

The LIM-only Proteins FHL2 and FHL3 Interact with α - and β -Subunits of the Muscle $\alpha_7\beta_1$ Integrin Receptor*

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FHL1, FHL2, and FHL3 are members of the four and one-half LIM domain protein subclass that are expressed in striated muscles. Here we show that FHL2 and FHL3 are novel $\alpha_7\beta_1$ integrin-interacting proteins. They bind both the α - and the β -subunit as well as different splice isoforms. The minimal binding sites for FHL2 and FHL3 on β_{1A} -chain overlap, whereas on α_{7A} and α_{7B} subunits they are situated adjacent. Determining the binding sites for integrins on FHL2 or FHL3 revealed that the suprastructure of the whole molecule is important for these associations, rather than any single LIM domain. Immunofluorescence studies with cells expressing full-length FHL proteins or their deletion mutants showed that FHL2 and FHL3 but not FHL1 colocalize with integrins at cell adhesion sites. Further, their recruitment to the membrane results from binding to either the α - or the β -chain of the integrin receptor. The association of FHL2 or FHL3 with integrin receptors neither influences attachment of cells to different substrates nor changes their migration capacity. However, in cardiac and skeletal muscles, FHL2 and FHL3, respectively, are colocalized with $\alpha_7\beta_1$ integrin receptor at the periphery of Z-discs, suggesting a role in mechanical stabilization of muscle cells.

Integrins are heterodimeric transmembrane molecules composed of an α - and a β -chain. The 18 α - and 8 β -subunits form at least 24 different integrin receptors (1, 2). Integrins provide cell adhesion to the extracellular matrix (ECM),¹ with the α/β -subunit combination determining the binding specificity. Apart from mediating cell adhesion, integrins ensure cell migration as well as transmitting proliferation and differentiation signals into cells (“outside-in signaling”). Changes in integrin ligand binding affinity also can be triggered through signals from the inside of cells (“inside-out signaling”) (3, 4).

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¹ The abbreviations used are: ECM, extracellular matrix; aa, amino acid(s); BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; GAL4-BD, GAL4 DNA-binding domain; GAL4-AD, GAL4 activation domain; GST, glutathione S-transferase; mAb, monoclonal antibody.

Since integrins do not have intrinsic enzymatic activity, it is of importance to characterize proteins that bind the cytoplasmic domains of integrins and transduce integrin-dependent signals. Many integrin-binding proteins have been described (reviewed in Refs. 1 and 2), some with structural properties, such as talin or F-actin; others, such as integrin-linked-kinase or focal adhesion kinase, are directly involved in integrin-mediated signaling. A third group of integrin-interacting proteins represent scaffolding or adaptor proteins (1, 2, 5, 6).

The $\alpha_7\beta_1$ integrin is a muscle tissue receptor, highly expressed on skeletal, cardiac, and smooth muscle cells (7). Several alternative splice forms of the α_7 chain have been described (8). Two of them, α_{7A} and α_{7B} , which differ only in their cytosolic parts, are differentially expressed during muscle development and regeneration (7, 9, 10). Since the binding specificity of α_{7A} and α_{7B} integrin isoforms to different ECM molecules seems to be the same, it is very likely that functional differences of these splice forms are specified via their binding ability to certain cytosolic proteins.

Most integrin-interacting proteins described bind to β -subunits (1, 2). Among those interacting with the α -chain, some possess a binding capacity restricted to particular α -chains, whereas others such as Mss4 or calreticulin may interact with several (11, 12). Finally, certain proteins (e.g. FHL2 and paxillin) interact with both α - and β -chains (6, 13).

FHL (four and one-half LIM domain proteins) proteins consist of four LIM domains and a single zinc finger at the NH₂ terminus. Since they lack other functional or structural domains, they belong to the LIM-only protein family. LIM domains are double zinc finger motifs, involved in a wide range of protein-protein interactions, regulating the function of cytoskeletal proteins, enzymes, or transcription factors (14, 15).

Five different FHL proteins have been described, FHL1–FHL4 and ACT. Whereas the expression of FHL4 and ACT is restricted to testis (16, 17), FHL1, FHL2, and FHL3 mainly occur in muscle cells (18, 19), although a broader expression pattern has been reported for FHL1 and FHL2 (19–21). As expected for proteins with repeating LIM domain structures, FHL proteins are able to interact with numerous cellular proteins. FHL2, the best studied member of the family interacts with transmembrane receptors (6, 22, 23), with cytosolic proteins having structural or other properties (21, 24, 25), with nuclear shuttling proteins such as β -catenin (26), or with transcription factors and their regulators (22, 27–30). In general, the regulation of transcriptional activity seems to be a common feature for all FHL proteins. FHL2 has been shown to promote C2C12 cell differentiation (26) and to function as a scaffolding protein in mature heart muscle, where it brings together pro-

teins with structural and enzymatic properties (24). Recently, FHL2 and FHL3 have been described as transmitting signals from the cell surface to the nucleus in a Rho-dependent manner (30), probably due to their binding to integrins or other cytoskeletal proteins. However, FHL2 null mutant mice have only a very mild heart phenotype, with a slightly altered hypertrophic response upon β -adrenergic stimulation (31, 32).

Here we show that all intracellular splice variants of the $\alpha_7\beta_1$ muscle integrin receptor interact with FHL2 and FHL3 but not with the highly homologous FHL1. FHL2 and FHL3 colocalize with $\alpha_7\beta_1$ integrin in focal adhesion complexes, which may occur through their binding to either the α - or the β -subunit. Furthermore, FHL2 and FHL3 colocalize with the $\alpha_7\beta_1$ integrin receptor at the periphery of Z-discs in cardiac and skeletal muscles, respectively.

EXPERIMENTAL PROCEDURES

DNA Constructs—All yeast two-hybrid pAS2-1 (Clontech, Palo Alto, CA) derivative constructs coding for fusion proteins of the GAL4 DNA-binding domain (GAL4-BD) and full-length or truncated intracellular parts of α_{7A} or α_{7B} integrin subunits were produced by PCR with appropriate primers and pCEP4- α_{7A} or pCEP4- α_{7B} plasmids as templates (33). The cDNA fragment coding for the complete intracellular part of human integrin α_7 was generated by reverse transcriptase-PCR using mRNA from 10.5-day-old mouse embryos as template and cloned in-frame into pAS2-1 vector using EcoRI/BamHI restriction sites. pAS2-1 yeast two-hybrid constructs, which code for fusion proteins of GAL4-BD and the complete intracellular part of human integrin α_1 , α_2 , α_{3A} , α_{3B} , α_5 , α_{6A} , α_{6B} , β_{1A} , and β_{1D} , were described elsewhere (6). cDNAs of complete murine α -actin and β -actin or of the cytosolic part of murine β -dystroglycan were derived by reverse transcriptase-PCR using mRNA from C2C12 myoblasts as template and cloned in-frame into the pAS2-1 GAL4-BD vector via NcoI/EcoRI or EcoRI/SalI restriction sites. cDNA fragments encoding the complete sequences of FHL1, FHL2, FHL3, or their deletion mutants were cloned in-frame into pACT2 vector (Clontech, Palo Alto, CA) as described previously (26).

For expression in mammalian cells, the cDNA fragment coding for complete cytoplasmic domain of integrin α_{7A} subunit was cloned in-frame into the GST tag vector pEBG as described (6). The full-length pCEP4- α_{7A} integrin construct was obtained by replacing the B-splice variant in the pCEP4- α_{7B} plasmid with the α_{7A} form. The Myc-tagged or GST-tagged FHL1, FHL2, and FHL3 constructs were obtained by cloning the appropriate inserts from pACT2 vector into pCS2+MT or pEBG eukaryotic expression plasmids. For generation of the EGFP-FHL2 construct, the FHL2 insert was released from pACT2 vector and inserted in-frame into the pEGFP-C1 vector (Clontech, Palo Alto, CA). For stable expression in C2C12 cells, the Myc-tagged FHL2 and FHL3 were released from pCS2+MT plasmids and recloned into the pBabe-neo vector. The correct in-frame cloning of the constructs was verified by sequencing.

Yeast Two-hybrid Screen—A human placenta cDNA library cloned into pACT2 yeast two-hybrid vector was used (MATCHMAKER™; Clontech). The cytoplasmic parts of murine integrin α_{7A} (aa 1104–1161) or α_{7B} -del1 (aa 1104–1171) in pAS2-1 were used as bait in two independent screens that were performed according to the protocol of Clontech. The aa numbers refer to NCBI sequence entry (accession number Y12380).

Direct Yeast Two-hybrid Interaction Assays—The yeast strain Y190 was cotransformed with the pAS2-1 plasmid containing the GAL4-BD fused with appropriate cDNAs as bait and with pACT2 plasmids containing cDNAs fused to GAL4-AD as prey. Transformants were grown on SD 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 quantification of the β -galactosidase activity, the transformants were first grown on SD medium lacking leucine and tryptophan and subsequently introduced into the liquid culture assay using *o*-nitrophenyl- β -D-galactoside as substrate. The β -galactosidase activity was calculated according to the protocol of Clontech.

Cell Culture and Antibodies—C2C12 muscle precursor cells were cultured at low densities in DMEM containing 20% fetal calf serum. HEK293 and NIH3T3 cells were cultured in DMEM with 10% fetal calf

serum. All cells were grown at 37 °C and 7.5% CO₂. C2C12 myoblasts stably expressing Myc-FHL2 or Myc-FHL3 were generated using a retroviral infection system as described in Ref. 26.

The following antibodies were used: mouse anti-Myc, clone 9E10; rabbit anti-Myc; and rabbit anti-GST (Sigma); mouse anti-integrin α_7 , clone 3C12 (recognizes the extracellular part of the receptor (34)); polyclonal rat anti- $\alpha_7\beta_1$ -integrin (recombinant extracellular $\alpha_7\beta_1$ integrin complex was used for immunization); rabbit anti-integrin α_{7B} ; and rabbit anti- β_{1A} (a gift of U. Mayer, Manchester, UK); mouse anti- β_{1D} (a gift of A. Sonnenberg, Amsterdam, The Netherlands), mouse anti-FHL2, clone F4B2-B11 (recognizes the NH₂-terminal half LIM domain); rabbit anti-FHL2 (epitope used for immunization: His-tagged aa 218–279); rabbit anti-FHL3 (epitope used for immunization: His-tagged aa 1–159); peroxidase-conjugated secondary antibodies for immunoblot analysis (Amersham Biosciences), and Cy2- and Cy3-conjugated goat anti-rabbit IgGs (Dianova, Hamburg, Germany).

Immunofluorescence Stainings—Cells were trypsinized and plated on coverslips, which had been precoated with 15 μ g/ml concentrations of various ECM proteins and blocked with 1% BSA. After attachment and spreading of cells (1–1.5 h), the coverslips were washed in PBS, 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% BSA/PBS. Binding of first antibodies was performed for 1 h at room temperature and was detected by species-specific fluorochrome-conjugated secondary antibodies as described (6, 26). When transfected cells were used, they were plated on coverslips 24 h after transfection. Immunostaining of a 10- μ m cryosection of muscle tissues was performed in the same manner. Tissues were taken from 8-week-old C57bl/6 mice and embedded in Tissue-Tec (Sakura Finetek, Zoeterwoude, The Netherlands) for cryosections. Fluorescence was monitored with a Zeiss Axioplan-2 microscope (Zeiss, Jena, Germany). Cell images were taken using a Hamamatsu digital camera and OpenLab Systems software (Improvision). Confocal images were generated with Leica DMR (Leica, Deerfield, IL) software, version 2.00.

GST Coprecipitation Assays and Immunoblotting— 2×10^5 HEK293 cells were plated on 6-well dishes 48 h before transfection, which was performed using Superfect™ transfection reagent (Qiagen, Hilden, Germany). 1 μ g of each cDNA plasmid was used per transfection, and the total amount (2 μ g/well) was equalized if necessary with appropriate empty expression vectors. 30 h later, cells were washed with PBS and lysed in IP buffer (137 mM NaCl, 25 mM Tris, pH 7.5, 2 mM MgCl₂, 10% glycerol, 0.5% Triton X-100) supplemented with 1 mM sodium vanadate, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin at room temperature for 10 min. The lysates were cleared by centrifugation at 13,000 $\times g$ for 10 min at 4 °C and incubated for 2 h at 4 °C with glutathione-Sepharose beads for GST pull-down analyses. The beads were subsequently washed two times with IP buffer. To reduce any nonspecific binding of proteins, Sepharose beads were blocked with 1% BSA in IP buffer before incubation with cell lysates. Samples were resolved by 10% SDS-PAGE, followed by electroblotting onto nitrocellulose membrane. The specific detection of proteins was performed with appropriate first antibodies and peroxidase-conjugated secondary antibodies using the ECL detection system (Amersham Biosciences).

Cell Attachment and Migration Assays—Attachment assays were performed as described elsewhere (33). Briefly, 96-well plates were coated overnight at 4 °C with different concentrations of laminin-1 or fibronectin and blocked thereafter with 1% BSA for 1 h at room temperature. Wells coated with only BSA served as negative controls. Cells were trypsinized and washed once in DMEM containing 0.5% BSA, and 5×10^4 cells were added in 50 μ l of DMEM with 0.5% BSA into each well. After incubation for 30 min at 37 °C, cells were washed three times with PBS using an automatic washer, and the amount of attached cells was measured by acidic phosphatase activity. Cell migration studies were performed mainly as described in Ref. 35. Briefly, 48-well plates were coated with laminin-1 or fibronectin and blocked with 1% BSA, and 10^5 cells in 0.5 ml of DMEM plus 10% fetal calf serum were plated into each well. To produce a cell-free “window,” a 0.6-mm thick steel plate was inserted into wells before seeding of cells and they were removed again after the cells have been attached to the bottom. This method has the advantage over the frequently used “scratch window” assay in that the substrate in the “window” is not destroyed. The migration was monitored by inverted microscopy at the times shown.

Purification of Extracellular Matrix Proteins—Murine laminin-1 was purified from the Engelbreth-Holm-Swarm tumor as described in Ref. 36. Human fibronectin was isolated from freshly frozen plasma according to Vuento *et al.* (37). Human vitronectin was purified from human plasma by heparin affinity chromatography according to the method of Yatohgo *et al.* (38). Rat tail collagen-1 was purchased from Roche

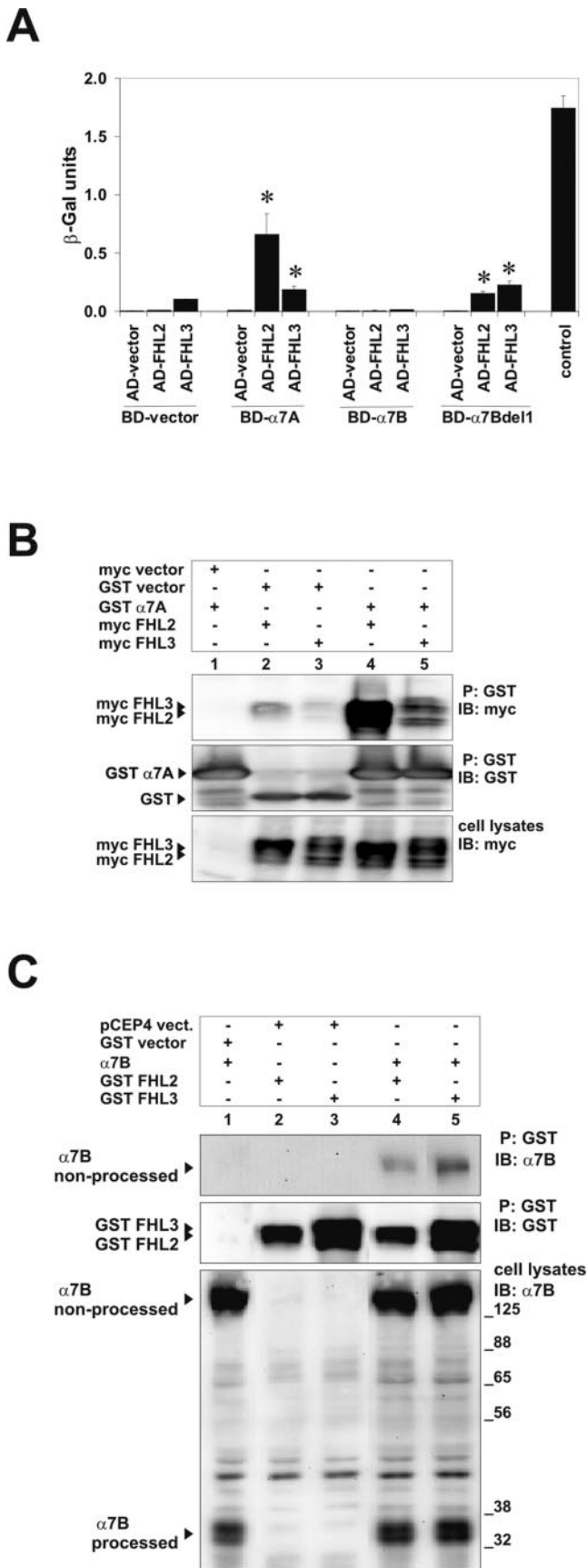


FIG. 1. Interaction of FHL2 and FHL3 with integrin α_7A and α_7B . A, yeast Y190 cells were transformed with GAL4 DNA-binding domain (BD) and GAL-4 activation domain (AD) chimeric constructs, and the β -galactosidase activity was measured using a liquid *o*-nitrophenyl- β -D-galactoside assay. The following parts of integrin α_7 subunits were used: aa 1104–1161 for α_7A , aa 1104–1180 for α_7B , and aa

Applied Science. The quality of purified proteins was verified by analyses of silver-stained polyacrylamide gels and by immunoblotting with appropriate specific antibodies. Only samples with no sign of degradation were used.

RESULTS

Identification of FHL2 and FHL3 as Novel Interaction Partners of α_7A and α_7B Integrin Subunits—To understand the functional properties of the muscle integrin receptor $\alpha_7\beta_1$, we searched for proteins binding to the different cytosolic splicing variants of the α -chain. The cytoplasmic part of α_7A (aa 1104–1161) and α_7B (aa 1104–1171) were fused to GAL4-BD and used as “bait” in two independent yeast two-hybrid screens. A human placenta cDNA library fused to the GAL4 activation domain (GAL4-AD) was used as a “prey” protein source. Two related members of the FHL protein subclass were isolated from the α_7A screen. Of 288 analyzed clones, 189 coded for FHL2 (also known as SLIM3 or DRAL), and one coded for FHL3 (alternative name SLIM2). In the α_7B screen, 2 of 31 positive clones also coded for FHL2. A direct yeast two-hybrid binding assay showed that both α_7A and α_7B integrin splicing variants specifically interacted with FHL2 and FHL3 but not with the GAL4-AD alone. Quantification of β -galactosidase activity further showed that α_7A /FHL2 binding is stronger than that of others and that α_7 /FHL interactions are several times weaker than that of p53/SV40 large T-antigen interaction, which was used as positive control (Fig. 1A). Our data also confirmed the already published transactivation property of FHL3 alone (30, 39).

A surprising situation was noticed for the binding to the α_7B cytoplasmic domain, since only a truncated form of the receptor lacking the 9 COOH-terminal amino acids showed an interaction with FHL2 and FHL3 but not the complete α_7B cytodomain (Fig. 1A). Similar results were obtained with several other α_7B -interacting proteins, and we were not able to identify any α_7B -binding proteins while screening placenta or heart cDNA libraries with the complete α_7B cytodomain. The inhibitory effect of the full-length α_7B cytosolic domain will be discussed later.

The specificity of interactions was confirmed in experiments with mammalian cells. Myc-tagged FHL2 and FHL3 proteins could be coprecipitated with GST-tagged α_7A cytoplasmic domain, when these chimeric proteins were coexpressed in HEK293 cells (Fig. 1B). The specificity of the observed bindings was clearly visible (Fig. 1B, upper panel, lanes 4 and 5) despite a weak binding of FHL proteins to glutathione-Sepharose beads (lanes 2 and 3). In a reciprocal pull-down assay with GST-tagged FHL2 and FHL3, we were able to coprecipitate the

1104–1171 for $\alpha_7B\Delta e11$. The experiment was repeated twice, and three different yeast transformants were used for each measurement. Control represents the interaction of p53 with SV40 large T-antigen proteins. The asterisks indicate significant differences to corresponding controls with BD vector only ($p < 0.01$ according to the *t* test). B, HEK293 cells were transiently transfected with cDNA constructs as indicated. After 30 h, GST- α_7A protein was precipitated (P) with glutathione-conjugated Sepharose beads from cell lysates. The coprecipitated FHL proteins were detected in 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-FHL2 and Myc-FHL3 analyzed by immunoblotting of equal amounts of total cell lysates with anti-Myc mAb. C, the presence of integrin α_7B chain (aa 1–1171) in GST-FHL2 or GST-FHL3 precipitates (P) was detected by immunoblotting (IB) with anti- α_7B rabbit polyclonal serum (upper blot). To ascertain the amount of precipitated FHL proteins, the blot was redeveloped with anti-GST serum (middle blot). The lower panel shows the equal expression levels of α_7B . 10 μ g of total cell lysate proteins were loaded per lane. The presence of the processed α_7B form indicates that the receptor was expressed on the cell surface.

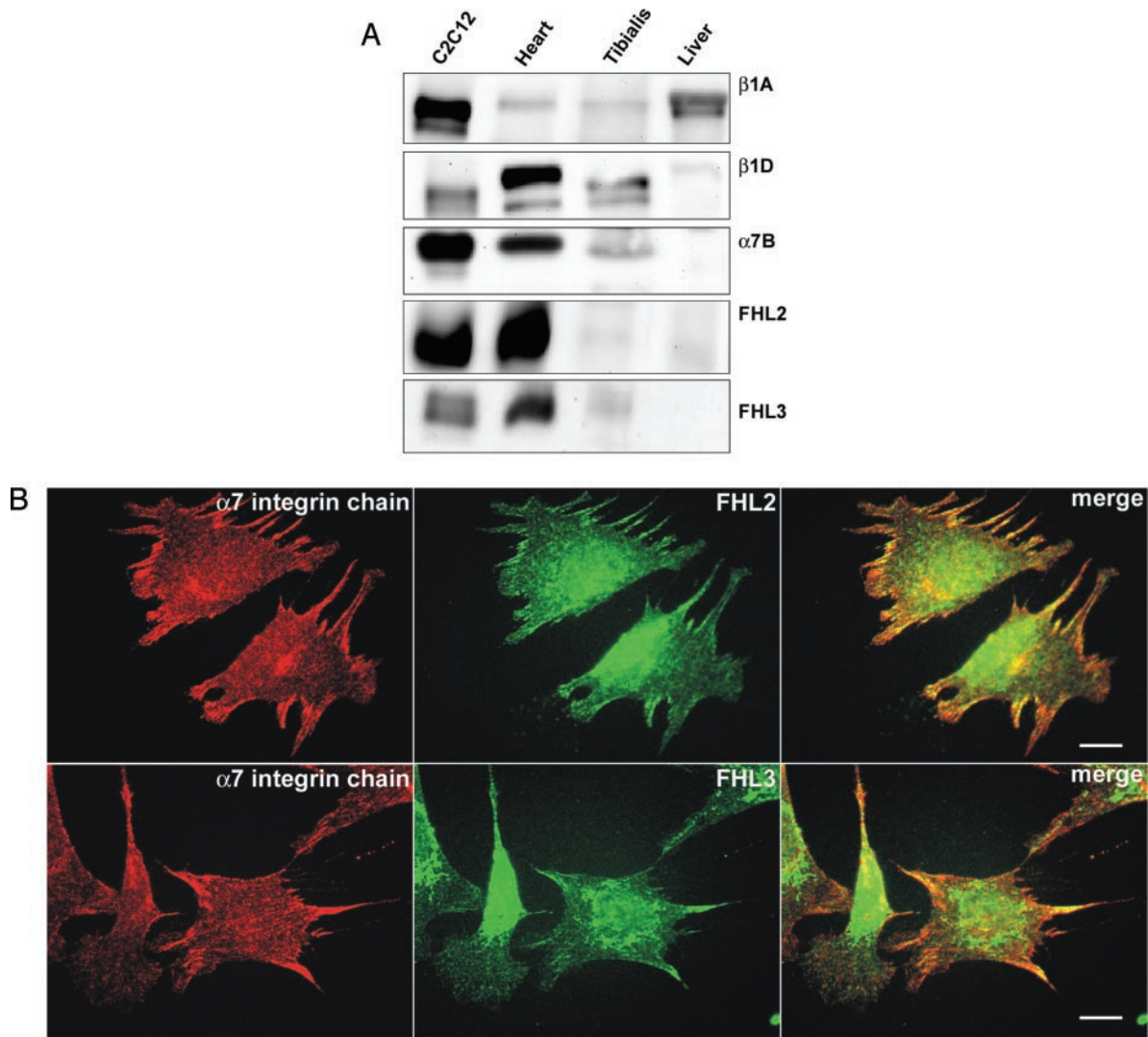


FIG. 2. Colocalization of FHL2 and FHL3 proteins with the muscle $\alpha_7\beta_1$ integrin receptor in myoblasts and skeletal muscles. *A*, C2C12 radioimmune precipitation assay buffer lysate and tissue samples lysed in reducing loading buffer were separated on a 10% acrylamide gel. For detection of integrin subunits, 20 μg of protein lysates were loaded. For revealing of FHL2, 10 μg of C2C12 protein and 30 μg of tissue protein were loaded, whereas for detection of FHL3, 20 and 60 μg , respectively, were used. *B*, C2C12 cells were set on laminin 1-coated coverslips (15 $\mu\text{g}/\text{ml}$) for 1.5 h and double immunostained for the integrin α_7 subunit with the 3C12 monoclonal antibody (*left panel, red*) and for FHL2 or FHL3 with rabbit polyclonal antibodies (*middle panel, green*). *C*, confocal microscopy images of the mouse heart. Sections were double immunostained with rabbit anti-FHL2 (*upper panels, red*) and polyclonal rat anti- $\alpha_7\beta_1$ integrin antibodies (*middle panels, green*). The *arrowheads* indicate colocalization of FHL2 with integrin $\alpha_7\beta_1$ at the plasmalemma. *n*, propidium iodide-stained nuclei. *Bars*, 4 μm . *D*, confocal microscopy images of the mouse tibialis anterior muscle. Sections were double immunostained with rabbit anti-FHL3 (*upper panels, red*) and polyclonal rat anti- $\alpha_7\beta_1$ integrin antibodies (*middle panels, green*). *Bars*, *right panels*, 20 μm ; *left panels*, 16 μm . Stainings with secondary antibodies only were used as negative controls (not shown).

$\alpha_7\beta_1$ integrin molecule consisting of the cytoplasmic and extracellular domains (Fig. 1C, lanes 4 and 5).

Fig. 2A shows that both $\alpha_7\beta_1$ integrin receptor and FHL2 and FHL3 proteins are expressed in myoblasts as well as in muscle tissues. However, the coprecipitation of endogenously expressed $\alpha_7\beta_1$ integrin and FHL proteins has been proved to be difficult, regardless of cell or tissue type we used (C2C12 myoblasts, skeletal or heart muscle) or lysis conditions we tried. We therefore analyzed the interaction of endogenous FHL proteins and α_7 integrin subunit with immunofluorescence technique in cells under conditions where the $\alpha_7\beta_1$ integrin receptor was functionally active. For this purpose, we used C2C12 mouse myoblasts, which endogenously express FHL2, FHL3, and the integrin $\alpha_7\beta_1$ subunit as a heterodimer with the β_{1A} (Fig. 2A and Ref. 40). The cells were plated on laminin-1, a substrate for $\alpha_7\beta_1$ integrin (41). The α_7 -specific monoclonal 3C12 antibody showed that the integrin receptor was preferentially localized at focal adhesion sites (Fig. 2B, *left panel* in *red*). The rabbit

polyclonal antibodies to both FHL2 and FHL3 revealed mainly the same immunostaining expression pattern (*middle panel* in *green*), and the merged images (*right panel*) demonstrated a colocalization of FHL2 and FHL3 with the integrin $\alpha_7\beta_1$ subunit.

We next analyzed whether FHL2 and FHL3 also colocalize with the $\alpha_7\beta_1$ integrin receptor in muscle tissues. Confocal microscopy analysis of FHL2 in cardiac and FHL3 in skeletal muscle sections (tibialis anterior) showed a costameric distribution pattern for both FHL proteins and for $\alpha_7\beta_1$ integrin (Fig. 2, C and D), which reflects the already published distribution of these proteins in Z-lines (20, 40, 42). Whereas FHL2 and FHL3 were mostly located at Z-discs, the $\alpha_7\beta_1$ receptor showed the highest distribution at the lateral plasmalemma of muscle fibers, exactly where FHL and integrin molecules colocalize (Fig. 2, C and D). The preferential localization of FHL3 in Z-discs was especially obvious while “scanning” along the vertical axes of cross-sectioned skeletal muscle with the help of the

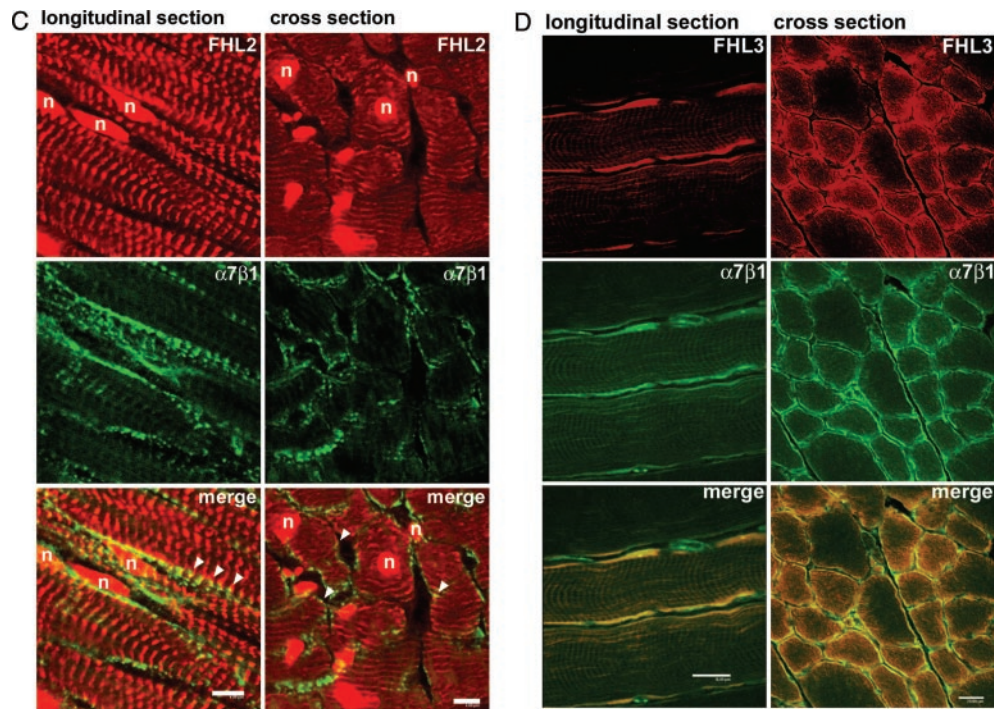


FIG. 2—continued

confocal microscope. Interestingly, FHL3 (Fig. 2D, left panel) did not show a uniform distribution in different muscle fibers.

Mapping of FHL Binding Sites on α_{7A} and α_{7B} Integrin Subunits—FHL2 and FHL3 are expressed concurrently in striated muscle, and since both proteins can bind α_{7A} and α_{7B} integrin splicing variants, it was of interest to know whether they use the same site on the integrin for interaction. To study this, a panel of deletion mutants of both integrin chains were created and tested for interaction with FHL2 and FHL3 in direct yeast two-hybrid assays. As shown in Fig. 3A, FHL2 and FHL3 still associate with α_{7A} subunit, when the last 10 COOH-terminal aa were deleted. However, whereas truncation of the following 13 aa abolished the binding of FHL3, deletion of an additional 12 aa was needed to abrogate the association with FHL2. A similar situation was detected in the case of α_{7B} deletion mutants (Fig. 3B), where again the minimal necessary binding sites for FHL2 and FHL3 were different. The site for FHL3 was always localized more toward to the COOH terminus than that for FHL2. Interestingly, the amino acid sequences of α_{7A} and α_{7B} representing the binding sites for the two LIM domain proteins do not share any common motifs.

FHL2 and FHL3, but Not FHL1, Bind to Intracellular Parts of Several Other Integrins—Here we describe for the first time that FHL3 associates with the α_7 integrin subunit. Previously, FHL2 has been shown to interact with several α - and β -integrin chains (6). In addition to FHL2 and FHL3, striated muscle tissues also express FHL1, a third member of the four and one-half LIM domain protein subfamily. We therefore extended our interaction analysis and tested whether all three muscle-expressed FHL proteins are able to bind diverse integrins (Table I). Whereas the binding patterns of FHL2 and FHL3 were similar, FHL1 did not show an interaction with any of the tested integrin subunits, despite its high similarity to the two other FHL proteins. Both FHL2 and FHL3 interact with the two related laminin-binding α_7 and α_3 integrin chains as well as with the β_1 splice forms, β_{1A} and β_{1D} . Only the strength of bindings varied between FHL2 and FHL3 according to the β -galactosidase test. Deletion analysis of the β_{1A} chain revealed that the binding sites for FHL2 and FHL3 overlapped

and were located at the COOH terminus.² Interestingly, FHL3 showed an ability to bind also to the α_v chain, which was not the case for FHL2 (Table I).

FHL2 and FHL3 interacted with all α - and β -chain splice variants of the $\alpha_7\beta_1$ integrin heterodimer, which connects muscle cells to the surrounding basement membrane via binding to laminin-2 and -4. An additional laminin-binding receptor abundantly expressed in muscle and connecting the cytoskeleton to the basement membrane is dystroglycan. However, interaction analysis with the cytosolic part of β -dystroglycan clearly demonstrated no binding to the FHL proteins (Table I).

To assure that only FHL2 and FHL3 but not FHL1 bind the major muscle $\alpha_7\beta_1$ integrin *in vivo*, we transiently transfected C2C12 myoblasts with Myc-tagged FHL proteins and analyzed their coexpression in cells that were adhered to laminin-1. Consistent with our yeast two-hybrid data, only FHL2 and FHL3 and not FHL1 showed a colocalization with the activated $\alpha_7\beta_1$ integrin receptor. Furthermore, only FHL2 and FHL3 but not FHL1 were present in $\alpha_7\beta_1$ integrin-mediated focal adhesion structures (Fig. 4).

Many LIM domain proteins associate with the actin cytoskeleton, either via direct binding to F-actin or via actin-associated proteins. In our immunofluorescence studies of FHL2 and FHL3, we noticed that they often localize along the F-actin cables as well as in focal adhesion structures (Figs. 4–6). However, we could not show any direct binding to either α - or β -actin molecules using the yeast two-hybrid system described (Table I).

FHL2 and FHL3 Localize to Cell Adhesion Complexes Independently of Cell Type and ECM Substrate—Since integrins are involved in the nucleation and clustering of cytosolic proteins after cells have attached to ECM, we next analyzed the importance of different matrices for the recruitment of FHL proteins into these structures. C2C12 myoblasts were plated on different ECM substrates and analyzed by immunofluorescence mi-

² T. Samson, N. Smyth, S. Janetzky, O. Wendler, J. M. Müller, R. Schüle, H. von der Mark, K. von der Mark, and V. Wixler, unpublished data.

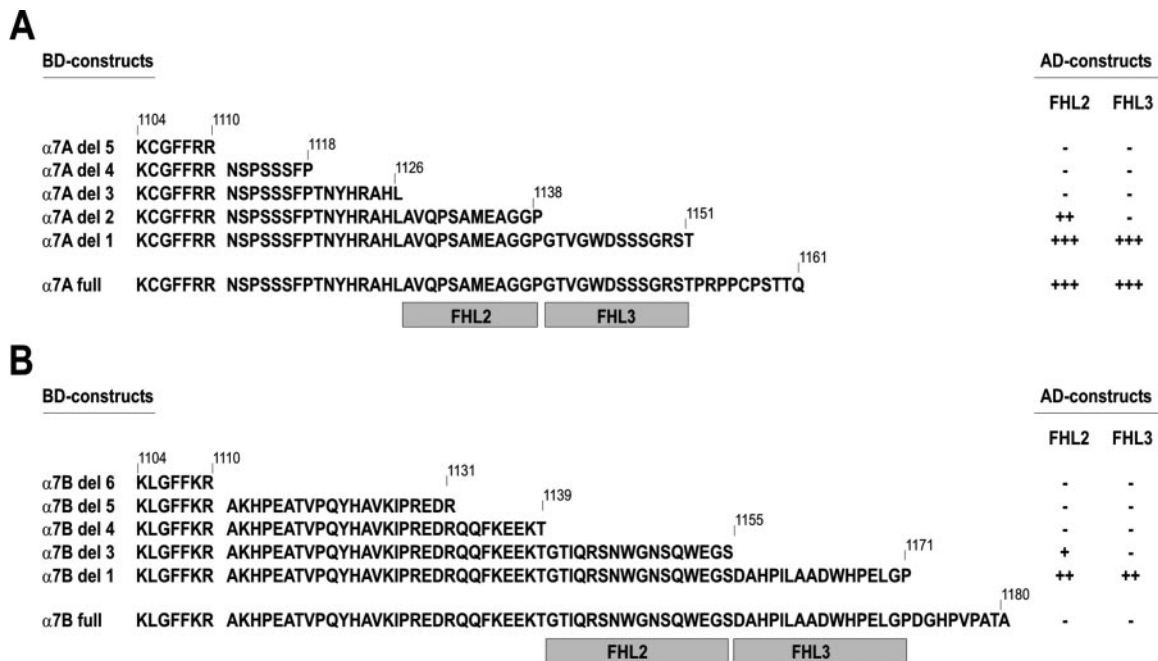


FIG. 3. Mapping of binding sites for FHL2 and FHL3 on α_7A and α_7B integrin subunits. Yeast Y190 cells were cotransformed with chimeric constructs of GAL4-BD and GAL4-AD. Protein-protein interactions were evaluated by a β -galactosidase filter assay as described under "Experimental Procedures." Sequences of cytoplasmic parts of integrin α_7 chains are shown. The numbers above the diagrams indicate aa sequences encoded by each construct. The membrane-proximal conserved region of integrin α_7A and α_7B subunits are separated from the unique COOH-terminal sequences by a space. The gray boxes below full-length sequences represent the binding sites for FHL2 or FHL3.

TABLE I

Specificity of interaction of FHL proteins with cytoplasmic parts of different integrin chains as well as β -dystroglycan and α - and β -actin

Yeast Y190 cells were cotransformed with GAL4-BD (BD) and GAL4-AD (AD) chimeric constructs. The interaction was evaluated using a β -galactosidase filter assay as described under "Experimental Procedures." The known interaction of FHL proteins with themselves was used as a positive control, and the empty GAL4-BD vector was used as a negative control.

| BD/AD cotransformation | FHL1 | FHL2 | FHL3 |
|------------------------|------|------|------|
| α_1 | - | - | - |
| α_2 | - | - | - |
| α_{3A} | - | +++ | ++ |
| α_{3B} | - | ++ | +++ |
| α_5 | - | - | - |
| α_{6A} | - | - | - |
| α_{6B} | - | - | - |
| α_7A | - | +++ | +++ |
| α_7B | - | ++ | ++ |
| α_v | - | - | ++ |
| β_{1A} | - | +++ | ++ |
| β_{1D} | - | ++ | +++ |
| β -Dystroglycan | - | - | - |
| α -Actin | - | - | - |
| β -Actin | - | - | - |
| FHL1 | - | - | - |
| FHL2 | - | +++ | +++ |
| FHL3 | - | +++ | +++ |
| BD vector | - | - | -/+ |

microscopy. Again only FHL2 and FHL3 but not FHL1 were localized in focal adhesion complexes (Fig. 5). The inability of FHL1 to locate to focal adhesions was independent of the tag we used (Myc or GST) and of whether a tag was located at the NH₂ or at the COOH terminus.² The FHL2 and FHL3 recruitment was also independent of the substrate on which the cells were plated (fibronectin, vitronectin, laminin-1, or collagen-1). Since integrin β_{1A} associates with diverse α -subunits, hence building receptors for the vast majority of ECM molecules, these results imply that the localization of FHL2 and FHL3 at

focal adhesion sites is mediated via binding to β_{1A} integrin chain. To exclude the possibility that FHL1 might be localized into focal adhesion structures via binding to other integrins or linker proteins not tested here or present in C2C12 myoblasts, we compared the subcellular localization of FHL proteins in cells representing different types of tissues. In addition to myoblasts, fibroblasts, endothelial, and epithelial cells were tested. Again, only FHL2 and FHL3 but not FHL1 showed a localization at focal adhesion sites, independent of the cell type and the substrate on which the cells were plated.²

We then questioned whether FHL proteins can locate into cell adhesion complexes simultaneously and whether they might be recruited there by heterodimerization with each other. Therefore, we investigated whether FHL proteins could form homo- and heterodimers in mammalian cells. GST- and Myc-tagged FHL proteins were coexpressed in HEK293 cells, and the existence of FHL dimers was analyzed by immunoblotting of GST precipitates. In agreement with the yeast two-hybrid data, FHL2 and FHL3 formed homodimers (Fig. 6A, lanes 7 and 12) and heterodimers (lanes 8 and 11) with each other. Surprisingly, FHL1 also showed a self-association (lane 2) and a heterodimerization with both FHL2 (lanes 3 and 6) and FHL3 (lanes 4 and 10), in contradiction to the yeast two-hybrid data (Table I). The FHL1 coprecipitation appeared robust, not being abrogated even under harsh washing with radioimmune precipitation assay buffer containing high salt concentrations or up to 15% methanol.² Whether the FHL1 interactions also occur in cells *in vivo* and whether they are direct or indirect we cannot conclude from these experiments.

Next we studied the colocalization of FHL proteins at the plasma membrane. C2C12 myoblasts were transiently transfected with expression plasmids coding for Myc-tagged FHL proteins and for enhanced green fluorescent protein-tagged FHL2. After plating cells on laminin-1, the subcellular distribution of expressed proteins was analyzed by immunofluorescence microscopy. Fig. 6B shows that FHL2 colocalizes in focal adhesion structures with itself and with FHL3, but not with FHL1. The data suggest that FHL proteins are recruited into

FIG. 4. FHL2 and FHL3 but not FHL1 are colocalized with the muscle $\alpha_7\beta_1$ integrin receptor. C2C12 myoblasts stably expressing ectopic integrin α_{7A} subunit in addition to the endogenous α_{7B} subunit were transiently transfected with Myc-tagged FHL1, FHL2, or FHL3 and set on laminin-1-coated coverslips (15 $\mu\text{g}/\text{ml}$) for 1.5 h. Cells were double-immunostained with anti- α_7 integrin mAb 3C12 (*left panel, red*) and rabbit polyclonal anti-Myc antibody (*middle panel, green*) and analyzed by fluorescence microscopy. Whereas FHL1 is distributed in the nucleus and cell body, FHL2 and FHL3 colocalize together with integrin α_7 at cell adhesion complexes. *Bar, $\sim 10 \mu\text{m}$.*

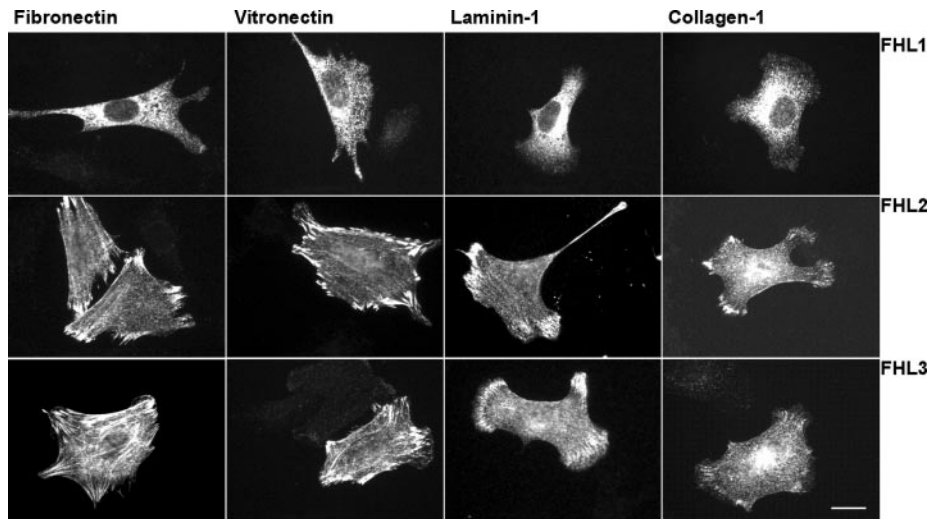
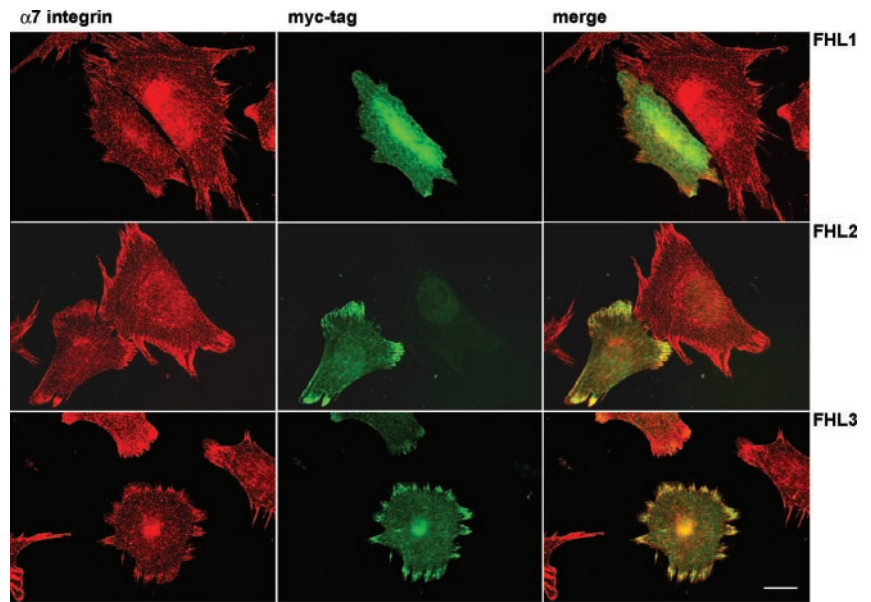


FIG. 5. FHL2 and FHL3 but not FHL1 are localized at cell adhesion structures independently of the substrate or the cell type. Mouse C2C12 myoblasts were transiently transfected with Myc-tagged FHL1, FHL2, or FHL3 cDNAs. 24 h later, they were trypsinized and plated for 1.5 h on coverslips coated with ECM substrates as indicated. The presence of FHL proteins at cell adhesion structures was verified by immunostaining with anti-Myc mAb. The location of FHL2 and FHL3 in the focal adhesions is not influenced by the availability of the used ECM molecules. FHL1 is not present in focal adhesions irrespective of which ECM substrate was provided. *Bar, $\sim 10 \mu\text{m}$.*

focal adhesion structures via direct association with integrins and not via binding to other proteins.

Determination of LIM Domains Important for FHL-Integrin and FHL-FHL Interactions—LIM domain proteins act as adaptors that exercise their function via binding to other proteins. It has been reported that a single or even a half LIM module may act as a protein-binding domain (43, 44). To understand the molecular mechanisms, we defined the interaction domains of FHL2 and FHL3 for their integrin binding partners and for homo- and heterodimerization. Using COOH- and NH₂-terminal deletion mutants of FHL2 and FHL3, we clearly showed that the suprastructure formed by more than one LIM domain is needed for binding to most of the tested proteins (Fig. 7). Indeed, deletion of the NH₂-terminal half LIM domain resulted in significant decrease or complete loss of interaction in certain cases. A combination of only two LIM domains (LIM 3 and 4) was sufficient for homodimerization of FHL2, albeit with lower efficiency (Fig. 7A). The combined structure of the first and second LIM domains of FHL3 was enough for FHL2/FHL3 heterodimerization (Fig. 7B). A single LIM domain was suffi-

cient for protein-protein association in only one case out of 16 FHL2-interacting proteins we tested: LIM2 of FHL2 for interaction with integrin α_{7A} (Fig. 7 and Refs. 6 and 26). Hence, the three-dimensional structure of whole FHL proteins seems to be of greater importance for functional integrity than the structure of a single LIM domain.

FHL Proteins Localize to Focal Adhesion Complexes via Binding to Both α - and β -Integrin Subunits—Integrins always occur *in vivo* as $\alpha\beta$ heterodimers. We showed here that FHL proteins are able to bind to both chains of an integrin receptor. To test whether the integrin-mediated recruitment of FHL proteins to the membrane compartment is due to the association only with the β -subunit or whether the interaction with the α -chain also supports this process, we transfected C2C12 myoblasts with FHL3 mutants harboring different integrin-binding properties according to the results shown in Fig. 7. The cells were subsequently plated on laminin-1 or fibronectin and analyzed by immunofluorescence microscopy. As expected, the FHL3(LIM1–4) mutant, which still binds to the β_{1A} integrin chain, was also localized in focal adhesions of cells attached on

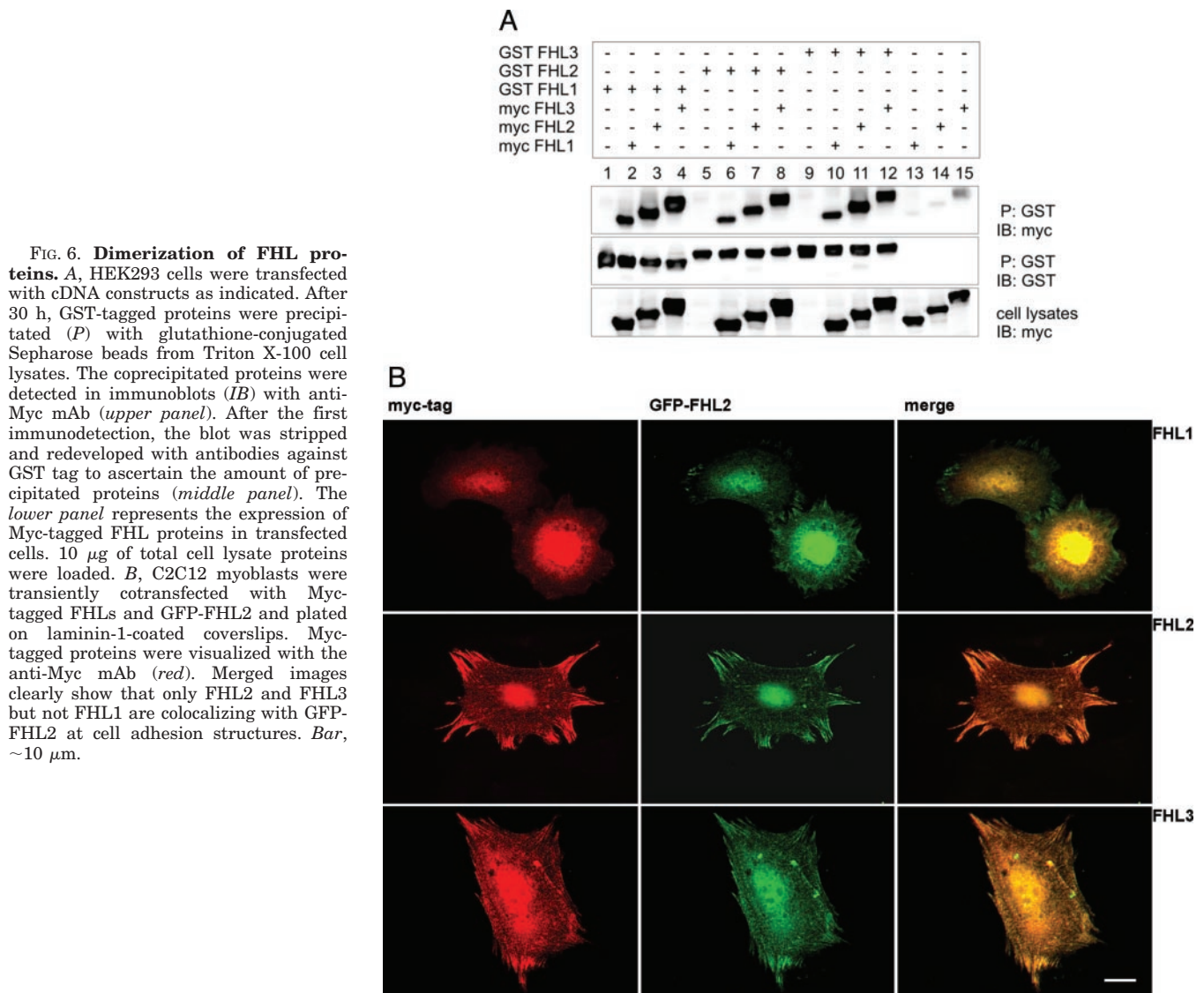


FIG. 6. Dimerization of FHL proteins. *A*, HEK293 cells were transfected with cDNA constructs as indicated. After 30 h, GST-tagged proteins were precipitated (*P*) with glutathione-conjugated Sepharose beads from Triton X-100 cell lysates. The coprecipitated proteins were detected in immunoblots (*IB*) with anti-Myc mAb (*upper panel*). After the first immunodetection, the blot was stripped and redeveloped with antibodies against GST tag to ascertain the amount of precipitated proteins (*middle panel*). The *lower panel* represents the expression of Myc-tagged FHL proteins in transfected cells. 10 μ g of total cell lysate proteins were loaded. *B*, C2C12 myoblasts were transiently cotransfected with Myc-tagged FHLs and GFP-FHL2 and plated on laminin-1-coated coverslips. Myc-tagged proteins were visualized with the anti-Myc mAb (*red*). Merged images clearly show that only FHL2 and FHL3 but not FHL1 are colocalizing with GFP-FHL2 at cell adhesion structures. *Bar*, $\sim 10 \mu$ m.

both substrates (Fig. 8, *upper panel*). The FHL3(LIM $\frac{1}{2}$ -2) truncated protein, which lost the binding capacity to all analyzed integrin chains, showed no membrane staining (Fig. 8, *lower panel*). The FHL3(LIM2-4) mutant, which does not interact with the β_1 integrins or FHL2, was located in focal adhesions only when the cells were attached to fibronectin but not to laminin-1 (Fig. 8, *middle panel*). C2C12 myoblasts do not endogenously express the α_{7A} integrin subunit but α_v . Therefore, the FHL3(LIM2-4) mutated protein might be recruited to the membrane only via the α_v -chain, a subunit of several fibronectin-binding receptors that do not bind laminin-1. Interestingly, the FHL3(LIM $\frac{1}{2}$ -2) mutant, which is still able to interact with FHL2 (Fig. 7B), is not presented in focal adhesions, despite the high expression level of endogenous FHL2 in C2C12 myoblasts. This supports the suggestion that only a direct binding to integrin chains assures the membrane localization of FHL proteins. Taken together, these data show that FHL proteins can be localized to the membrane compartment via binding to either β - or α -subunits of an integrin receptor and that this recruitment occurs directly and cannot be mediated via other FHL proteins.

FHL Proteins Do Not Influence Attachment and Migration Behavior of C2C12 Myoblasts—A major task of integrins is adhesion of cells to ECM proteins and supporting cell migration. Since FHL proteins bind not only particular α -chains but

also the ubiquitously present β_{1A} integrin chain, we further tested the influence of FHL proteins on these particular integrin functions. We created C2C12 cell lines stably expressing Myc-tagged FHL proteins (Fig. 9A). Seeding these cells on plates coated with increasing amounts of laminin 1 clearly showed no difference between FHL-expressing and control cells (Fig. 9B). The same results were obtained when these cells were plated on fibronectin as a substrate or when FHL-overexpressing HEK293 cells were used in analogous experiments.² Similarly, no change in migration velocity was measured in C2C12 myoblasts (Fig. 9C) or in HEK293 cells overexpressing FHL proteins, regardless of the substrate on which they were plated.² This indicates that FHL2 and FHL3 do not influence cell attachment and migration, despite their binding to α - and β -integrin chains and their presence in focal adhesions.

DISCUSSION

We identified FHL2 and FHL3 as proteins that specifically interact with the muscle integrin $\alpha_7\beta_1$ receptor. In addition to this, the two LIM-only proteins FHL2 and FHL3 showed a much broader integrin binding capacity. In detail, they bind to α_{7A} , α_{7B} , α_{3A} , and α_{3B} subunits, to α_v (FHL3 only), and, surprisingly, also to the β_1 chains of the integrin receptors. FHL2 and FHL3 are four and one-half LIM domain adaptor proteins. They are protein-protein interaction molecules, assembling

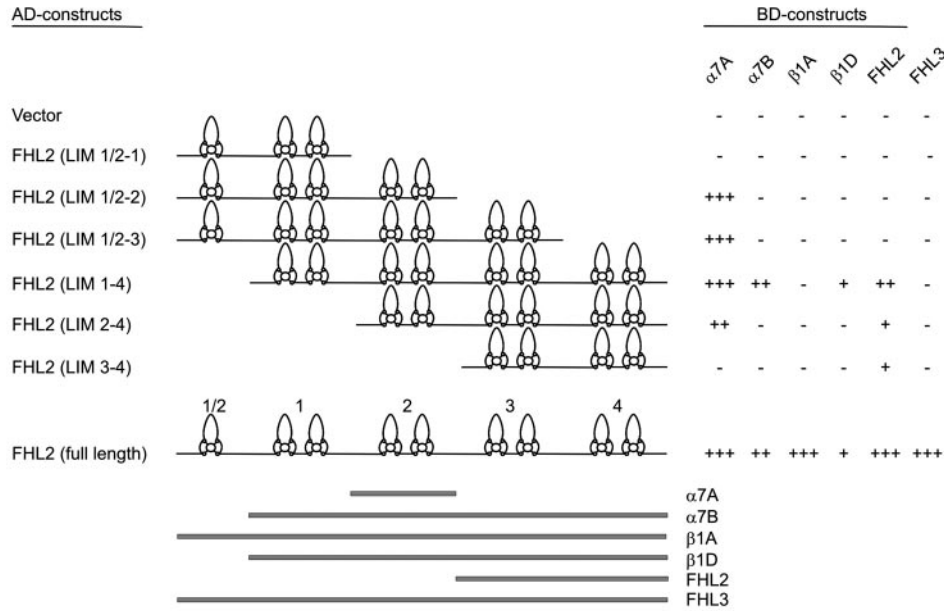
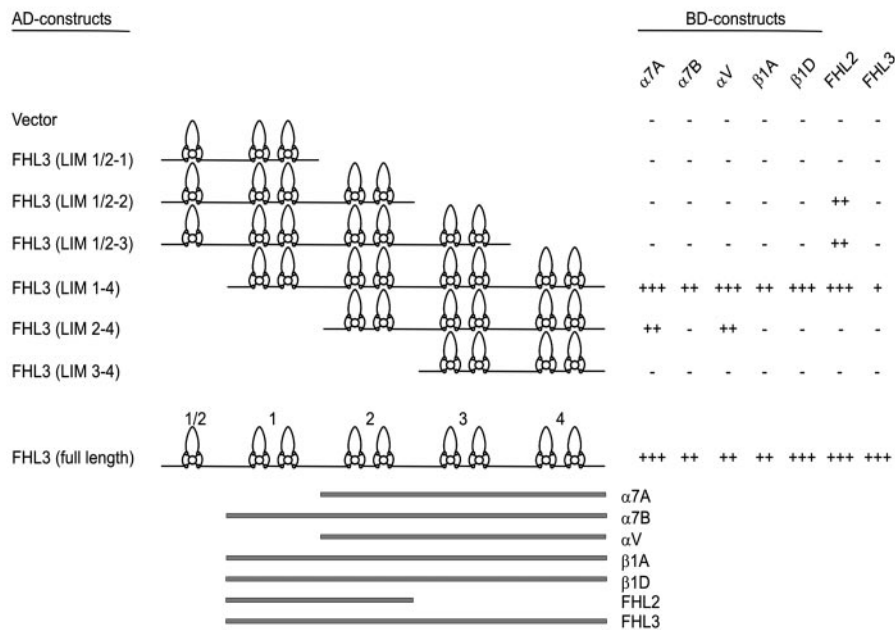
A**B**

FIG. 7. Determination of minimal binding sites within FHL2 and FHL3 for integrin subunits and for homo- and heterodimerization. Yeast Y190 cells were cotransformed with deletion mutants of either GAL4-AD-FHL2 (A) or GAL4-AD-FHL3 (B) and with GAL4-BD-cDNA constructs of their numerous interacting proteins as indicated. The interactions were evaluated by a β -galactosidase filter assay as described under "Experimental Procedures." The gray boxes below full-length sequences of FHL2 or FHL3 depict combinations of LIM domains needed for association with particular interaction partners.

multiple protein complexes (44, 45). The high homology between FHL2 and FHL3 and the similar tissue distribution may explain their comparable binding spectrum with respect to integrin chains, and indeed both bind the COOH terminus (containing the conserved NPXY motif) of the β_1 integrin. However, FHL2 and FHL3 use different sequence motifs for association with the α -chains, thus indicating that any specific functional consequences of the interaction with integrins are determined by α - rather than β -subunits.

Interestingly, despite the more central location of binding sites for FHL2 and FHL3 proteins on the α_7B sequence, our

yeast two-hybrid interaction tests were always negative when the full-length α_7B cytodomain (aa 1104–1180) was used. Only the truncated form of α_7B lacking the 9 most COOH-terminal amino acids (DGHPVPATA) showed a stable and reproducible interaction with both FHL2 and FHL3. This COOH-terminal region may be a negative regulator, since in several previous yeast two-hybrid screens with two different cDNA libraries we failed to isolate any interacting proteins when the full-length α_7B cytodomain was used as "bait."² However, the nucleotide exchange factor Mss4 that interacts with the membrane-proximal conserved region of integrin α -chains also associated with

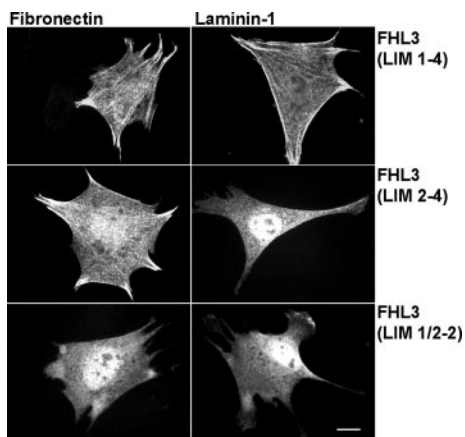


FIG. 8. FHL proteins localize to focal adhesion complexes via binding to either α - or β -integrin subunits. C2C12 myoblasts were transiently transfected with indicated expression constructs. After attachment on fibronectin- or laminin-1-coated coverslips, the cells were analyzed by immunofluorescence microscopy as described in the legend to Fig. 5 and under "Experimental Procedures." Bar, $\sim 10 \mu\text{m}$.

α_{7B} (12).² This interaction was only weak with the full-length but very strong when the truncated α_{7B} cytodomain was used. Due to these findings, we used the α_{7B} part containing amino acids 1104–1171 in the current yeast two-hybrid screen. One explanation for this interaction behavior could be that the COOH-terminal region interacts with the more membrane proximal part of the molecule, forming a loop and inhibiting binding of other proteins to α_{7B} as well as activation of the $\alpha_{7B}\beta_1$ integrin. Conformational changes induced through binding of the receptor to ECM (outside-in signaling) or through binding of particular cytosolic protein(s) (inside-out signaling) might unfold the α_{7B} chain and activate the integrin receptor. A similar situation has recently been proposed for $\alpha_{11b}\beta_3$ integrin (46). Interestingly, a possible change in conformation of the α_{7B} cytodomain upon binding to laminin has been suggested already by Song *et al.* (47). Whether the α_{7B} -interacting proteins isolated in our screen have an influence on the conformational state of the integrin receptor is currently under investigation.

LIM domains are double zinc finger motifs that fold independently and act as protein binding interfaces. In terms of this, we expected that particular LIM domains would be responsible for binding to different integrin chains. However, our analysis of FHL2 and FHL3 deletion mutants did not support this assumption. In most cases, deletion of any LIM domain resulted in decreasing or even complete abrogation of protein-protein association. Despite the ability to form homo- and heterodimers, the scaffolding capacity of FHL proteins seems to be more limited than it was assumed originally (43, 44). In view of the numerous interaction partners, described here and elsewhere, we suggest that the association with most of them may take place only in a distinctive and exclusive manner with rather fewer interactions occurring for any individual molecule than originally believed.

The presence of FHL proteins in focal adhesion structures has been noted in earlier reports (6, 20, 48, 49). However, this is the first detailed molecular analysis of this phenomenon. We show that FHL2 and FHL3 but not FHL1 bind to diverse α - and β -subunits of integrin receptors. These interactions are direct and specifically responsible for location of FHL2 and FHL3 at cell adhesion sites. Truncation of any part of FHL molecules not only affected the interaction with integrins but also weakened or abrogated the focal adhesion localization of FHL proteins. The study of FHL3 truncated mutants further confirmed that the recruitment into cell adhesion sites is integrin-depend-

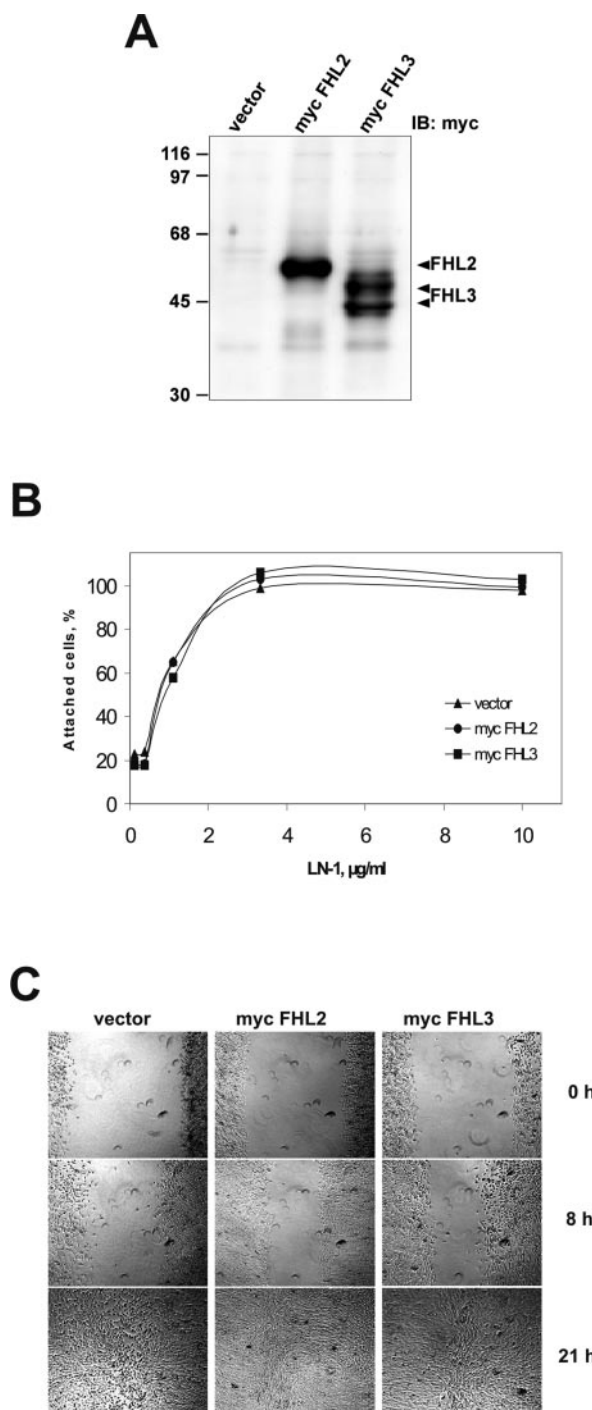


FIG. 9. FHL2 and FHL3 proteins do not influence attachment and migration of C2C12 myoblasts. A, $10 \mu\text{g}$ of total C2C12 cell lysate proteins were separated on 10% SDS-PAGE. The expression of ectopic FHL proteins in C2C12 sublines selected for G418 resistance was verified by immunoblotting (IB) with anti-Myc 9E10 mAb. The Myc-FHL3 protein appears as a doublet. B, logarithmically growing cells were trypsinized and washed once with DMEM plus 0.5% BSA, and 5×10^4 cells were plated in DMEM plus 0.5% BSA into microtiter wells previously coated with increasing amounts of purified laminin 1. Cell adhesion was measured as described under "Experimental Procedures." Each measurement was done in triplicate. The complete experiment was repeated three times, and mean values are presented. C, 1×10^5 cells were plated into 48-well plates precoated with laminin 1 as described under "Experimental Procedures," and pictures of cell-free "windows" were taken at the times indicated.

ent and showed that it may also occur via binding to α -chain only. Moreover, we could show that despite the ability to form dimers, the FHL proteins cannot be recruited into focal adhe-

sions via interaction with each other, since only the FHL2 and FHL3 truncated forms that bind integrins were located with them at the membrane. Consistent with the fact that they associate with the ubiquitously expressed β_{1A} subunit, FHL2 and FHL3 were presented at cell adhesion sites in all tested cell types independent of the substrate on which the cells were attached. The highly related FHL1 protein was not localized in focal adhesions under any conditions we tested, despite its ability to coprecipitate with other FHL proteins. This is in agreement with the inability of FHL1 to associate with integrins and the finding that FHL-FHL interactions are unable to lead to focal adhesion recruitment. However, it is in contrast with the variable staining pattern described for FHL1 when overexpressed in Cos7 or Sol8 cells by Brown *et al.* (49–51). This was claimed to depend upon the activation state of the integrin receptors, but here we clearly show that activation of integrins (independent of substrates and cell types we used) does not alter the cytoplasmic distribution of FHL1.

It is not clear why FHL2 and FHL3 should bind to both subunits of a particular heterodimeric receptor. By analogy to talin, simultaneous binding of an FHL protein to integrin α - and β -chains possibly ensures separation of them from each other, leading thus to stable activation of the receptor (3). However, neither FHL2 nor FHL3 overexpression altered cell attachment or cell migration, two functions that need activated integrins. Further expression of FHL proteins in cells, including expression of FHL2 in rhabdomyosarcoma, where it is naturally down-regulated, did not change the quality of immunofluorescence staining of integrin receptors or their binding partners such as vinculin or paxillin.²

Both α_{7A} and α_{7B} subunits are highly concentrated at myotendinous and neuromuscular junctions of adult muscles, and α_{7B} is additionally distributed at sarcolemma of muscle cells (52, 53). Together with the β_{1D} subunit, they connect the cytoskeleton and contractile elements to the ECM (53), enabling the transformation of contracting forces into movement. The major structures that resist the mechanical forces are the sarcomeric Z-discs and myotendinous junctions. In cardiomyocytes, only the α_{7B} isoform is expressed, which is present as a heterodimer with the β_{1D} chain at costamers and intercalated discs (40). Our immunofluorescence studies of muscle tissue let us assume that the association of FHL proteins to the $\alpha_7\beta_1$ integrin receptors plays a role in mechanical stabilization of muscle cells. Indeed, the $\alpha_7\beta_1$ was colocalized at costamers with FHL2 in heart and with FHL3 in skeletal muscles. A dual binding of FHL2 and FHL3 to both α - and β -integrin chains might provide a stronger connection of the receptor to the cell interior than only a single interaction. A binding of FHL2 and FHL3 to actin molecules has recently been published (42), which would suggest that the LIM-only proteins directly link the cytoskeleton to the ECM via binding to integrins and actin. In contrast, our yeast two-hybrid experiments with monomeric α or β G-actin molecules failed to confirm this observation. However, we cannot exclude the possibility that FHL2 and FHL3 do bind to F-actin. The immunofluorescence staining of myoblasts or other cell types supports this suggestion.

An additional important function of integrins is their involvement in the formation of the ECM. Our preliminary results on studying the influence of the overexpression of FHLs on fibronectin matrix assembly or on matrix metalloproteinases secretion suggest that they have little effect.² Whether they might regulate the expression of other ECM proteins (*e.g.* laminins) is not known. Taking into account the Rho-dependent shuttling of FHL2 and FHL3 into the nucleus and their transcriptional regulation properties (30), this is an intriguing assumption and is currently under investigation.

In conclusion, we identified and characterized in depth for the first time proteins that associate with all spliced isoforms of the $\alpha_7\beta_1$ muscle integrin receptor and showed that they are codistributed at plasmalemma in adult muscles. Given that FHL2 and FHL3 are adaptor proteins with numerous interaction partners as well as nuclear shuttling proteins with transcriptional activities, the elucidation of precise molecular mechanisms underlying these interactions will open new insights into the role of the muscle integrin receptor.

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