Def-6, a Guanine Nucleotide Exchange Factor for Rac1, Interacts with the Skeletal Muscle Integrin Chain α7A and Influences Myoblast Differentiation*

Thomas Samson[‡§1], **Carola Will**[§], **Alexander Knoblauch**[§], **Lisa Sharek**[‡], **Klaus von der Mark**[¶], **Keith Burridge**[‡2], **and Viktor Wixler**[§2]

From the ‡*Department of Cell and Developmental Biology, Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599 , the* §*Institut für Molekulare Virologie, Zentrum für Molekularbiologie der Entzündung, Universitätsklinikum Münster, 48149 Münster, Germany, and the* ¶*Experimentelle Medizin I, Nikolaus-Fiebiger Zentrum, Universität Erlangen-Nürnberg, 91054 Erlangen, Germany*

Integrin α7β1 is the major laminin binding integrin receptor of muscle cells. The α7 chain occurs in several splice isoforms, of which α7A and α7B differ in their intracellular domains only. The fact that the expression of α7A and α7B is tightly regulated during skeletal muscle development suggests different and distinct roles for both isoforms. However, so far, functional properties and interacting proteins were described for the α7B chain only. Using a yeast two-hybrid screen, we have found that Def-6, a guanine nucleotide exchange factor for Rac1, binds to the intracellular domain of the α7A subunit. The specificity of the Def-6-α7A interaction has been shown by direct yeast two-hybrid binding assays and coprecipitation experiments. This is the first description of an α7A-specific and -exclusive interaction, because Def-6 did not bind to any other tested integrin cytoplasmic domain. Interestingly, the binding of Def-6 to α7A was abolished, when cells were cotransfected with an Src-related kinase, which is known to phosphorylate Def-6 and stimulate its exchange activity. We found expression of Def-6 was not only restricted to T-lymphocytes as described thus far but in a more widespread manner, including different muscle tissues. In cells, Def-6 is seen in newly forming cell protrusions and focal adhesions, and its localization partially overlaps with the α7A integrin receptor. C2C12 myoblasts overexpressing Def-6 show a delay of Rac1 inactivation during myogenic differentiation and abnormal myotube formation. Thus, our data suggest a role for Def-6 in the fine regulation of Rac1 during myogenesis with the integrin α7A chain guiding this regulation in a spatio-temporal manner.

Integrins are heterodimeric cell surface receptors that bind to components of the surrounding extracellular matrix (ECM)[3] or to other cell surface molecules. They are composed of two non-covalently linked transmembrane polypeptides (α- and β-chain). So far 18 α- and 8 β-chains have been described, which form at least 24 integrin receptors with different substrate-binding specificities determined by the individual combination of α- and β-chains. Besides forming a mechanical link between the ECM and the cytoskeleton, integrins regulate a broad variety of signaling events that influence cellular functions such as proliferation, cell differentiation, and apoptosis (1). Consequently, the cytoplasmic domains of integrin receptors play key roles in the transduction of "outside-in" as well as "inside-out" signals (2–6). They have been found to interact with an array of proteins that are part of signaling cascades or that provide linkage to the actin cytoskeleton (7).

Integrin α7β1 is mainly expressed in muscle tissues (8, 9), and the receptor exclusively binds to laminins (10). Muscle precursor cells (myoblasts) use the α7β1 receptor to adhere to and migrate on laminin (11), and laminin-containing ECM increases the differentiation potential of myoblasts (12). The importance of this kind of cell-matrix linkage in muscles becomes obvious when one considers the phenotypes of mouse null mutants, which lack either the major laminin isoform in muscles (laminin α2 chain as part of laminin-211)[4] (13) or the integrin α7 chain (14). In both cases, the animals suffer from severe postnatal muscular dystrophy and muscle wasting.

The integrin α7 chain exists in different splice isoforms (8, 15, 16). The α7X1 and α7X2 splice variants differ in the extracellular putative β-propeller domain, a region between the homology repeats III and IV (17). This leads to different binding abilities of the receptor for certain laminin isoforms (10). However, the physiological relevance of these diverse binding affinities is not known.

The splice isoforms α7A and α7B have different amino acid sequences within the intracellular tail. Interestingly, the expression of α7A and α7B during muscle development and regeneration is tightly regulated, and this regulation pattern is highly conserved among different mammalian species (15, 18, 19).

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[1] To whom correspondence should be addressed: Dept. of Cell and Developmental Biology, Lineberger Comprehensive Cancer Center, CB#7295, University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-966-5783; Fax: 919-966-3015; E-mail: thomas_samson@med.unc.edu.

[2] Both authors contributed equally to this work.

[3] The abbreviations used are: ECM, extracellular matrix; GEF, guanine nucleotide exchange factor; RT, reverse transcription; DBD, DNA-binding domain; AD, activation domain; GST, glutathione *S*-transferase; DHL, *dbl*-homology-like.

[4] Nomenclature of laminin isoforms according to Aumailley *et al.* (63).

Undifferentiated myoblasts express the α7B splice isoform only, whereas α7A expression is up-regulated after induction of myotube formation (15). Interestingly, α7 splice variants are also regulated during muscle regeneration after injury (20). All these observations strongly suggest an important function for the α7A chain during differentiation of myoblasts into myotubes. The cytoplasmic tail of the α7 chain does not influence the attachment ability of α7β1 expressing cells to laminin (21). Therefore the physiological relevance of the different intracellular parts is most likely due to different abilities of α7A and α7B to interact with intracellular proteins. Thus they might function in alternative signaling events during muscle differentiation and regeneration or provide additional mechanical linkage to the cytoskeleton.

Rho GTPases are molecular switches regulating a variety of cellular processes, like endocytosis, cell-cycle progression, differentiation, and gene transcription (22). Members of the Rho GTPase family cycle between an activated GTP-bound state and an inactivated form in which the GTP has been hydrolyzed to GDP. Guanine nucleotide exchange factors (GEFs) promote the exchange of GDP for GTP, thereby activating Rho GTPases.

Rho GTPases were shown to be strongly involved in the proper differentiation of myoblasts (23). Specifically, the tight regulation of Rac1 seems to be important for myogenesis. During normal differentiation of myoblasts the overall endogenous pool of active Rac1 significantly decreases. However, Rac1 activity is never shut off completely but remains at a basal level during differentiation (24). In fact, some steps of myogenesis seem to require locally restricted Rac1 activity (25). The mechanisms regulating these complex patterns of Rac1 activity during myogenesis are still poorly understood.

In the present study, we report a novel interaction between the cytoplasmic tail of the integrin α7A chain and Def-6 (also known as: IBP, SLAT). The Def-6-related protein Swap-70 also binds to the α7A cytoplasmic domain, albeit with a lower affinity. Def-6 and Swap-70 were recently described as a novel group of GEFs for Rho GTPases (26, 27). Their interactions with α7A are highly specific and exclusive, because no other integrin cytoplasmic domain showed binding to these proteins. Interestingly, we found that tyrosine phosphorylation of Def-6 abolishes this interaction. Our data implicate Def-6 in the tight regulation of Rac1 activity during myoblast differentiation.

EXPERIMENTAL PROCEDURES

DNA Vectors—All yeast two-hybrid pAS2-1 (Clontech) derivate constructs coding for fusion proteins of the GAL4-DNA binding domain and full-length or truncated intracellular parts of integrin subunits have been described elsewhere (28). cDNA fragments encoding the complete sequences of *Def-6* (NCBI accession number gi:142375804) and *Swap-70* (NCBI gi:40789274) or their deletion mutants were generated by RT-PCR from murine spleen RNA. The RT-PCR fragments were subsequently cloned in-frame into pACT2 vector (Clontech).

pEBG-derived expression constructs containing GST-α7A or GST-α7B and their deletion mutants for expression in mammalian cells have been described previously (28). To express Def-6 in mammalian cells, the Def-6 cDNA insert was subcloned from the pACT2 vector into pEF1-Myc/his (Invitrogen),

pCS2+MT (29), or pBabe-neo (30). pBabe-puro constructs containing the cDNA for the complete α7A or α7B chain have been already described elsewhere (28).

To generate pBabe-neo/-puro-derived retroviruses, GPE86-cells (31) were stably transfected with FuGene6 (Roche Applied Science). Supernatants from these cells were collected and used for the infection of C2C12 cells. 48 h later, selection of infected cells was started with 1 mg/ml G418 or 4 μg/ml puromycin.

Yeast Two-hybrid Analysis—The initial yeast two-hybrid screen to identify novel intracellular binding partners of the α7Aβ1 integrin receptor has been described elsewhere (28). Briefly, a human placenta cDNA library (MATCHMAKER™, Clontech) was screened for proteins that interact with the cytoplasmic part of the α7A integrin chain. The intracellular part of the murine integrin α7A subunit (amino acids 1104–1161) was cloned in the pAS2-1 vector and used as bait (amino acids positions refer to NCBI accession code gi:3378242).

For direct yeast two-hybrid analysis, the yeast strain Y190 was cotransformed with the pAS2-1 plasmid containing the GAL4-DNA binding domain (DBD) fused with appropriate cDNAs as bait and with pACT2 plasmid containing cDNAs fused to GAL4-activation domain (AD) as prey. Transformants were grown on synthetic defined medium lacking leucine, tryptophan, and histidine in the presence of 25 mM 3-amino-1,2,4-triazole. On day 6 the colonies were tested for the *lacZ* reporter gene activity in a β-galactosidase filter assay. The interaction was scored as negative (−) when no blue colonies were visible after 8 h, and scored as: weak (+), intermediate (++), or strong (+++) when blue colonies became visible after 8, 4, or 1 h, respectively. For relative quantification of the β-galactosidase activity, the transformants were first grown on synthetic defined medium lacking leucine and tryptophan and subsequently introduced into the liquid culture assay using *ortho*-nitrophenyl-β-D-galactopyranoside as substrate. The measured β-galactosidase activity within one experiment was calculated relative to a positive control, which was set as 100%.

Cell Culture—HEK293, NIH3T3, and HeLa cells were cultured in Dulbecco's modified Eagle's medium-high glucose supplemented with 10% fetal bovine serum. C2C12 myoblasts were cultured at low densities in growth medium: 1:1 mixture of Dulbecco's modified Eagle's medium-low glucose and F-12/Ham medium, supplemented with 20% fetal calf serum. To induce differentiation, growth medium was replaced by differentiation medium (Dulbecco's modified Eagle's medium-high glucose, 2% horse serum). All cells were grown in a 37 °C and 5% CO_2 incubator.

Antibodies—The following antibodies were used: mouse-anti-Myc, clone 9E10 (32), rabbit-anti-GST, and anti-slow skeletal myosin monoclonal antibody (NOQ7.5.4D, Sigma), mouse-anti-sarcomere myosin (clone MF-20, Developmental Studies Hybridoma Bank, University of Iowa), mouse-anti-phosphotyrosine (clone PY99, Santa Cruz Biotechnology), mouse-anti-integrin α7, clone 3C12 (recognizes the extracellular part of the receptor) (21, 33), rabbit-anti-integrin α7A (a gift of Ulrike Mayer, Manchester, UK), mouse anti-Rac1 (BD Biosciences), peroxidase-conjugated secondary antibodies for immunoblot analysis (Amersham Biosciences), and secondary fluorescent antibodies (Dianova and Molecular Probes).

Polyclonal antibodies against Def-6 were raised against a bacterially expressed GST fusion protein that contained amino acids 410–630 of murine Def-6. The final serum was precleared with GST-loaded glutathione-Sepharose beads (Amersham Biosciences). Subsequently, Def-6 antibodies were affinity-purified using GST-Def-6-amino acids 410–630-conjugated CNBr-Sepharose (Amersham Biosciences). The specificity of the purified antibodies was tested by immunoblotting cell lysates from Def-6-transfected cells *versus* non-transfected cells (Fig. 4*C*, *last two lanes*).

Immunofluorescence Staining of Cells—Cells were cultured on coverslips as indicated in the figure legends. Before staining, the cells were washed in phosphate-buffered saline, fixed for 15 min with 2% paraformaldehyde at room temperature, permeabilized with 0.2% Triton X-100 for 2 min, and subsequently blocked with 1% bovine serum albumin in phosphate-buffered saline. The cells were incubated at room temperature for 1 h with primary antibody and were detected by species-specific fluorochrome-conjugated secondary antibodies. Fluorescence images were taken with a Axioplan-2 microscope (Zeiss). Images shown in Fig. 5 (*F* and *G*) were taken with a Zeiss 510 Meta laser-scanning confocal microscope.

Determination of Myotube Fusion Index—C2C12 cells were grown and differentiated on coverslips. On day 3 after the switch to differentiation medium, cells were fixed and stained with 4′,6-diamidino-2-phenylindole and anti-sarcomere myosin antibody MF-20. Photos were taken from random fields (20× objective), and nuclei were counted per field. The fusion index was calculated as the ratio of nuclei in differentiated myotubes *versus* the total amount of nuclei per field. Myotubes were defined as MF-20-positive cells, which contain more than three nuclei. At least four fields were counted per experiment.

Coprecipitation Assays and Immunoblotting—2×10^5 HEK293 cells were plated in 6-well dishes 48 h before transfection with FuGene6 transfection reagent (Roche Applied Science). 1 μg of each cDNA plasmid was used per transfection. The total DNA amount was equalized with appropriate empty expression vectors. 30 h after transfection the cells were washed with phosphate-buffered saline and lysed in IP buffer (25 mM Tris, pH 7.5, 137 mM NaCl, 2 mM MgCl$_2$, 10% glycerol, 0.5% Triton X-100, 1 mM sodium vanadate, 5 μg/ml leupeptin, 5 μg/ml aprotinin) for 10 min. The lysates were cleared by centrifugation at 13,000 × g for 10 min at 4 °C. To reduce nonspecific binding of proteins, Sepharose beads were blocked with 1% bovine serum albumin in IP buffer before incubation with cell lysates. For GST pulldown experiments, the cleared supernatants were incubated for 1.5 h at 4 °C with GSH-Sepharose beads (Amersham Biosciences). For immune precipitations, Protein G-Sepharose beads (Amersham Biosciences) were used instead. The beads were subsequently washed twice with IP buffer, boiled in sample buffer, and separated on 10% SDS-PAGE and immunoblotted as indicated. To detect the peroxidase-conjugated secondary antibodies, an ECL detection system (Pierce) was used.

For detection of phosphorylated Def-6 protein in cell lysates, C2C12 cells were lysed in 2× SDS gel sample buffer and boiled for 5 min, to denature all proteins completely. Subsequently the samples were diluted 20-fold with phospho-IP buffer (150 mM NaCl, 20 mM Tris, pH 7.6, 1% Triton X-100, 1% sodium deoxycholate, 1 mM sodium vanadate, 5 μg/ml leupeptin, 5 μg/ml aprotinin) to a final concentration of 0.1% SDS. Tyrosine-phosphorylated proteins were then precipitated using PY-99 antibody and Protein G-Sepharose beads. The beads were washed four times with phospho-IP buffer before being analyzed by immunoblotting with anti-Def-6 polyclonal antibody.

Rac1 Activity Assay—Activity assays for Rac1 were performed as previously described (34). Briefly, the cells were lysed in lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM MgCl$_2$, 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). The lysates were cleared by centrifugation at 13,000 × g for 4 min at 4 °C. The supernatants were incubated for 30 min at 4 °C with glutathione-Sepharose beads previously loaded with 30 μg of GST-tagged PBD (Rac1/cdc42 binding domain of PAK1). Subsequently the beads were washed four times in lysis buffer, boiled in sample buffer, and separated by 15% SDS-PAGE followed by immunoblotting.

RT-PCR—For the isolation of total RNA from cells or tissues, TRIzol (Invitrogen) was used. mRNA was isolated from the total RNA using Oligotex (Qiagen) and reverse transcribed with the First-strand-cDNA synthesis Kit (Roche Applied Science). For qualitative expression analysis the High Fidelity PCR System (Roche Applied Science) was used with the following primers: Def-6, ACCATGGCCCTGCG-CAAGGAGCTG/TGGTGCTGGATCCAGTTTTTC; Itga7, CTCTACAGCTTTGATCGTGCAGC/AAACCACTGGA-CTGGTACAGCAC; and glyceraldehyde-3-phosphate dehydrogenase, ATCACTGCCACCCAGAAGAC/ATGAGGT-CCACCACCCTGTT.

RESULTS

Both Def-6 and Swap-70 Bind Specifically to the Cytoplasmic Part of the Integrin α7A Subunit—To identify novel proteins that specifically interact with the cytoplasmic part of the α7A integrin subunit, we performed a yeast two-hybrid screen. A human placenta cDNA library was screened with the complete intracellular domain of the α7A chain (amino acids 1104–1161) as bait. Out of 288 isolated clones, 7 coded for Def-6 and 2 for Swap-70. Both proteins have recently been described as GEFs for the Rho GTPase Rac1 (26, 27). Def-6 and Swap-70 differ from all other GEFs of the Dbl family in that the pleckstrin homology domain is located N-terminal to a *dbl*-homology-like (DHL) domain, which shows a low homology to the DH domain of other GEFs like Vav and Tiam1 (26, 35).

To test the specificity of the interactions, direct yeast two-hybrid analyses were carried out. Def-6 and Swap-70 were expressed in yeast Y190 cells as AD fusion proteins and tested for interaction with DBD fusions of several different integrin cytoplasmic domains (Table 1). Interestingly, with the exception of α7A, none of the 11 tested α-integrin subunits showed binding to either Def-6 or Swap-70. Also α7B, the splice counterpart of α7A chain, did not interact with these GEF proteins. According to previous reports, the nine most C-terminal amino acids of the α7B chain seem to have an inhibitory effect on the interaction with other proteins (15, 28). But even a deletion mutant of the α7B cytoplasmic tail, which lacks these nine amino acids (α7B$_{del1}$), did not bind to Def-6 or Swap-70. Fur-

TABLE 1

Specificity of interaction of Def-6 and Swap-70 with cytoplasmic domains of different integrin chains

Yeast Y190 cells were cotransformed with GAL4-DNA-BD (DBD) and GAL4-AD (AD) chimeric constructs. The interaction was evaluated using a β-galactosidase filter assay as described under "Experimental Procedures."

DBD	AD		
	Def-6	Swap-70	AD vector
α1	−	−	−
α2	−	−	−
αIIb	−	−	−
α3A	−	−	−
α3B	−	−	−
α4	−	−	−
α5	−	−	−
α6A	−	−	−
α6B	−	−	−
α7A	+ + +	+ +	−
α7B	−	−	−
α7B$_{del1}$	−	−	−
β1A	−	−	−
β1D	−	−	−
DBD vector	−	−	−

thermore, the cytoplasmic domains of neither β1A nor β1D integrin subunits interacted with these GEF proteins. Nonspecific binding of Def-6 and Swap-70 to the DBD alone could be excluded, because there was no interaction detectable between the DBD and AD-Def-6 or AD-Swap-70 (Table 1).

To test the relative binding strengths of Def-6 and Swap-70 to the α7A cytoplasmic domain, quantitative yeast two-hybrid analysis were performed. The known strong interacting protein pair p53 and SV40 large T-antigen was used as a positive control and set as 100%. According to the relative β-galactosidase activity, Def-6 bound to α7A about one order of magnitude stronger compared with that interaction between Swap-70 and α7A (Fig. 1*A*, Def-6/α7A: 232% ± 43%; Swap-70/α7A: 13.8% ± 3.6%). Again, no interaction was detected between Def-6 or Swap-70 and the DBD protein alone.

Next we tested if these interactions could occur in mammalian cells. HEK293 cells were cotransfected with plasmids coding for the expression of GST-tagged α7A- or α7B-cytoplasmic tails and Myc-tagged Def-6 or Swap-70. GST-tagged α7-cytodomains were precipitated and analyzed by immunoblotting for the coprecipitation of Myc-Def-6 or Myc-Swap-70. Def-6 coprecipitated only with GST-α7A, but not with GST-α7B$_{del1}$ or GST alone (Fig. 1*B*, *top panel*). Myc-Swap-70 showed the same coprecipitation behavior, except that the degree of binding to GST-α7A was much lower (Fig. 1*B*, *long exposure blot*). Thus the results from coprecipitation experiments correlate well with those obtained by the quantitative yeast two-hybrid data.

Taken together, these studies show that Def-6 and Swap-70 interact with the cytoplasmic domain of the integrin α7A chain, but not with any other integrin tested, including α7B and β1 chains. As the A splice isoform of the α7 integrin subunit occurs exclusively in differentiating and mature skeletal muscle cells, these results suggest a muscle specific role for this interaction.

Mapping of the Interaction-mediating Protein Domains and Amino Acid Sequences—As both Def-6 and Swap-70 bind to the α7A integrin chain, it was of interest to test whether they use identical binding sites on the α7A chain. To study this, a series of deletion mutants of the α7A cytoplasmic domain was created
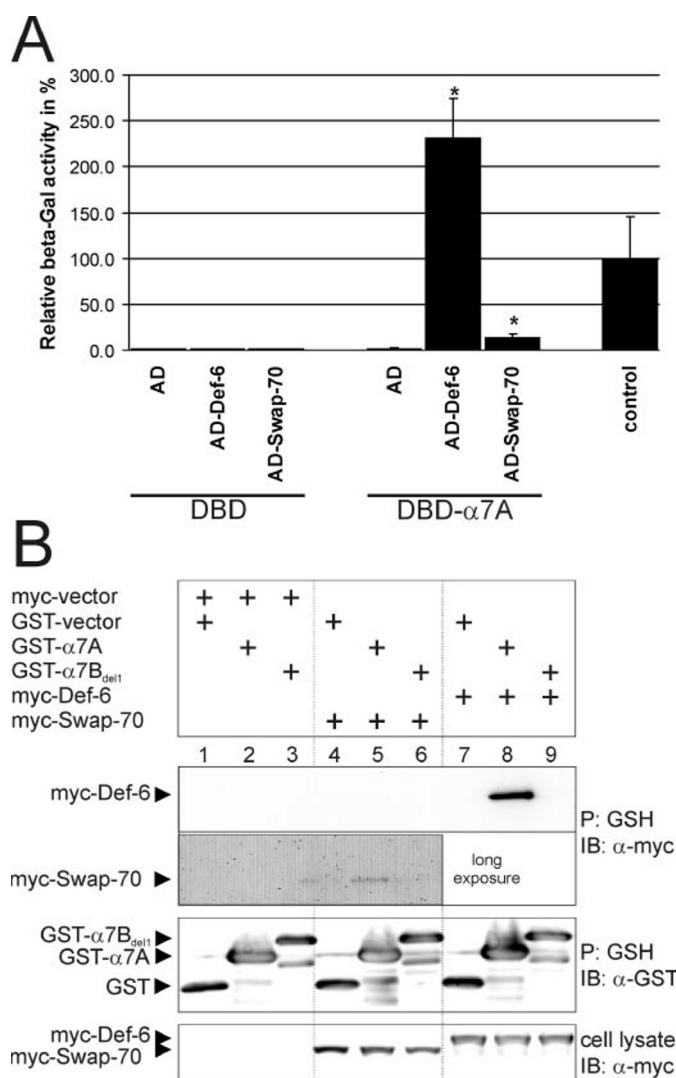
FIGURE 1. **Interaction of Def-6 and SWAP70 with the cytoplasmic domain of the integrin α7A chain.** *A*, yeast Y190 cells were transformed with GAL4-DBD and GAL4-AD chimeric constructs. Induced β-galactosidase activity was measured using a liquid *o*-nitrophenyl-β-D-galactosidase assay. Three different yeast transformants were used for each measurement. The control represents the interaction of p53 with SV40 large T-antigen, which was set as 100% of induced β-galactosidase activity. The *asterisks* indicate significant differences to corresponding controls with DBD vector only ($p < 0.05$ according to the *t* test). *B*, HEK293 cells were transiently transfected with cDNA constructs as indicated. After 40 h, GST proteins were precipitated (*P*) with glutathione-conjugated Sepharose beads (*GSH*) from cell lysates. The coprecipitated proteins were detected by immunoblots (*IB*) with monoclonal anti-Myc antibody (*upper blot*). The same blot was stripped and subsequently redeveloped with a rabbit anti-GST antibody (*middle blot*). The *lower panel* shows the expression of Myc-Def-6 and Myc-Swap-70 analyzed by immunoblotting of equal amounts of total cell lysates with anti-Myc monoclonal antibody.

and used for direct yeast two-hybrid interaction assays with Def-6 and Swap-70, respectively. As shown in Fig. 2*A* both proteins bind to the full-length α7A cytoplasmic domain and to the α7A$_{del1}$ deletion mutant, which lacks the last 10 C-terminal amino acids. Deletion of an additional 13 amino acids (α7A$_{del2}$), or more, abrogated the binding of Def-6 and Swap-70. These data indicate that both Def-6 and Swap-70 use the same amino acid motif of the α7A chain for interaction which is at least 13 amino acids (GTVGWDSSSGRST) in length.

Def-6 and Swap-70 show an identical arrangement of protein subdomains: an N-terminal EF-hand motif (EF), a central PH

FIGURE 2. **Determination of the interacting amino acid motifs and protein domains.** Yeast Y190 cells were cotransformed with DBD and AD chimeric constructs. The interaction was evaluated using a β-galactosidase filter assay as described under "Experimental Procedures." *Numbers* in the figures refers to amino acid positions in the corresponding protein. *A*, interaction of different α7A deletion mutants with Def-6 and Swap-70. The *gray box* indicates the minimal amino acid motif of the α7A chain for the binding to Def-6 and SWAP70. *B*, interaction of different deletion mutants of Def-6 and Swap-70 with the intracellular part of the integrin α7A chain.

domain, and a C-terminal DHL domain (26, 27, 35). So far, none of these subdomains have been found to bind to integrin receptors. To investigate if one of these subdomains is sufficient for interaction with α7A or whether the complete protein structure is required, a series of deletion mutants for both Def-6 and Swap-70 was generated and tested for binding ability to the α7A cytoplasmic domain in direct yeast two-hybrid assays. Interestingly, only full-length Def-6 and Swap-70 interacted with the α7A cytoplasmic domain. No single subdomain or combination of domains led to an interaction (Fig. 2B).

Taken together, these experiments show that Def-6 and Swap-70 bind to the same amino acid motif on the α7A cytoplasmic domain. Both Def-6 and Swap-70 only interacted as full-length proteins with the α7A chain, suggesting that the three-dimensional structure of the proteins influence their interaction with the α7A cytoplasmic domain. Events that change the conformation of these proteins might serve to regulate the interaction between Def-6 or Swap-70 and the α7A subunit. Because the binding specificity and features of Def-6 and Swap-70 for the integrin α7A chain were exactly the same with Def-6 binding much stronger to the α7A chain (Figs. 1 and 2), we focused our further studies on Def-6 only.

The GEF-activating Phosphorylation of Def-6 Abolishes Its Binding Capacity to α7A Cytodomain—It was previously reported that Def-6 exists in two different conformations. Normally, an intramolecular interaction keeps the protein in a closed conformation, thereby inhibiting its GEF activity. Phos-

phorylation of the tyrosine residue Y210 by a Src-related kinase and binding to cell membrane-associated phosphatidyl inositol 3,4,5-trisphosphate relieves the intramolecular interaction and activates the GEF (26). Our interaction analysis of Def-6 mutants showed that the entire protein structure is needed for binding to the α7A cytodomain. To test whether the conformational change of Def-6 induced by Y210 phosphorylation influences the interaction with the α7A intracellular domain, two Def-6 mutants were generated. The mutant Y210F cannot be phosphorylated due to the lack of a hydroxyl residue on the aromatic amino acid. In contrast, the mutant Y210E mimics phosphorylation due to the negative charge of the glutamate (phosphomimetic). These mutants were used for quantitative direct yeast two-hybrid assays. As shown in Fig. 3A, the binding of α7A to the Def-6-Y210F mutant was comparable to that of wild-type Def-6 protein (Def-6 wild type: 232% ± 43%; Y210F: 159% ± 83%). In contrast, the Def-6-Y210E mutant did not show any interaction with the α7A cytodomain (Y210E: 3.7% ± 1.0%; AD only: 1.9% ± 0.2%), suggesting that the phosphorylation of Def-6 on Y210 abolishes association with the α7A chain.

To confirm these observations in a mammalian cell system, we expressed these mutants or the wild-type Def-6 as Myc-tagged proteins along with the GST-tagged α7A or GST alone in HEK293 cells and performed pulldown assays. The presence of bound Def-6 in the GST precipitates was checked via immunoblotting (Fig. 3B). In accordance with the results obtained in the yeast two-hybrid experiments, only the Def-6 wild-type
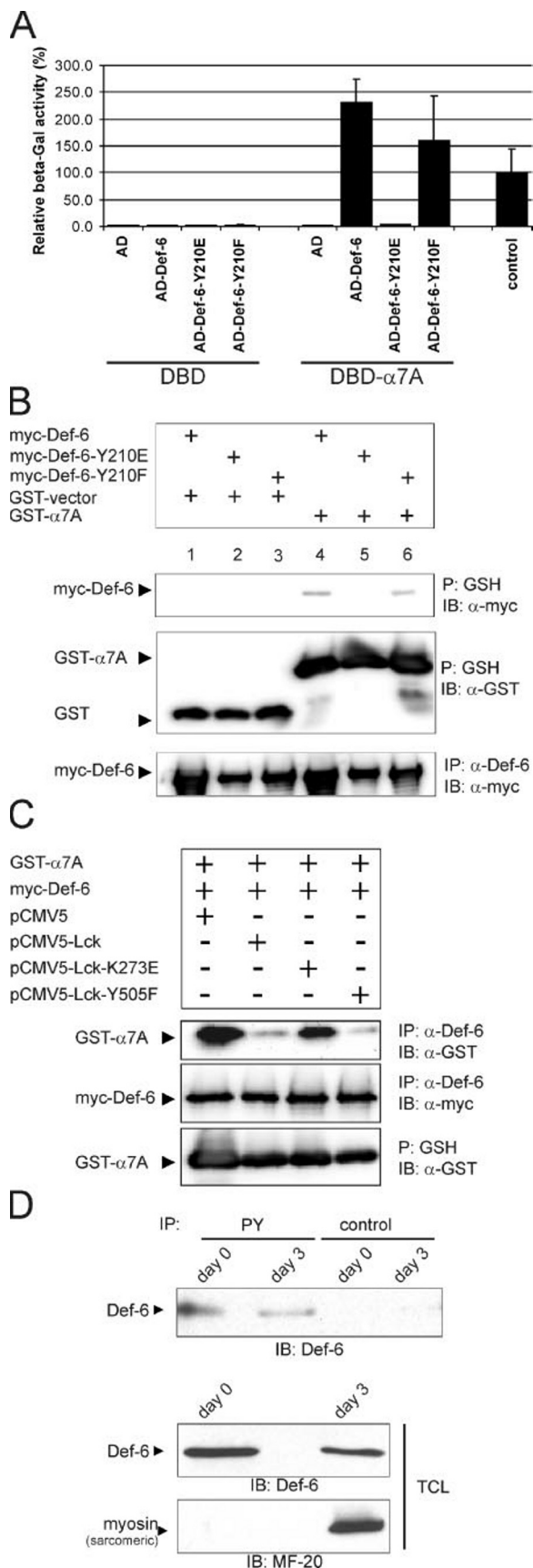
FIGURE 3. **Influence of phosphorylation of Def-6-Y210 on α7A binding.** *A*, the interaction capacities of Def-6 point mutations (Def-6-Y210F and Y210E) with the cytoplasmic domain of the integrin α7A chain were analyzed by

protein and the Def-6-Y210F mutant associated with α7A, but not the phosphomimetic Def-6-Y210E mutant.

We next tested if the tyrosine phosphorylation of the native Def-6 molecule also abolishes the binding to the α7A cytodomain. The Src-related kinase Lck has been shown to phosphorylate Def-6 on tyrosine Y210 (26). Hence, cells were transfected with plasmids coding for Myc-Def-6, GST-α7A, and Lck. Three different variants of Lck were used, wild type (Lck), dominant-negative (Lck-K273E), and constitutively active (Lck-Y505F) (36). As expected, GST-α7A could only be precipitated by Myc-Def-6 from cells that coexpressed dominant negative Lck-kinase or no Lck-kinase. In contrast, if wild-type Lck or the constitutive active variant were coexpressed, the amount of coprecipitated GST-α7A was strongly reduced (Fig. 3*C*).

In summary, the combination of direct yeast two-hybrid and coprecipitation assays suggests that Y210 phosphorylation of Def-6 abolishes its binding to the cytoplasmic domain of the α7A integrin chain. These data imply that only the non-active GEF is able to bind to α7A, because the phosphorylation of Y210 was previously shown by others to stimulate exchange activity of Def-6 (26).

Def-6 Expression in Tissues and the C2C12 Cell Line—To date, Def-6 function has mainly been analyzed in lymphocytes (26, 37–39), and no consistent data of Def-6 expression in other tissues or cell types are available. Because the α7A splice isoform is expressed exclusively in differentiating myogenic cells or in terminally differentiated skeletal myotubes (8, 19, 40), it was of interest to know whether Def-6 is endogenously expressed in these cell types. To analyze this, RT-PCRs were performed with mRNA preparations from spleen and skeletal muscle originating from an adult c57/Bl6 mouse. The spleen RNA was used as a positive control and resulted in a *Def-6* RT-PCR product of the expected size. Interestingly, a clear and specific *Def-6* PCR product was also amplified from the skeletal muscle mRNA preparation (Fig. 4*A*). In a parallel PCR reaction, the existence of α7A mRNA was checked with primers, which amplify both α7A and α7B but result in different PCR fragments of different length. As expected, α7A as well as α7B

quantitative yeast two-hybrid assays (performed as described in Fig. 1*A*). *B*, HEK293 cells were transiently transfected with cDNA constructs for GST or GST-α7A and Myc-Def-6 or its mutants (Y210F and Y210E) as indicated. GST proteins were precipitated (*P*) and coprecipitated proteins were detected in immunoblots (*IB*) with anti-Myc antibody (*upper blot*). The same blot was stripped and subsequently redeveloped with a rabbit anti-GST antibody (*middle blot*). The *lower panel* shows the expression of Myc-Def-6 (or its mutants) analyzed by immunoprecipitating (*IP*) Def-6 from equal amounts of total cell lysates and immunoblotting with anti-Myc antibody. *C*, HEK293 cells were transiently transfected with cDNA constructs for GST-α7A, Myc-Def-6 and one of three different forms of Lck: Lck wild type, Lck-K273E (dominant negative), and Lck-Y505F (constitutive active). Def-6 protein was immunoprecipitated and coprecipitated GST-proteins were detected in immunoblots with polyclonal anti-GST antibody (*upper blot*). The same blot was stripped and subsequently redeveloped with anti-Myc antibody (*middle blot*). The *lower panel* shows the expression of GST-α7A analyzed by precipitating GST-proteins from equal amounts of total cell lysates and immunoblotting with anti-GST antibody. *D*, C2C12 cultures were harvested as myoblasts and day 4 myotubes. After lysis and denaturing treatment of the lysate, immunoprecipitations were performed. Def-6 was detected when tyrosine-phosphorylated proteins were precipitated (*PY*, antibody: PY99) but not when a control antibody was used (control, antibody: 9E10). *Panels* at the *bottom* show immunoblots of total cell lysates (1:500 of input) stained for Def-6 and the myogenic differentiation marker sarcomere myosin (antibody: MF20).
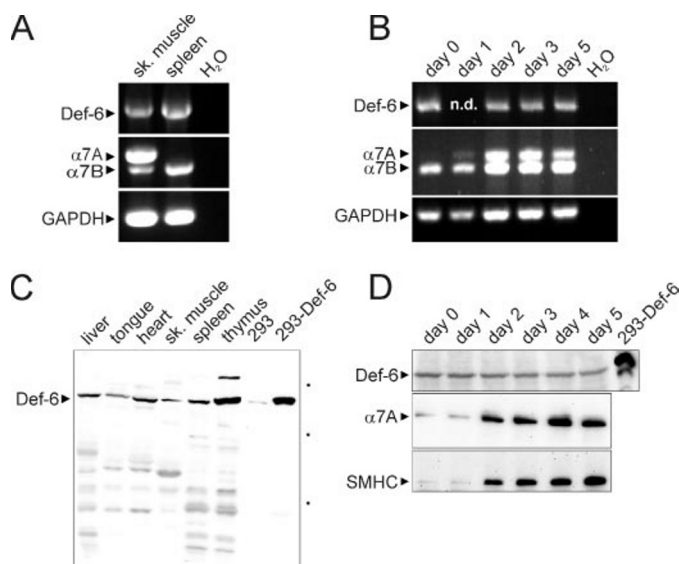
FIGURE 4. **Endogenous expression of Def-6.** Mouse tissues and C2C12 cells were analyzed for expression of Def-6. *A* and *B*, different mRNA preparations were analyzed by RT-PCR. The different primer combinations led to the amplification of specific PCR fragments of Def-6, α7 integrin chain or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The α7 primers anneal to both splice variants (α7A and α7B), resulting in different lengths of the PCR product. The C2C12 cultures were harvested at different times after the switch to differentiation medium (day 0). *C* and *D*, radioimmune precipitation assay lysates from different mouse tissues and C2C12 cells were analyzed by immunoblotting (used antibodies are indicated). HEK293 cells, which overexpressed Def-6 protein, were used as a positive control for the anti-Def-6 serum. *Dots* in *C* indicate 32, 56, and 88 kDa. 50 μg of total protein was loaded per *lane*. *n.d.*, not determined.

chains were found in muscle tissue according to previous reports (19). α7B mRNA was also found in spleen tissue, which probably originates from smooth muscle cells of blood vessels expressing only this splice variant of the integrin α7 chain (18).

To exclude that the detected Def-6 mRNA originated from peripheral blood T-cells, which may have been isolated together with muscle tissue, RT-PCR analyses were also applied on mRNA preparations from a myogenic tissue culture system. We used the myogenic cell line C2C12, which differentiates within several days after growth factor depletion to contractile myotubes (41). Def-6 mRNA was detected in proliferating myoblasts (Fig. 4*B*, day 0) as well as in differentiating myotubes (days 2–5). Sequencing of the RT-PCR products from C2C12 mRNA further confirmed the Def-6 sequence identity of the PCR product.[5] This clearly shows that Def-6 is indeed expressed in muscle cell types. Data presented in Fig. 4*B* further demonstrate that the expression of Def-6 during muscle differentiation is not regulated, like the expression of the integrin α7A subunit. The undifferentiated C2C12 myoblasts express the α7B splice isoform only, whereas the α7A chain is up-regulated after induction of differentiation (Fig. 4*B* and Ref. 18).

To extend these expression studies to the protein level, a polyclonal rabbit serum was raised against Def-6 and subsequently affinity-purified. The specificity of this serum was confirmed by immunoblotting lysates from HEK293 cells overexpressing Def-6 protein (Fig. 4*C*, *last two lanes*). Compared with

lysate from non-transfected HEK293 cells, this serum detects a strong signal at ~75 kDa, which corresponds to the molecular mass of murine Def-6 (37). Lysates of different mouse tissues were then analyzed by immunoblotting with this antibody (Fig. 4*C*). As expected, the highest expression of Def-6 protein was detected in thymus and spleen. Interestingly, Def-6 protein was also present in all organs tested, including different muscle tissues (heart, tongue, and skeletal muscle (Fig. 4*C*)). Additionally, lysates from differentiating C2C12 cells were analyzed for Def-6 protein. In accordance to the RT-PCR data, the expression of Def-6 protein was not changed during the differentiation (Fig. 4*D*). The proper differentiation of the C2C12 cells was confirmed by immunoblot analyzes of the same lysates with antibodies against the muscle-specific marker "slow myosin heavy chain" and the A splice isoform of the α7 integrin chain. Taken together, these results demonstrate that Def-6 is endogenously expressed in several mouse tissues and cells, including skeletal muscle and in the myoblast cell line C2C12.

Next we questioned if endogenous Def-6 protein in C2C12 myoblasts is phosphorylated and if this phosphorylation changes during myogenic differentiation. We performed immunoprecipitations of tyrosine-phosphorylated proteins from C2C12 cells. To avoid a potential coprecipitation instead of a direct precipitation, the immunoprecipitation was performed after a denaturing treatment of the cell lysates. Indeed, Def-6 was found to be tyrosine-phosphorylated in myoblasts and myotubes at a low level, because it was detected in the precipitates by immunoblotting of the precipitates with polyclonal Def-6 antibody (Fig. 3*D*). The relative amount of phosphorylated Def-6 changed little from myoblasts to myotubes. Specificity of the immunoprecipitation was confirmed by using the 9E10 antibody as a control, by which Def-6 was not precipitated.

Def-6 Localizes at Cell Protrusions and Focal Adhesions—To investigate the subcellular localization of Def-6, different cell types (NIH-3T3, HeLa, and C2C12) were transfected with expression constructs for Def-6 and subsequently analyzed by immunofluorescence microscopy (Fig. 5). The protein was found to localize to the edge of lamellipodial cell protrusions (Fig. 5*A*) and to focal adhesions (Fig. 5*B*).

Next we wanted to study whether the intracellular localization of Def-6 is influenced by the α7Aβ1 integrin receptor in myogenic cells. We first tried using the polyclonal anti-Def-6 antibody, but this resulted in inconclusive staining of C2C12 myoblasts.[5] Despite a well detectable band of endogenous Def-6 protein in immunoblotting analysis of C2C12 lysates (Fig. 4*D*), its expression seemed to be below the level of detection for immunofluorescence applications. To overcome this problem, Def-6 protein was stably overexpressed in C2C12 cells (Fig. 5*C*, *left panel*). Because undifferentiated myoblasts express only integrin α7Bβ1, the α7A subunit was also expressed by a retroviral gene transfer in Def-6-overexpressing C2C12 cells. Myoblasts infected with retroviruses for α7B expression served as a control. The expression of the integrin α7A chain was confirmed by immunoblotting (Fig. 5*C*, *right panel*). These cells were plated on coverslips precoated with laminin-111, an ECM substrate for the α7β1 receptor. The immunofluorescence analysis of these cells revealed that Def-6 localizes to focal adhe-

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[5] T. Samson, C. Will, A. Knoblauch, L. Sharek, K. von der Mark, K. Burridge, and V. Wixler, unpublished observation.
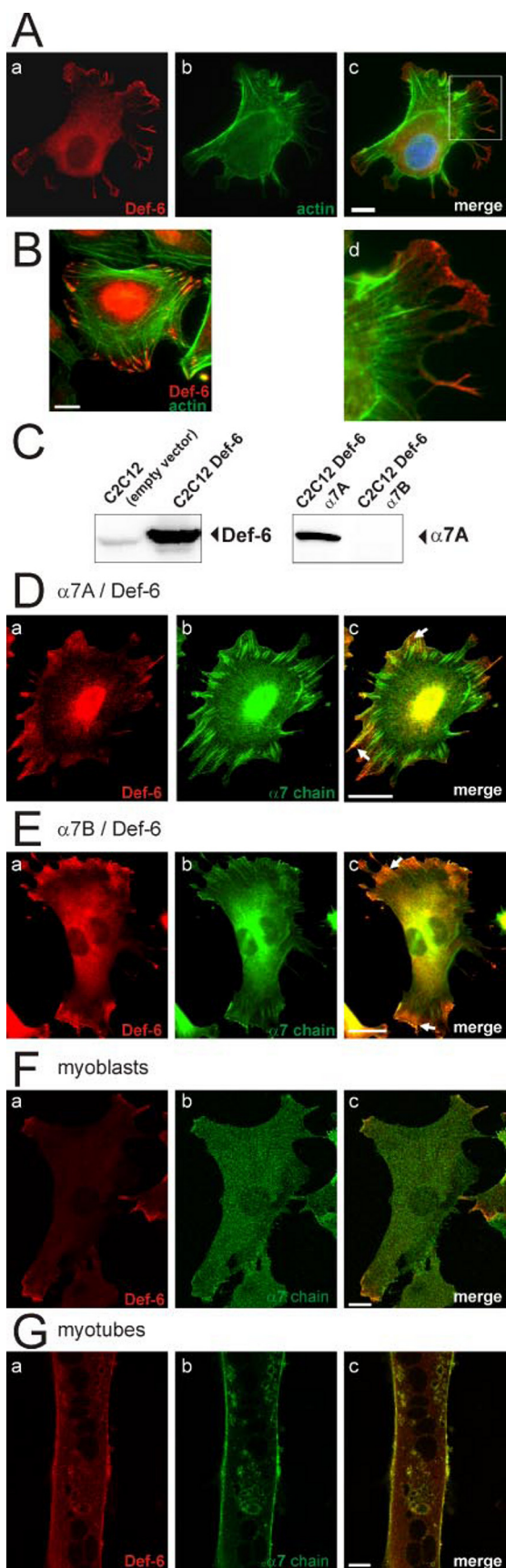
FIGURE 5. **Subcellular localization of Def-6.** *A* and *B*, different cell types were transiently transfected with a Def-6 expression construct, plated on coverslips, and stained for immunofluorescence. *A*, NIH-3T3 fibroblast plated on

sions and lamellipodia, as seen in fibroblasts (NIH-3T3) and epithelia cells (HeLa). Further, in these structures Def-6 localization partially overlaps with laminin-binding α7β1 integrin. The same observations were noted for α7A- and α7B-expressing cells (Fig. 5, *D* and *E*).

Taken together, these results show that Def-6 localizes to lamellipodia and focal adhesions. This distribution is independent of the α7A integrin chain, because it was observed also in cells that do not express this integrin chain. However, when α7A and Def-6 are present within one cell, they partially colocalize in focal adhesions.

To study where Def-6 localizes in C2C12 cells during the differentiation from myoblasts to myotubes we used the Def-6-overexpressing C2C12 cells (Fig. 5*C*, *left panel*). The cells were plated on uncoated coverslips and subsequently induced to differentiate. In undifferentiated myoblasts we again detected Def-6 in cellular protrusions (Fig. 5*F*). In myotubes Def-6 localized to the sarcolemma where a clear colocalization with the integrin α7 chain could be observed (Fig. 5*G*). It is worth noting that the analyzed myotubes (day 4 after induction of differentiation) express endogenously the integrin α7A chain.

Def-6 Influences Differentiation of C2C12 Myoblasts—During differentiation of myoblasts expression of the α7A integrin chain is gradually up-regulated. Because Def-6 binds to the cytoplasmic tail of α7A, we next tested if myogenic differentiation of the Def-6-overexpressing C2C12 cells is affected. C2C12 cells, which were infected with an empty-vector virus, expressed only the endogenous level of Def-6 and served as a control (Fig. 5*C*, *left panel*). These cells were plated on 6-well dishes, and differentiation was induced after 24 h. Progression of morphological changes in the differentiating cultures were monitored by microscopy (Fig. 6*A*). Empty vector-infected C2C12 cells aligned and fused normally like uninfected cells. Fusion of control cells started to occur on day 2 after growth factor deprivation. On day 5 they showed a high density of parallel arrays of thin and mainly unbranched myotubes. Def-6-overexpressing myoblasts also began to fuse on day 2, but in contrast to empty vector-infected cells they tended to lose contact to the tissue culture dish, which resulted in lower cell density. In addition, the myotube formation of Def-6-overexpressing cells was severely impaired. A subset of these cells formed strongly branched and very thick myotubes after 3 days (Fig. 6, *B* and *C*). Nevertheless, the control cells as well as the Def-6-overexpressing cells underwent myogenic differentiation, because sarcomeric myosin was expressed in both cases (MF-20 staining, Fig. 6*B*). Measuring the thickness of fused myotubes

laminin-111; *a*, Def-6; *b*, actin; *c*, merged image; *image d* represents a higher magnification of the frame in *image c*. *B*, HeLa cell; *red*, Def-6; *green*, actin. *C*, C2C12 myoblasts were stably transduced with an expression construct for Def-6 (*left panel*). These Def-6-overexpressing cells were subsequently transduced with expression constructs for the integrin chain α7A or α7B. Expression of α7A was verified by immunoblotting (*right panel*). *D* and *E*, cells were plated on laminin-111-coated coverslips. *D*, α7A/Def-6-expressing C2C12 cells. *E*, α7B/Def-6-expressing C2C12 cells. Cells in *D* and *E* were stained for Def-6 (*D*, *panel a* and *E*, *panel a*) and the α7 integrin chain (*D*, *panel b* and *E*, *panel b*). Merged images are shown in *D*, *panel c* and *E*, *panel c*. The *white arrows* indicate regions of partial colocalization. *F* and *G*, Def-6-overexpressing C2C12 cells were fixed, stained either as myoblasts (*F*) or as differentiated myotubes (*G*), and analyzed using confocal microscopy. *Panels*: *a*, Def-6; *b*, α7 integrin chain; and *c*, merged image. The *white bars* represent 10 μm.
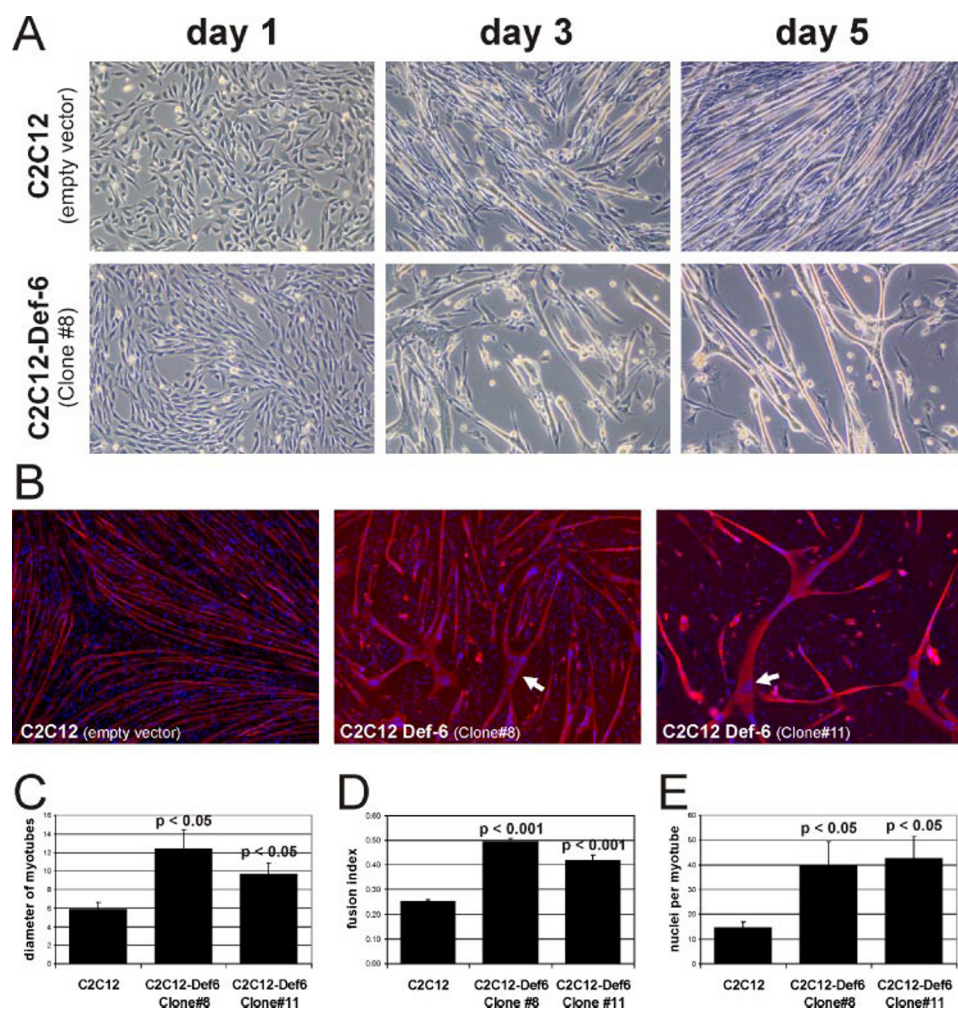
FIGURE 6. Def-6 overexpression impairs differentiation of C2C12 myoblasts. *A*, myogenic differentiation of C2C12-control or Def-6-overexpressing C2C12 cells was induced by switching the culture medium to differentiation medium (day 0). Phase micrographs of differentiating cells were taken at different times (days 1, 3, and 5). Def-6-overexpressing C2C12 cells formed arrays of unparallel and branched myotubes and cells tended to detach from the tissue culture dish during differentiation. *B*, differentiating cultures were fixed on day 3 and stained with 4′,6-diamidino-2-phenylindole and the myogenic differentiation sarcomeric myosin (MF-20). The *arrows* indicate clusters of nuclei in myotubes originating from Def-6 overexpressing C2C12 cells. *C–E*, different parameters of differentiating C2C12 control cells and two different Def-6-overexpressing clones. Def-6-overexpressing C2C12 cells developed myotubes with increased average diameter (*C*) and number of nuclei per myotube (*E*). The fusion index of Def-6-overexpressing C2C12 cells was also increased (*D*). Data in *C–E* are mean ± S.E.

(starting at day 1), Def-6-overexpressing cells delayed this down-regulation. During days 1–3 of the differentiation, the Rac1.GTP levels were elevated compared with control cells. Only later the Rac1.GTP level gradually decreased to that of the control cells. Control immunoblots for the myogenic differentiation markers sarcomere myosin and α7A integrin chain confirmed that both cell lines underwent myogenesis (Fig. 7*B*).

Taken together, these experiments show that overexpression of Def-6 in C2C12 myoblasts impairs the down-regulation of Rac1 activity, causing dysregulated formation of unusually thick and branched myotubes.

DISCUSSION

Here we report a specific interaction between the Rac1 GEF Def-6 and the cytoplasmic domain of the integrin chain α7A, which is a splice isoform expressed exclusively in skeletal muscle tissues. This interaction was found to be inhibited by a tyrosine phosphorylation of Def-6, which was described to stimulate the GEF activity of this protein. Further, we show a widespread tissue distribution of Def-6, including different muscle types and the C2C12 myoblast cell line. The protein localizes to cellular protrusions and focal adhesions where it overlaps with α7β1 distribution. Overexpression of Def-6 in C2C12 myoblasts leads to elevated Rac1.GTP levels and altered myogenic differentiation.

revealed that those originating from Def-6-overexpressing C2C12 cells had about twice the diameter compared with control myotubes (Fig. 6*C*). In addition, differentiated Def-6-overexpressing C2C12 cultures exhibited an unusually high fusion index (Def-6-overexpressing cells: fusion index > 0.4; control cells: fusion index = 0.26; Fig. 6*D*), and such myotubes had an increased amount of nuclei (Def-6-overexpressing myotubes: ∼40 nuclei; control myotubes: ∼15 nuclei; Fig. 6*E*). The nuclei were often accumulated in clusters at branch points of the myotubes (*arrows* in Fig. 6*B*).

These results show that Def-6 affects the differentiation and myotube formation of C2C12 cells. Because Def-6 has been shown to be an Rac1 GEF (26) and because high Rac1 activity in C2C12 cells impairs myoblast differentiation (24), we measured Rac1.GTP levels in Def-6-overexpressing cells during differentiation (Fig. 7*A*). Although the level of Rac1.GTP in control C2C12 cells decreased soon after the switch to differentiation medium

The process of muscle development and regeneration is a complex cascade of numerous cell functions, which finally lead to the formation of myofibers. Undifferentiated myoblasts proliferate and migrate on ECM substrates (12, 42), align with each other, and eventually fuse to form multicellular myotubes (43). This process is accompanied by a cell cycle arrest and expression of muscle-specific genes (44). Both, Rac1 and α7β1 integrin were described to affect differentiation and maintenance of muscles (9, 23). Because the integrin α7A chain is a splice isoform exclusively expressed in skeletal muscle cells *and* Def-6 is a GEF for Rac1, this interaction could provide a skeletal muscle-specific signaling link between integrin α7β1 and the Rho GTPase Rac1.

Integrin α7β1 was shown to be important for development and homeostasis of muscles, because mice deficient for the integrin α7 chain show both a partial embryonic lethality and
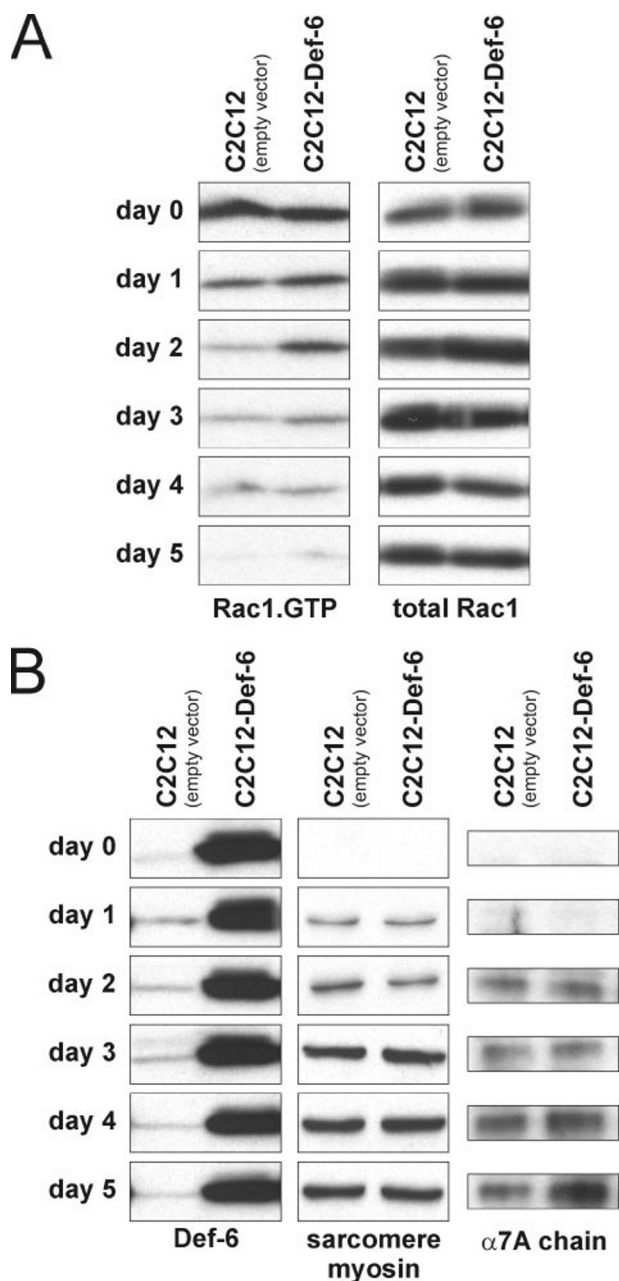
FIGURE 7. **Elevated Rac1.GTP levels in Def-6-overexpressing C2C12 myoblasts during differentiation.** *A*, the amount of Rac1.GTP in differentiating C2C12 cultures was determined at different times by precipitating Rac1.GTP from total cell lysates using GST-PBD (*left panel*) as described under "Experimental Procedures." The *panel* on the *right* shows immunoblotting of equal amounts of total cell lysates. Compared with empty vector-infected C2C12 cells, Def-6-overexpressing cells had a delayed decrease of Rac1.GTP levels during differentiation. Only later (days 4 and 5), Def-6-overexpressing cells also decreased the amount of activated Rac1.GTP. *B*, immunoblots that show expression of Def-6, sarcomeric myosin (MF20), and α7A integrin in differentiating C2C12 control and Def-6-overexpressing cells.

muscular dystrophy in adult animals (14). Interestingly, integrin α5β1 is up-regulated in α7-deficient mice (45); however, the compensation is obviously incomplete, because severe muscle wasting can be observed in these animals. This may be at least partly due to the fact that α5β1 is a fibronectin receptor instead of a laminin receptor. In addition, the intracellular domains of the α5 and both α7 splice isoforms are different, which implies diverse intracellular protein interactions and sig-

naling events. So far, all experiments attempting to elucidate the signaling capacity of the α7 intracellular domain were performed with α7B-expressing cells or in myoblasts where the exact expression of splice isoforms was not analyzed. Mielenz and coworkers (21) found that α7B interacts with a p130[CAS]/crk complex. The muscle integrin-binding protein was shown to interact with α7B, thereby regulating laminin matrix deposition and paxillin signaling (46). FHL2 and FHL3 bind to α7A and α7B, but not to α5 (28), which may be one reason for the failure of integrin α5 compensation in α7-deficient mice. FHL2 and FHL3 were also found to bind to actin (47), which suggests that they may link α7A and α7B to the actin cytoskeleton. All this could be necessary to provide a more stable connection between integrins and the cytoskeleton, to resist the strong mechanical forces acting on muscle cells. Interestingly, the muscle-specific splice variant β1D was also shown to provide a stronger link to the actin cytoskeleton (48). However, although FHL2 and FHL3 are the only proteins found to bind to the α7A cytoplasmic domain, these interactions are not exclusive, because other integrin cytoplasmic domains (including α7B) also interact with FHL2 and FHL3 (28, 29). No functional data about a specific and exclusive role for α7Aβ1 have yet been obtained, and the question why muscles express α7A and α7B splice variants has not been answered so far. Likewise, the tissue distribution and subcellular localization of α7A does not give a conclusive indication about a specific function. Except for the finding that the α7A splice isoform only occurs in differentiated skeletal muscles (19), α7A and α7B always codistribute within the muscle cell, for example at myotendinous and neuromuscular junctions (49). So far, α7A has not been reported to localize exclusively without α7B at any cellular compartment of muscle cells.

Our studies started with the goal of identifying proteins that bind specifically to the α7A isoform. The finding that Def-6 and Swap-70 bind to the intracellular domain of the α7A receptor is the first description of exclusive and specific interactions with this integrin chain. We were able to confirm this interaction by direct yeast two-hybrid assays and coprecipitation experiments from transfected mammalian cells. Our interaction studies clearly show an α7A-specific binding of Def-6, because it did not bind to α-chains, which form integrin receptors for the majority of ECM ligands: α1β1 and α2β1 as collagen receptors, α3β1, α6β1, and α7β1 as laminin receptors, and α5β1 and α4β1 as fibronectin receptors. Finally, the interaction specificity for α7A *versus* α7B was further proven by analyzing a deletion mutant of α7B, which lacks a putative C-terminal inhibitory domain (15). We have previously shown that this deletion mutant interacts strongly with FHL2 and FHL3 (28), but here we did not find binding to Def-6 or Swap-70. Most integrin intracellular binding proteins have been found to interact with β chains (7). Interestingly, Def-6 and Swap-70 did not show binding to the β1A isoform or the muscle specific isoform β1D. Within our interaction studies we also tried to coprecipitate Def-6 with the α7A chain from cell lysates of C2C12 myotubes, which endogenously expressed both proteins. However, because interactions between intracellular pro-

teins and cytoplasmic domains of integrins are often very weak, these attempts did not lead to conclusive results.[5]

Def-6 and Swap-70 represent a novel family of Rho-GEFs that have recently been described (26, 27). The classic arrangement of DH/PH domains is inverted in these GEFs. In addition, the degree of homology of the Def-6 and Swap-70 DH-like domains to other DH domains is not significant, and a potential coiled-coil stretch was identified within this region (50). So far, Def-6 was reported to be mainly associated with functions of lymphocytes with highest expression in T-cells and T-cell homing organs (26, 37, 38). Here we confirmed the high expression of Def-6 in the thymus as a T-cell compartment (Fig. 4C), but additionally we detected Def-6 mRNA and protein expression in all tested organs, including muscle tissues (heart, skeletal muscle, and tongue) and the C2C12 myoblast cell line. After initial reports that expression of Swap-70 is almost exclusively restricted to B-cells (51), it turned out that this protein is present in a more widespread manner (52) with a signaling function upstream of Rac1 (27). Likewise, our RT-PCR analysis also revealed Swap-70 expression in skeletal muscle tissue.[5] Similarly, our expression analysis of Def-6 also showed a more general distribution, indicating that it functions in cell types other than T-cells. Mavrakis and coworkers (35) have already suggested endogenous expression of Def-6 in NIH-3T3 fibroblasts.

Rac1 activity levels and its downstream signaling effects during myoblast differentiation are still an active field of discussion and controversy (23). Overall, Rac1 activity has to decrease for proper myoblast differentiation, because overexpression of constitutive active Rac1 in C2C12 myoblasts leads to impairment of differentiation (24). However, several steps during myogenesis seem to require Rac1 activity in certain compartments of the cell. Rac1.GTP is required for the formation of acetylcholine clusters, and overexpression of constitutively active Rac1 increases the clustering of acetylcholine receptors on myotubes (25). Expression of muscle-specific genes in the nuclear compartment were shown to be regulated by Rac1, even though the results are inconsistent concerning the mode of regulation (53, 54). In founder cells of forming muscles in *Drosophila*, Rac1 becomes specifically aggregated at fusion sites (55). In summary, all these findings indicate that Rac1 regulates certain locally restricted steps of differentiation, even though the net Rac1 activity level decreases in differentiating myoblasts.

It is thus interesting that we found an interaction of a Rac1 GEF and a muscle-specific integrin, which is regulated during myogenesis. Indeed, focal adhesions containing α7A also contained Def-6, even though we observed Def-6 localization to focal adhesions independently of α7A as well. Thus, subcellular localization of Def-6 must be guided by interactions with other proteins or binding of the Def-6-PH domain to phosphatidylinositol phosphates. Def-6 membrane localization was previously shown to be dependent on phosphatidylinositol 3-kinase activity (35). Because Def-6 does not bind to α7A when it is tyrosine-phosphorylated, this interaction could provide a mechanism to recruit inactive Def-6 to certain areas of the cell.

Because α7A is up-regulated during differentiation, it might act to sequester inactive Def-6, thereby contributing to the general decrease in Rac1 activity that accompanies myogenesis. Alternatively, by recruiting Def-6 to specific sites where this integrin is concentrated, the association between α7A and Def-6 may allow local activation of Rac1 at these sites, for example by phosphorylation of Def-6 by Src family kinases.

Interestingly, the Src family kinase Yes is up-regulated and activated in differentiating myoblasts (56), and we indeed detected tyrosine-phosphorylated Def-6 in C2C12 myoblasts and myotubes. However, the amount of phosphorylated Def-6 was low, and the relative total amount of phosphorylated Def-6 did not change dramatically from myoblasts to myotubes, implying that Def-6 phosphorylation probably occurs only in a small area of the cell.

Down-regulation of Rac1 was shown to be essential for establishing the cell cycle exit of differentiating myoblasts (24). Overexpression of constitutive active Rac1 in differentiating myoblasts delays the cell cycle exit, and the resulting myotubes are much thicker. Interestingly, when we overexpressed Def-6 in C2C12 myoblasts, we observed the same phenotype. The myotubes were much thicker compared with control cells, and the number of nuclei was increased. The Rac1.GTP level in differentiating C2C12 myoblasts overexpressing Def-6 was initially higher compared with control cells. By day 5 it decreased to a level comparable to that found in control cells. This observation strongly suggests that the GEF activity of Def-6 is normally down regulated in differentiating myoblasts, because the overall Def-6 amount is not changed (Fig. 4D). Due to the high amount of Def-6 protein in overexpressing cells, this fine regulation is probably delayed, because mechanisms that normally down-regulate Def-6 GEF activity or the localization of Def-6 are overstrained.

To further analyze the role of Def-6 during myoblast differentiation, we also attempted to generate Def-6 knockdown cell lines. To date a successful knockdown of Def-6 has not been reported, and we did not get a reliable and efficient reduction of Def-6, despite numerous attempts using different RNA interference sequences and transfection methods.[5] Similar difficulties with knocking down a different GEF (GEFT) in C2C12 myoblasts have been reported (57). The mouse knock-out models of both Def-6 and Swap-70 genes have been generated (58–60), but the published reports have focused on lymphocyte-specific functions only. Whether muscle functions are affected in these mice has not been reported, and it might be that a mild muscle phenotype was not yet detected. On the other hand, it is possible that Def-6 and Swap-70 are functionally redundant, because both have GEF activity on Rac1, and both can bind to the same amino acid sequence in the cytoplasmic domain of α7A. Indeed, our PCR analysis also revealed Swap-70 in C2C12 myoblasts.[5] Redundancy seems to be a general feature of GTPase regulators, as it was reported for the Vav GEF family (61) as well as the GTPase-activating proteins Bcr and Abr (62).

In summary, we have found that Def-6 and Swap-70 bind to the cytoplasmic part of the α7A integrin chain, which is the first description of a specific interaction with this integrin splice variant. Our data suggest that Def-6 as a GEF for

Rac1 has a role during formation of myotubes. Def-6 and its activity have to be precisely regulated for proper differentiation of myoblasts. The interaction between α7A and Def-6 could serve as a mechanism to locally restrict Def-6 GEF activity within differentiating cells. We hope to clarify this question by employing recently developed FRET-based biosensor technologies, which allow measurement of locally restricted Rac1 activity.

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