. Bar, 25 μm.

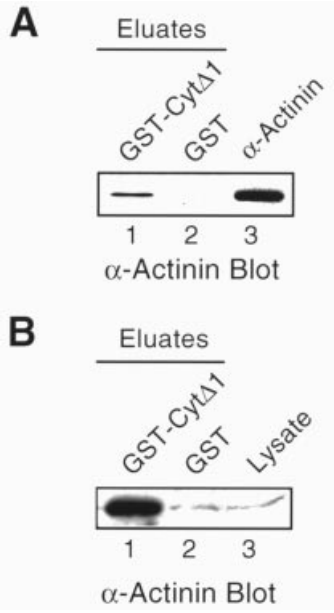

Figure 5 Interaction between ADAM 12 cytoplasmic domain and α-actinin-1 under non-denaturing conditions

(**A**) Interaction between ADAM 12 cytoplasmic domain and chicken gizzard α-actinin. Purified chicken gizzard α-actinin (500 μl, 0.1 mg/ml) was incubated with GST-CytΔ1 or GST alone immobilized on GSH columns, followed by washing the columns, eluting with SDS gel-loading buffer (200 μl), subjecting the eluate to SDS/PAGE, and Western blotting with anti-α-actinin antibody. Lanes 1 and 2 contain 20 μl of the eluates from the GST-CytΔ1 and GST columns respectively; lane 3 contains 2 μl of the α-actinin solution before loading on the columns. (**B**) Interaction between ADAM 12 cytoplasmic domain and endogenous α-actinin from C2C12 cells. Extract from C2C12 cells (10 ml) was applied on to GST-CytΔ1 or GST columns, the columns were washed and eluted with SDS gel-loading buffer (200 μl). The eluates were analysed by SDS/PAGE and Western blotting with anti-α-actinin antibody. Lanes 1 and 2 contain 20 μl of eluates from the GST-CytΔ1 and GST columns respectively; lane 3 contains 20 μl of the cell extract before loading on the columns.

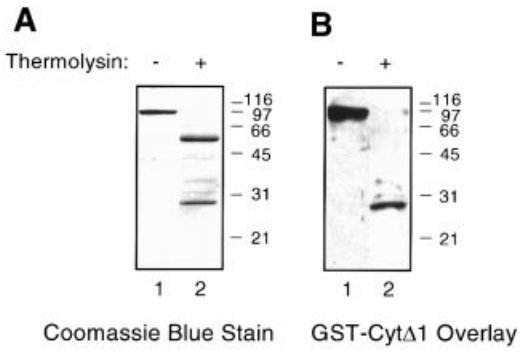

Figure 6 Localization of the ADAM 12 binding site in α-actinin-1

Purified chicken gizzard α-actinin incubated without (lane 1) or with thermolysin (lane 2) was subjected to SDS/PAGE and stained with Coomassie Blue (**A**) or transferred to a nitrocellulose membrane and incubated with GST-CytΔ1 protein (100 μg/ml). (**B**) GST-CytΔ1 bound to α-actinin was visualized by incubation of the membrane with goat anti-GST antibody and then with HRP-conjugated anti-goat IgG antibody.

was concentrating in non-differentiated C2C12 cells (Figure 3), we examined whether α-actinin-1 could directly interact with the cytoplasmic domain of ADAM 12. First, different regions of ADAM 12 cytoplasmic domain were expressed in *E. coli* as GST-fusion proteins, purified, and subjected to a blot overlay assay with α-actinin purified from chicken gizzard. This preparation of α-actinin does not contain α-actinin-2 (as skeletal muscle α-actinins are not expressed in smooth muscle tissue) and is mainly composed of α-actinin-1 [16]. The CytΔ2 and CytΔ4 constructs were non-overlapping fragments of ADAM 12 corresponding to amino acids 814–903 and 743–813 respectively; CytΔ3 construct contained the C-terminal 58 amino acids of ADAM 12 (residues 846–903; Figure 1). Although all the recombinant proteins were successfully expressed in *E. coli* and purified as soluble proteins, we consistently observed significant degradation of GST-CytΔ1, GST-CytΔ2 and GST-CytΔ4 proteins that occurred prior to their purification. Nevertheless, the position of the major bands observed in gels after protein purification corresponded well with the predicted molecular masses of the recombinant proteins (Figure 4A, see the Experimental section for an estimation of the molecular masses).

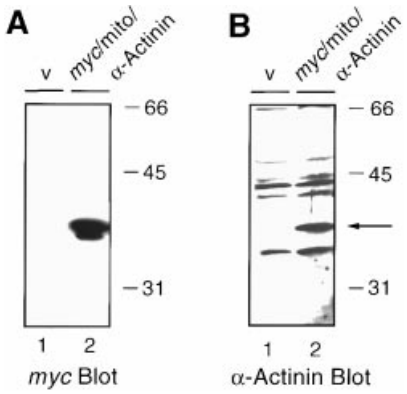

Figure 7 Expression of the 27 kDa N-terminal fragment of mouse α-actinin-1 in C2C12 cells using a mitochondrial targeting system

C2C12 cells were transfected with an expression vector encoding the 27 kDa domain of mouse α-actinin-1, containing an N-terminal mitochondrial targeting sequence and a C-terminal *c*-Myc tag (lane 2) or the same vector without the α-actinin-1 insert (lane 1). Expression of the recombinant protein was analysed 2 days later by subjecting the extracts from transfected cells to SDS/PAGE and Western blotting with anti-Myc antibody (**A**) or anti-α-actinin antibody (**B**).

Binding of chicken gizzard α-actinin to the GST fusion proteins depended on the ionic strength of the buffer. Under the conditions most commonly used by other researchers, i.e., in the presence of 50 mM NaCl, all four recombinant proteins, including GST fusions of non-overlapping fragments CytΔ2 and CytΔ4, interacted with α-actinin. In addition, two major degradation products of GST-CytΔ1 and GST-CytΔ2, with molecular masses approx. 4 kDa smaller than the masses of the full size proteins, also bound α-actinin (Figure 4B). In contrast, in the presence of 150 mM NaCl, binding of α-actinin to the CytΔ4 construct (containing a C-terminal truncation of ADAM 12 cytoplasmic domain) was dramatically reduced, whereas binding to CytΔ1, CytΔ2 and CytΔ3 fragments was not changed (Figure 4C). This suggested that the major binding site for α-actinin-1, which may be functional under physiological ionic strength conditions, was located within the last 58 residues of ADAM 12.

Interaction between α-actinin-1 and ADAM 12 cytoplasmic domain under non-denaturing conditions

To determine whether α-actinin-1 can bind to the cytoplasmic domain of ADAM 12 under non-denaturing conditions, the GST-CytΔ1 protein was immobilized on a GSH–Sepharose

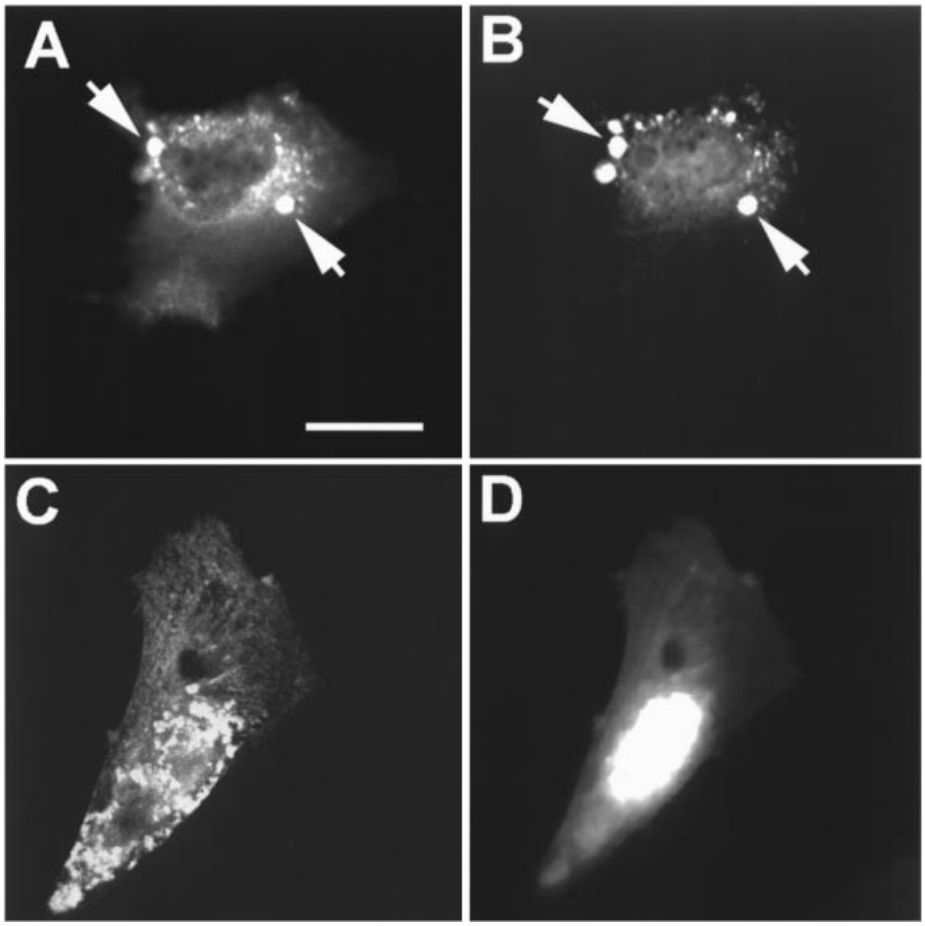

Figure 8 Recruitment of ADAM 12 cytoplasmic domain to the mitochondrial surface by mitochondria-targeted 27 kDa N-terminal domain of α-actinin-1

C2C12 cells were co-transfected with a vector encoding the 27 kDa N-terminal domain of mouse α-actinin-1, containing an N-terminal mitochondrial-targeting sequence and a C-terminal *c*-Myc tag, and GFP-Cyt (**A**, **B**) or GFP (**C**, **D**). Cells were stained with mouse anti-Myc antibody and rhodamine-conjugated anti-mouse IgG antibody to visualize the recombinant α-actinin-1 fragment (**A**, **C**) or analysed by direct fluorescence microscopy to visualize GFP-Cyt (**B**) and GFP (**D**). Arrowheads indicate the sites of co-localization of GFP-Cyt and mitochondria-targeted fragment of α-actinin-1. Bar, 25 μm.

column and incubated either with purified chicken gizzard α-actinin or with the extract from C2C12 cells containing endogenous mouse α-actinin. As shown in Figure 5(A), chicken gizzard α-actinin bound to GST-CytΔ1 immobilized on a column, but not to GST alone, suggesting that the two proteins directly interacted under non-denaturing conditions. Moreover, endogenous α-actinin from C2C12 myoblasts, represented mainly by α-actinin-1 [14,15], was retained much more efficiently on a GST-CytΔ1 affinity column than on a GST column (Figure 5B), consistent with an interaction between ADAM 12 and mouse α-actinin-1 *in vitro*.

Identification of the ADAM 12-binding site in α-actinin-1

To map the binding site for ADAM 12, α-actinin was cleaved with thermolysin into two well-characterized fragments of 27 kDa and 53 kDa. The 27 kDa N-terminal fragment has been previously shown to interact with actin [13], zyxin [17,18] and cysteine-rich protein 1 [19], whereas the 53 kDa fragment bound to β1 integrin [20], L-selectin [21], *N*-methyl-D-aspartate receptor [22], Rho effector kinase PKN [23] and actinin-associated LIM protein [24]. In the blot overlay assay, GST fusion protein harbouring the CytΔ1 fragment of ADAM 12 bound to the 27 kDa fragment of α-actinin and it did not bind to the 53 kDa fragment (Figure 6).

α-Actinin-1-dependent recruitment of ADAM 12 in living cells

To assess whether the interaction between ADAM 12 cytoplasmic domain and α-actinin-1 might be responsible for localization of GFP-CytΔ1 fusion protein to the cytoskeleton, we have targeted the 27 kDa ADAM 12-binding domain of α-actinin-1 to ectopic sites that did not contain endogenous α-actinin and investigated whether the GFP-Cyt protein was recruited to the same sites. C2C12 cells were co-transfected with GFP-Cyt and the 27 kDa N-terminal fragment of mouse α-actinin-1 containing an N-terminal mitochondrial targeting sequence and a C-terminal *c*-Myc tag. Western blotting of the extracts from transfected cells using anti-Myc or anti-α-actinin antibodies confirmed expression of an intact form of the recombinant protein of approx. 37 kDa (predicted molecular mass of 35 kDa) (Figure 7). Double staining of α-actinin-1-transfected cells with anti-c-Myc antibody and MitoTracker Red CMXRos, a mitochondria-specific dye, demonstrated that the recombinant α-actinin-1 fragment co-localized

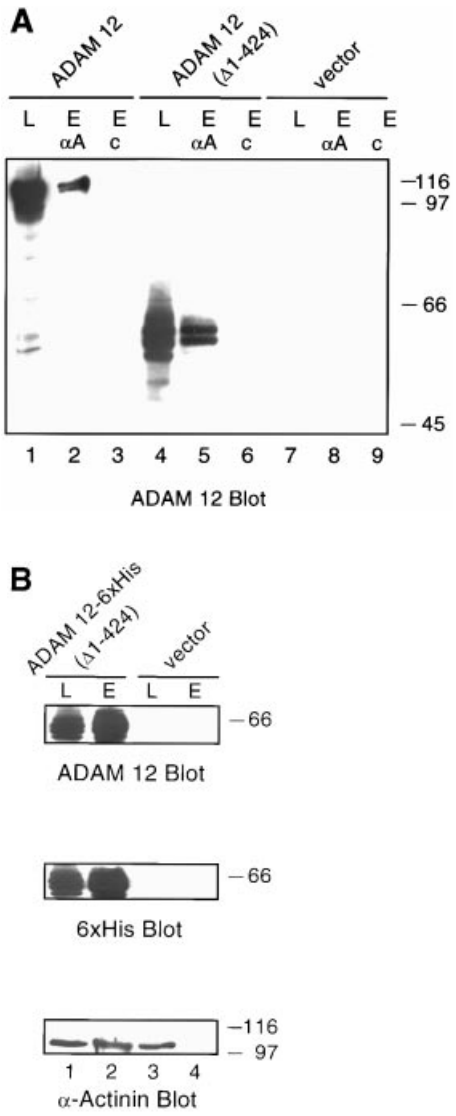

Figure 9 Interaction of a transmembrane form of ADAM 12 with α-actinin-1 *in vitro* and *in vivo*

(**A**) COS-7 cells were transiently transfected with an expression vector encoding the full size ADAM 12 (lanes 1–3), ADAM 12 (Δ1–424) (lanes 4–6), or with a vector without insert (lanes 7–9). Cell lysates (4 ml) were loaded on affinity columns containing α-actinin covalently attached to Sepharose beads or control Sepharose columns without α-actinin, followed by washing the columns and elution with SDS gel-loading buffer (600 μl). The cell lysates (lanes 1, 4 and 7; 40 μl each), the eluate from α-actinin columns (lanes 2, 5 and 8; 30 μl each), and the eluates from control columns (lanes 3, 6 and 9; 30 μl each) were analysed by SDS/PAGE and Western blotting with anti-ADAM 12 antibody. (**B**) C2C12 cells were transfected with a vector containing ADAM 12 (Δ1–424) with a His_6-tag at the C-terminus (lanes 1, 2) or vector only (lanes 3, 4). At 40 h after transfection, cell lysates (10 ml) were applied on Ni^{2+}-affinity columns, followed by washing the columns, eluting with imidazole-containing elution buffer (300 μl), and subjecting the eluates to SDS/PAGE and Western blotting with anti-ADAM 12 antibody (upper panel), anti-His_6-tag antibody (middle panel), or anti-α-actinin antibody (lower panel). Lanes 1 and 3 contain 20 μl of the cell lysates, lanes 2 and 4 contain 30 μl of the eluates.

with mitochondria (results not shown). Mitochondria of the transfected cells tended to form extensive clusters, a phenomenon that was observed before by other investigators [18]. As shown in Figure 8, co-transfection of C2C12 cells with the mitochondria-targeted α-actinin-1 resulted in the recruitment of GFP-Cyt, but not GFP alone, to the mitochondrial surface. This was consistent with the interaction between the 27 kDa domain of α-actinin-1 and ADAM 12 cytoplasmic domain *in vivo* and the ability of α-actinin-1 to target ADAM 12 to specific cellular locations.

Interaction between a transmembrane form of ADAM 12 and α-actinin-1 *in vitro* and *in vivo*

To determine whether a transmembrane ADAM 12 can interact with α-actinin-1, we have transfected COS-7 cells with an expression vector encoding either the full size ADAM 12 or ADAM 12 (Δ1–424) fragment extending from the disintegrin to the cytoplasmic domain and containing an exogenous secretion signal (see the Experimental section). ADAM 12 (Δ1–424) construct (residues 425–903; Figure 1) lacked the pro- and metalloprotease-domains, but contained the disintegrin-like/cysteine-rich region that had been previously shown to mediate cell–cell adhesion [9,10] and corresponded to a form of ADAM 12 that stimulated myoblast differentiation and fusion [4]. Both ADAM 12 and ADAM 12 (Δ1–424) gave rise to several protein bands in Western blots that ranged from 105 to 120 kDa for ADAM 12 (predicted molecular mass of 95 kDa) and from 55 to 70 kDa for ADAM 12 (Δ1–424) (predicted molecular mass of 52 kDa; Figure 9A), which might have been a result of different protein glycosylation. Chicken gizzard α-actinin was covalently attached to CNBr-activated agarose and incubated with extracts from the transfected cells. As shown in Figure 9(A), both the full size ADAM 12 and ADAM 12 (Δ1–424) were retained on the α-actinin, but not on the control columns, indicating that the transmembrane forms of ADAM 12 were capable of binding to α-actinin-1 *in vitro*.

To determine whether a transmembrane form of ADAM 12 can bind to α-actinin-1 in intact muscle cells, we expressed ADAM 12 (Δ1–424) containing a C-terminal His_6-tag in C2C12 cells, and examined whether the endogenous α-actinin, represented mainly by α-actinin-1 [14,15], co-purified with the His_6-tagged ADAM 12 (Δ1–424) protein on a Ni^{2+}-affinity resin. Recombinant ADAM 12 (Δ1–424) was detected in C2C12 cell lysates by immunoblotting using anti-ADAM 12 or anti-His_6-tag antibody (Figure 9B). α-Actinin from ADAM 12 (Δ1–424)-transfected cells, but not from mock-transfected cells, was retained on a Ni^{2+}-affinity column together with ADAM 12 (Δ1–424) (Figure 9B), suggesting that the two proteins formed a complex *in vivo*. The amount of α-actinin that co-purified with ADAM 12 (Δ1–424) constituted only a small fraction ($< 1\%$) of the total amount of α-actinin present in cells. This is consistent with the fact that most of the cellular α-actinin that is associated with cytoskeleton resides within the cell body, with only a minor amount located in the sub-membrane compartment.

DISCUSSION

The present study reports a direct interaction between ADAM 12 and α-actinin-1. The cytoplasmic domain of mouse ADAM 12 bound to chicken gizzard α-actinin (composed mainly of α-actinin-1 and devoid of α-actinin-2 [16]) and mediated an interaction between a transmembrane form of ADAM 12 and endogenous α-actinin (i.e. α-actinin-1 [14,15]) in intact C2C12 cells. In myoblasts, the cytoplasmic domain of ADAM 12 localized to α-actinin-1-rich sites such as actin stress fibres and, to a lesser extent, focal adhesions. Importantly, targeting of α-actinin-1 to ectopic sites in C2C12 cells resulted in the recruitment of ADAM 12 cytoplasmic domain to the same sites, providing strong evidence that the two proteins interacted directly *in vivo*.

Recently [12], the cytoplasmic domain of ADAM 12 has been found to interact with a skeletal muscle-specific isoform of α-actinin, α-actinin-2. While α-actinin-1 and -2 are closely related

(approx. 80 % identity), they appear to have distinct functions and do not co-localize *in situ* [14,15]. This suggests that α-actinin-1 and -2 engage in isoform-specific protein–protein interactions, although they both bind to ADAM 12. Since ADAM 12 promotes myoblast differentiation and/or fusion and since α-actinin-2 is expressed after cell fusion, whereas α-actinin-1 persists throughout the differentiation [14], the interaction of ADAM 12 with α-actinin-1 reported in the present study may be of particular relevance for the understanding of ADAM 12 function in myogenesis.

Although several proteins have been shown to bind directly to α-actinin, a consensus α-actinin-binding sequence is not known. Actinin-associated LIM proteins interact with α-actinin through a PDZ motif [24]. Association of α-actinin with phosphoinositide 3-kinase is mediated through a proline-rich region in the enzyme [25]. For the transmembrane receptors, the presence of clusters of cationic residues seems to be the only common feature of the α-actinin-binding sites [26]. We have mapped the major site of interaction with α-actinin-1 to the C-terminal 58-amino acid fragment of ADAM 12. This fragment comprises two proline-rich regions (residues 847–858 and 882–889), and these are possible candidates for the α-actinin-1-binding sites in ADAM 12. Interestingly, α-actinin-2 seemed to bind to two different sites in ADAM 12 cytoplasmic domain: one site located in the 28-amino acid membrane proximal region and another one in the C-terminal 133-amino acid fragment of ADAM 12 [12]. The former region contains a cluster of positively charged residues (amino acids 729–742) that has been postulated to constitute the major α-actinin-2-binding site. This cluster was not included, however, in the GST or GFP fusion proteins used in the studies presented here. Specifically, it was not present in the GST-CytΔ4 construct also lacking the major site of interaction with α-actinin-1 located in the C-terminal 58-amino acid region (CytΔ3) of ADAM 12 (Figure 1). Nevertheless, GST-CytΔ4 bound to α-actinin-1, although less strongly than GST-CytΔ3, suggesting that the cluster of positively charged residues located in the vicinity of the membrane was not required for the interaction with α-actinin-1.

The second α-actinin-2-binding site located at the C-terminus of ADAM 12 seemed to require the entire 133-amino acid sequence to maintain its interaction with α-actinin-2 [12]. This was supported by the observation that while the intact 133-amino acid fragment (residues 771–903) interacted with α-actinin-2 in the yeast two-hybrid assay, the 75-amino acid (residues 771–845) and the 58-amino acid (residues 846–903) non-overlapping fragments failed to support this interaction [12]. In the present report, we demonstrate that the C-terminal 58-amino acid fragment of ADAM 12 is sufficient for interaction with α-actinin-1.

In summary, it appears that the interaction between ADAM 12 and α-actinin-1 and -2 is complex and may involve multiple sites in ADAM 12, each of which may have different binding specificities for individual α-actinin isoforms, and even different biological functions. Clearly, more studies are needed to identify the exact positions of the residues in ADAM 12 that participate in α-actinin-1 and -2 binding and to determine the interplay between the individual binding sites.

α-Actinin is a rod-shaped antiparallel homodimer (a subunit molecular mass of approx. 100 kDa). Each α-actinin monomer contains an N-terminal globular domain, four spectrin-like repeats that mediate dimerization, and a C-terminal region containing two EF-hand motifs [13]. It has been postulated that the interaction with ADAM 12 occurred in a region that was located between the third spectrin-like repeat and the C-terminus of α-actinin-2 [12]. This prediction was based on the sequences of the α-actinin-2 clones that bound to the cytoplasmic domain of ADAM 12 in yeast two-hybrid screen. Contrary to the above prediction, we have mapped the site of interaction with ADAM 12 by direct biochemical analysis to the 27 kDa N-terminal domain of α-actinin-1 (Figure 6). Moreover, expression of this domain in C2C12 cells using a mitochondrial targeting system resulted in the recruitment of ADAM 12 cytoplasmic domain to the mitochondria, which further supported the localization of the ADAM 12 binding site within the 27 kDa N-terminal domain of α-actinin-1. Our results suggest that α-actinin-1 and -2 may utilize different motifs to interact with ADAM 12. Interestingly, the 27 kDa N-terminal domain of α-actinin-1 that binds to ADAM 12 has been previously known to mediate interactions with the actin cytoskeleton, rather than provide linkages with transmembrane proteins. The 27 kDa N-terminal domain binds to F-actin and cytoskeletal proteins zyxin [17,18] and cysteine-rich protein 1 [19]. The three transmembrane receptors that interact with α-actinin and for which the binding sites in α-actinin have been identified, i.e. integrin β1 [20], L-selectin [21], and *N*-methyl-D-aspartate receptor [22], associate with the central spectrin-like repeats of α-actinin.

A number of other cell-adhesion receptors have been previously documented to interact with α-actinin-1 and actin-based cytoskeleton. These receptor-cytoskeletal linkages have been often critical for receptor clustering in the plane of the plasma membrane, modulation of ligand affinity, or signalling events triggered inside the cell [27–29]. The interaction between ADAM 12 and α-actinin-1 points to a potential role for the cytoskeleton in ADAM 12-mediated cell–cell adhesion or cell signalling during myoblast differentiation and/or fusion.

We thank Dr Daniel L. Boyle, Microscopy and Image Processing Facility at Kansas State University, for assistance with confocal microscopy. This work was supported by National Institutes of Health Grant AR45787. This is contribution 00-292-J from the Kansas Agricultural Experiment Station.

REFERENCES

1 Schlöndorff, J. and Blobel, C. P. (1999) Metalloprotease-disintegrins: modular proteins capable of promoting cell-cell interactions and triggering signals by protein-ectodomain shedding. J. Cell Sci. **112**, 3603–3617
2 Black, R. A. and White, J. M. (1998) ADAMs: focus on the protease domain. Curr. Opin. Cell Biol. **10**, 654–659
3 Wolfsberg, T. G. and White, J. M. (1996) ADAMs in fertilization and development. Dev. Biol. **180**, 389–401
4 Yagami-Hiromasa, T., Sato, T., Kurisaki, T., Kamijo, K., Nabeshima, Y. and Fujisawa-Sehara, A. (1995) A metalloprotease-disintegrin participating in myoblast fusion. Nature (London) **377**, 652–656
5 Kurisaki, T., Masuda, A., Osumi, N., Nabeshima, Y. and Fujisawa-Sehara, A. (1998) Spatially- and temporally-restricted expression of meltrin α (ADAM12) and β (ADAM19) in mouse embryo. Mech. Dev. **73**, 211–215
6 Gilpin, B. J., Loechel, F., Mattei, M.-G., Engvall, E., Albrechtsen, R. and Wewer, U. (1998) A novel, secreted form of human ADAM 12 (meltrin α) provokes myogenesis *in vivo*. J. Biol. Chem. **273**, 157–166
7 Inoue, D., Reid, M., Lum, L., Krätzschmar, J., Weskamp, G., Myung, Y. M., Baron, R. and Blobel, C. P. (1998) Cloning and initial characterization of mouse meltrin β and analysis of the expression of four metalloprotease-disintegrins in bone cells. J. Biol. Chem. **273**, 4180–4187
8 Abe, E., Mocharla, H., Yamate, T., Taguchi, Y. and Manolagas, S. C. (1999) Meltrin-α, fusion protein involved in multinucleated giant cell and osteoclast formation. Calcif. Tissue Int. **64**, 508–515
9 Iba, K., Albrechtsen, R., Gilpin, B. J., Loechel, F. and Wewer, U. (1999) Cysteine-rich domain of human ADAM 12 (meltrin α) supports tumor cell adhesion. Am. J. Pathol. **154**, 1489–1501
10 Zolkiewska, A. (1999) Disintegrin-like/cysteine-rich region of ADAM 12 is an active cell adhesion domain. Exp. Cell Res. **252**, 423–431
11 Loechel, F., Gilpin, B. J., Engvall, E., Albrechtsen, R. and Wewer, U. M. (1998) Human ADAM 12 (Meltrin α) is an active metalloprotease. J. Biol. Chem. **273**, 16993–16997

12 Galliano, M. F., Huet, C., Frygelius, J., Polgren, A., Wewer, U. M. and Engvall, E. (2000) Binding of ADAM 12, a marker of skeletal muscle regeneration, to the muscle-specific actin-binding protein, α-actinin-2, is required for myoblast fusion. J. Biol. Chem. **275**, 13933–13939
13 Critchley, D. R. and Flood, G. (1999). α-Actinins. In Guidebook to the Cytoskeletal and Motor Proteins (Kreis, T. and Vale, R., eds.), 2nd edn., pp. 24–27, Oxford University Press, Oxford
14 Endo, T. and Masaki, T. (1984) Differential expression and distribution of chicken skeletal- and smooth-muscle-type α-actinins during myogenesis in culture. J. Cell Biol. **99**, 2322–2332
15 Tokuue, Y., Goto, S., Imamura, M., Obinata, T., Masaki, T. and Endo, T. (1991) Transfection of chicken skeletal muscle α-actinin cDNA into non-muscle and myogenic cells: Dimerization is not essential for α-actinin to bind to microfilaments. Exp. Cell Res. **197**, 158–167
16 Arimura, C., Suzuki, T., Yanagisawa, M., Imamura, M., Hamada, Y. and Masaki, T. (1988) Primary structure of chicken skeletal muscle and fibroblast α-actinins deduced from cDNA sequences. Eur. J. Biochem. **177**, 649–655
17 Crawford, A. W., Michelsen, J. W. and Beckerle, M. C. (1992) An interaction between zyxin and α-actinin. J. Cell Biol. **116**, 1381–1393
18 Reinhard, M., Zumbrunn, J., Jaquemar, D., Kuhn, M., Walter, U. and Treub, B. (1999) An α-actinin binding site of zyxin is essential for subcellular zyxin localization and α-actinin recruitment. J. Biol. Chem. **274**, 13410–13418
19 Pomies, P., Louis, H. A. and Beckerle, M. C. (1997) CRP1, a LIM domain protein implicated in muscle differentiation, interacts with α-actinin. J. Cell Biol. **139**, 157–168

Received 28 February 2001/24 April 2001; accepted 17 May 2001

20 Otey, C. A., Pavalko, F. M. and Burridge, K. (1990) An interaction between α-actinin and the β1 integrin subunit *in vitro*. J. Cell Biol. **111**, 721–729
21 Pavalko, F. M., Walker, D. M., Graham, L., Goheen, M., Doerschuk, C. M. and Kansas, G. S. (1995) The cytoplasmic domain of L-selectin interacts with cytoskeletal proteins via α-actinin: receptor positioning in microvilli does not require interaction with α-actinin. J. Cell Biol. **129**, 1155–1164
22 Wyszynski, M., Lin, J., Rao, A., Nigh, E., Beggs, A. H., Craig, A. M. and Sheng, M. (1997) Competitive binding of α-actinin and calmodulin to the NMDA receptor. Nature (London) **385**, 439–442
23 Mukai, H., Toshimori, M., Shibata, H., Takanaga, H., Kitagawa, M., Miyahara, M., Shimakawa, M. and Ono, Y. (1997) Interaction of PKN with α-actinin. J. Biol. Chem. **272**, 4740–4746
24 Xia, H., Winokur, S. T., Kuo, W. L., Altherr, M. R. and Bredt, D. S. (1997) Actinin-associated LIM protein: identification of a domain interaction between PDZ and spectrin-like repeat motifs. J. Cell Biol. **139**, 507–515
25 Shibasaki, F., Fukami, K., Fukui, Y. and Takenawa, T. (1994) Phosphatidylinositol 3-kinase binds to α-actinin through the p85 subunit. Biochem. J. **302**, 551–557
26 Heiska, L., Kantor, C., Parr, T., Critchley, D. R., Vilja, P., Gahmberg, C. G. and Carpén, O. (1996) Binding of the cytoplasmic domain of intercellular adhesion molecule-2 (ICAM-2) to α-actinin. J. Biol. Chem. **271**, 26214–26219
27 Burridge, K. and Chrzanowska-Wodnicka, M. (1996) Focal adhesions, contractility, and signaling. Annu. Rev. Cell Dev. Biol. **12**, 463–518
28 Hall, A. (1998) Rho GTPases and the actin cytoskeleton. Science (Washington, D.C.) **279**, 509–514
29 Kusumi, A., Suzuki, K. and Koyasako, K. (1999) Mobility and cytoskeletal interactions of cell adhesion receptors. Curr. Opin. Cell Biol. **11**, 582–590