The integrin α7β1 is the major laminin-binding integrin in skeletal, heart, and smooth muscle and is a receptor for laminin-1 and -2. It mediates myoblast migration on laminin-1 and -2 and thus might be involved in muscle development and repair. Previously we have shown that α7β1 integrin regulates cell adhesion and migration. Deletion of the cytoplasmic domain did not affect assembly of the mutated α7β1-integrin regulating cell adhesion and migration. Deletion of the cytoplasmic domain did not affect assembly of the mutated α7β1-integrin regulating cell adhesion and migration. Deletion of the cytoplasmic domain did not affect assembly of the mutated α7β1. The motility of these cells on the laminin-1/E8 fragment, however, was significantly reduced to the level of mock-transfected cells; lamellipodia formation and polarization of the cells were also impaired. Adhesion to the laminin-1/E8 fragment induced tyrosine phosphorylation of the focal adhesion kinase, paxillin, and p130CAS as well as the formation of a p130CAS-Crk complex in wild-type α7β1-transfected cells. In α7β1-null cells, however, the extent of p130CAS tyrosine formation was reduced and formation of the p130CAS-Crk complex was impaired, with unaltered levels of p130CAS and Crk protein levels. These findings indicate adhesion-dependent regulation of p130CAS and Crk complex formation by the cytoplasmic domain of α7β1 integrin after cell adhesion to laminin-1/E8 and imply α7β1-controlled lamellipodia formation and cell migration through the p130CAS-Crk protein complex.

During muscle repair, undifferentiated muscle precursor cells, so-called satellite cells, are activated and migrate to sites of damaged muscle along the basement membranes of pre-existing muscle fibers to close the wound by proliferating and fusing (1, 2). In vitro, skeletal myoblasts have been shown to migrate on laminin (LN)1 1 (3), the laminin-1/E8 fragment that is the major laminin-binding integrin and in nervous tissue (32, 33). The extracellular and the intracellular domains of integrin α7β1 undergo developmentally regulated splicing (34–36); myoblasts express the cytoplasmic splice variant B and the extracellular splice variants X1 and X2.

Blocking integrin α7β1 antibodies inhibit the migration of myoblasts on laminin-1 and laminin-2, suggesting that α7β1 is responsible for myoblast migration on laminin (5, 29). Integrin α7β1 is mainly expressed in skeletal, smooth, and cardiac muscle (32), but also in some glioblastoma and melanoma cells (30, 31) and in nervous tissue (32, 33). The extracellular and the intracellular domains of integrin α7β1 undergo developmentally regulated splicing (34–36); myoblasts express the cytoplasmic splice variant B and the extracellular splice variants X1 and X2.

FITC, fluorescein isothiocyanate; DMM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; TBST, Tris-buffered saline plus Tween 20.

This paper is available on line at http://www.jbc.org
X. After myotube formation, the cytoplasmic splice variant A and C and the extracellular splice variant X2 become up-regulated. The α7 chain is post-translationally cleaved into a 97-kDa fragment and a 35-kDa fragment (sizes for the B splice variant), which contains a large piece of the extracellular part (~25 kDa) (11). In the mature integrin, the fragments remain disulfide-linked. Transfection of integrin α7 into α7-deficient cells induces cell migration specifically on laminin-1 and -2 (5, 37, 38).

In this study we investigated the role of the cytoplasmic domain of the α7 subunit in laminin-induced signaling. We deleted the cytoplasmic domain of α7 and transfected 293 cells with a construct encoding the extracellular splice variant X2 (α7X2; see X2⁎) to elucidate the role of the α7 cytoplasmic domain in terms of heterodimer formation, surface expression, integrin α7-mediated cell attachment, migration, and p130Cas/Crk coupling. Deletion of the α7 cytoplasmic domain did not affect receptor assembly or activity, as assessed by the ability of the mutant receptor to confer cell attachment. In contrast, cell migration, lamellipodia formation, and formation of the p130Cas/Crk signaling complex were reduced, highlighting a role for the α7 cytoplasmic domain in signal transduction.

**MATERIALS AND METHODS**

**Chemicals**—Chemicals were from Sigma or Roth (Karlsruhe, Germany) if not stated otherwise.

**Antibodies**—The affinity-purified polyclonal antibody U4 directed against a peptide of the integrin α7B cytoplasmic domain was kindly provided by Dr. Ulrike Mayer (39) and diluted 1:2000 for Western blotting. The rabbit antibody 242 E (directed against the α7 extracellular domain; Ref. 37) was diluted 1:200. The monoclonal anti-α7 mAbs 3C12 and 6A11 have been described previously (38), and the monoclonal anti-α7 mAb 5A6 will be described elsewhere.2 Anti-p130Cas, anti-Crk, anti-Erk, and anti-Shc antibodies used for Western blotting (goat anti-rabbit-peroxidase and anti-vinculin mAb 7F9) were generous gifts of Dr. Alexey Belkin, Grove, PA) and diluted 1:5000 and 1:20,000, respectively. FITC (fluorescein isothiocyanate)-labeled secondary antibodies were from Amer-Sham-Biocross (Braunschweig, Germany). FITC-phalloidin was from Molecular Probes (Leiden, Netherlands).

**Deletion of the Integrin α7 Cytoplasmic Domain**—The integrin α7X2 expression vector pCEF4α7X2 (38) was digested with NheI and HindIII, which removed the cDNA segment encoding for the cytoplasmic domain except for the first two membrane-proximal amino acid residues (Lys-Leu). Ends were filled with Klenow polymerase, and the plasmid was religated, which resulted in a stop codon after the residues Lys-Val. Plasmid DNA was purified according to the manufacturer’s instructions (Qiagen, Hilden, Germany) and digested with restriction enzymes.

**Cell Culture and Transfection**—293HEK-EBNA cells were obtained from Invitrogen (Groningen, Netherlands) and cultured in DMEM/F-12 (Life Technologies, Inc.) containing 5% fetal calf serum (FCS; S0215-Lot 290706, Life Technologies, Inc.) and 50 μg/ml of streptomycin, 50 μg/ml of penicillin, and 50 units of Penicillin/ml (Life Technologies, Inc.), 250 μg/ml G418 (Calbiochem, Bad Soden, Germany). Cells were kept in a humidified atmosphere containing 7.5% CO2. For certain experiments, cells were serum-starved by washing twice in serum-free medium and keeping them in serum-free medium for 24 h. The medium was replaced with serum-free medium 2 h before experiments, and for block of protein biosynthesis cycloheximide was added at a concentration of 25 μM and applied for 2 h. Trypsin was stopped with 1 mg/ml soybean trypsin inhibitor (Sigma) and 1% BSA (Sigma) in DMEM/F-12 under these conditions. For transfection, 105 HEK293-EBNA cells were seeded on 60-mm dishes and grown for 16 h. Cells were washed twice with PBS and once with OptiMEM (Life Technologies, Inc.). Cells were incubated with 600 μl of OptiMEM containing 10 μg of plasmid DNA and 15 μl of Lipofectin (Life Technologies, Inc.) for 6 h and then additionally 3 ml of DMEM/F-12 were added for 16 h. Medium was changed after 48 h, and cells were selected and maintained in culture medium containing 300 μg/ml hygromycin B (Roche Molecular Biochemicals, Mannheim, Germany).

**FACS Analysis**—FACS analysis was performed as described previously (37). Briefly, cells were trypsinized, washed, and resuspended in FACS-PBS (5% FCS in PBS containing 0.02% sodium azide) (2×10⁶ cells/ml) to 2×10⁶ cells/ml. Cells were incubated with primary antibodies (GoH3, 2 μg/ml; TS2/16, 3C12, 5A6, and 6A11, hybridoma supernatant) for 30 min on ice. Cells were washed twice with FACS-PBS and incubated with FITC-labeled secondary antibodies (1:200) for 30 min on ice, washed twice with FACS-PBS and fixed in 1% p-formaldehyde in PBS. FACS analysis was performed with a Coulter cytometer.

**Cell Migration Assay**—Flasks (25 cm²; Falcon) were coated with PBS-diluted PL (20 μg/ml) or LN-1/E8 fragment (2 μg/ml) for 1 h at 37 °C with a volume of 1.5 ml/25-cm² flask. Flasks were washed twice with PBS and blocked with 1% heat-denatured (30 min; 80 °C) BSA (Sigma; A7030) in PBS for 30 min at 37 °C and again washed twice with PBS. Cells were trypsinized, washed, and plated at a density of 2000 cells/cm² in 10 ml HEPES (pH 7.4)-buffered DMEM/F-12 containing anti-biotics and 5% FCS. The flask were allowed to equilibrate in a 7.5% CO2-containing atmosphere at 37 °C for 1 h, the lid was closed air-tight, and the cells were placed in a humidified chamber at 37 °C for 36 h. Cells were visualized with a Leitz ICM-405 microscope (Oberkochen, Germany). Migration was monitored by time-lapse video microscopy as described previously (37). Briefly, cells were filmed under low illumination with a CCD camera (JVC) connected to a time lapse video recorder triggered by an external timer. Pictures were taken every 2 min, and cells were recorded for >12 h. For analysis of cell migration, a set of 12 pictures in 1 h steps was imported in the MicroDraw program and cells were tracked manually by connecting the centers of the cell bodies of individual cells. Tracks of cells were digitalized and converted to pixels, which were converted to micrometers after calibration.

**Immunofluorescence Microscopy**—Cells were washed quickly three times with ice-cold PBS and fixed in 3.7% p-formaldehyde in PBS for 15 min at 4 °C, washed three times in PBS and permeabilized with 0.5% Triton X-100 in PBS for 30 min at room temperature. Cells were again washed twice with PBS and fixed for 30 min at room temperature with 3% BSA in PBS. FITC-phalloidin was diluted 1:1000 in 3% BSA in PBS and applied for 1 h at room temperature. Samples were washed three times for 5 min in PBS, mounted, and examined with a Zeiss Axioskop microscope equipped with a 63× oil immersion objective (numerical aperture 1.40).

**Cell Lysis, Immunoprecipitation, and Western Blot Analysis**—Cell lysis was performed in two ways, depending on the application. Cells were washed once with ice-cold PBS and lysed with 1 ml of buffer of 107 cells. Condition A (LN-1/E8 chromatography) consisted of 50 mM N-octylglycoside, 300 mM NaCl, 25 mM Tris/HCl, pH 7.4, 1 mM MnO4, 1 mM CaCl2, 1 mM N-ethylmaleimide (Merck, Darmstadt, Germany), and 5 μg/ml of PMSF (phenylmethylsulfonyl fluoride, Sigma)). Condition B (coimmunoprecipitations) was 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM sodium vanadate, 50 mM sodium fluoride, 1 mM PMSF, 5 mM EDTA. Cells were scraped into ice-cold lysis buffer and allowed to lyse for 30 min on a shaking platform at 4 °C, and the lysate was spun down at 10,000 g for 15 min at 4 °C. The protein concentration of the supernatant was determined by UV absorption at 240 nm.

For immunoprecipitations samples were adjusted to 1 ml of lysis buffer with precleared with equilibrated Protein G-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) for 30 min at 4 °C by rotation. After centrifugation appropriate antibodies were added to the supernatant, the mixture was rotated for 1 h, and 20–50 μl of equilibrated Protein G-Sepharose were added for an additional 1 h. If not stated otherwise, antibodies were used at a concentration of 5 μg/ml of protein. Coimmunoprecipitations were performed the same way, but for 2 h. The immune complexes were retained by short pulse centrifugation, washed three times in lysis buffer (for condition A: lysis buffer containing 25 mM N-octylglycoside), and resuspended in 2×SDS sample buffer (41). If not otherwise indicated, SDS-PAGE was performed with 10% gel. Proteins were transferred to nitrocellulose (0.2 μm, Schleicher & Schuell, Dassel, Germany) for 1.5 h at 1 mA/cm² by semidy blotting and a discontinuous buffer system. Blots were stained with Ponceau S and blocked with 3% BSA in TBST (0.1% Tween 20, 25 mM Tris/HCl, pH 7.4, 150 mM NaCl) for 1 h at room temperature. Antibodies were diluted in blocking solution, and incubated with the blot for 1 h at room temperature or for 16 h at 4 °C.

2 H. von der Mark, manuscript in preparation.
Blots were washed four times for 10 min each with TBST, developed with peroxidase-conjugated secondary antibodies and chemiluminescence, and exposed to Kodak XAR-5 films (Eastman Co.). For stripping, blots were washed twice with distilled water, incubated two times for 10 min with 0.1% glycine, 0.5 M NaCl, 0.1% Tween 20, 2% β-mercaptoethanol, pH 2.5, neutralized extensively with TBST, and blocked again.

Cell Surface Biotinylation—Cells were washed once in PBS containing 5 mM EDTA and released from plates with 5 mM EDTA in PBS. After washing three times with ice-cold PBS, pH 8.0, cells were adjusted to 25 × 10^6/ml and nonmembrane permeable NHS-LC-Sulfobiotin (Pierce) was added to a final concentration of 0.5 mg/ml. Surface biotinylation was carried out for 30 min at room temperature on a shaking platform, and the reaction was stopped by three washes with ice-cold PBS, 10 mM Tris/HCl, pH 8.0. Cells were lysed under condition A.

LN-1/E8-Sepharose Chromatography—The LN-1/E8 fragment used throughout this study was a generous gift of Dr. Rainer Deutzmann (University of Regensburg, Regensburg, Germany). It was coupled to CNBr activated Sepharose CL 4-B according to the manufacturer's instructions (Amersham Pharmacia Biotech), which resulted in ~1 mg of LN-1/E8 fragment/ml of Sepharose. For purification of recombinant integrin α7β1 complexes from transfected 293 cells, LN-1/E8-Sepharose was equilibrated in lysis buffer A by three washes and 200 μl of a 1:3 suspension was added to surface-biotinylated cell extracts from 10^7 cells. The mixture was rotated for 4 h at 4 °C and washed four times in lysis buffer A containing 25 mM N-acytlyglycyranside by short centrifugation at 200 × g and resuspension in 1 ml of wash buffer. The recombinant protein complexes were eluted in a volume of 50 μl with 5 mM EDTA in 25 mM N-acytlyglycyranside, 300 mM NaCl, 25 mM Tris/HCl pH 7.4, 1 mM N-ethylmaleimide, 1 mM PMSF by vortexing and centrifugation at 200 × g. Eluted proteins were subjected to Western blot analysis and probed with streptavidin-peroxidase complex (1:5000, Amersham Pharmacia Biotech)., LN-1/E8-Sepharose was recycled by repeated washes in 1 × NaCl, 5 mM EDTA, 20 mM Tris/HCl, pH 7.4 and stored at 4 °C in TBS containing 0.1% sodium azide.

Cell Attachment Assay—Plates (96 wells; Nunc, Denmark) were coated with 100 μl of protein solution/well for 1 h at 37 °C with coating concentrations as indicated under "Results." Plates were then washed twice with PBS and blocked with 1% heat-denatured (30 min; 80 °C) BSA (Sigma; 7030). Cells were trypsinized and washed either in DMEM containing 0.1% heat-denatured BSA, added to the cell suspension (1.3 × 10^5 cells/ml) and blocked again. The mixture was rocked at 4 °C for 30 min. 5% N-mercaptoethanol, pH 2.5, neutralized extensively with TBST, and blocked again.

RESULTS

Heterodimerization and Surface Expression of α7β1—Do Not Require the Cytoplasmic Domain of Integrin α7X2—We reported previously that integrin α7X2B,3 α7X2A, and α7X2C induce cell migration specifically on laminin-1 and its E8 fragment (LN-1/E8) when expressed ectopically in nonmotile, integrin α7-negative 293HEK cells (38). To elucidate the role of the α7B cytoplasmic domain, a truncation mutant lacking the cytoplasmic domain except of the two first membrane-proximal residues (Lys-Leu) was constructed and transfected in HEK293-EBNA cells. Transfected 293α7X2B and 293α7X2cyt cells were magnetically sorted with anti-α7 mAb 3C12 for high surface expression levels (Fig. 1) and scanned by FACS analysis with two different anti-α7 mAbs, a nonblocking (3C12) as well as a blocking antibody (6A11). The wild-type and mutant cells were rather homogenous and displayed similar surface expression levels of integrin α7 (Fig. 1A and Table 1), indicating that deletion of the cytoplasmic domain of integrin α7X2 does not interfere with cell surface presentation. 293α7X2cyt cells displayed reduced surface levels of integrin α7, thus behaving similarly to the wild-type receptor (37). Immunoblot analysis confirmed deletion of the cytoplasmic domain (Fig. 1B, lanes 3, 6, and 9) and demonstrated that the total α7 expression level was not reduced after deletion of the α7 cytoplasmic domain (compare Fig. 1B, lanes 2 and 3). These data suggest that the truncated protein does not differ from the wild-type protein with respect to stability. However, some degradation products were observed in both 293α7X2B and 293α7X2cyt cells (Fig. 1B, lanes 2, 3, and 8) and a large part of the transfectected integrin was not processed (Fig. 1B, lane 8). Both findings are probably due to overexpression of α7.

Overexpression of integrin α7 leads to down-regulation of α6 and other integrin α subunits, but not the β1 chain, on the cell surface of 293 cells (38). As integrins are being transported to the cell surface as αβ heterodimers (11), all integrin α7 on the cell surface should be associated with the β1 chain. To verify that both transfected α7 chains form heterodimers with the endogenous β1 chain, cells were surface-biotinylated, and lysates were either adsorbed with LN-1/E8 fragment Sepharose or immunoprecipitated with β1 mAB TS2/16 or anti-α7 mAB 3C12 (Fig. 1C). Transfected wild-type and deleted integrin α7 chains were specifically retained by LN-1/E8-Sepharose as complex with endogenous β1 chains, Fig. 1C, lanes 5 and 9). No significant binding of proteins to LN-1/E8-Sepharose was detected in lysates from vector-transfected (mock-transfected) cells (Fig. 1C, lane 2). In the mock-transfected cells anti-β1 precipitated various other integrin chains (Fig. 1C, lane 3, arrowheads), but not α7. In α7-transfected cells, anti-α7 co-precipitated β1 and vice versa (Fig. 1C, lanes 6, 7, 10, and 11). This indicated that the integrin α7 cytoplasmic domain is not essential for α7 heterodimerization, processing, surface presentation, or ligand binding.

The Integrin α7 Cytoplasmic Domain Is Not Essential for Cell Attachment—Comparison of mock-transfected cells and 293α7X2B and 293α7X2cyt cells revealed a dose-dependent cell attachment to LN-1/E8 fragment (Fig. 2A). Both α7 variants enhanced binding of 293 cells to LN-1/E8 fragment as compared with the mock-transfected cells. Attachment of both 293α7X2B (38) and 293α7X2cyt cells to LN-1/E8 fragment, but not to PLL could be fully blocked with integrin α7 blocking mAB 6A11 (Fig. 2B), while attachment to LN-1 was only blocked by 50% owing to a α7-independent cell binding site in the laminin-1 E1 fragment (4, 43). Thus, the truncated α7X2B receptor

3 Wild-type transfected cells are named 293α7X2B, and cells carrying the deletion are named 293α7X2cyt.
FIG. 1. Characterization of α7 protein expression in 293α7X2B and 293α7Δcyt cells. A, FACS analysis of mock-transfected (Mock) and immunomagnetically sorted 293α7X2B and 293α7Δcyt cells. Cells were stained with a nonblocking anti-α7 mAb (3C12), a blocking anti-α7 mAb (6A11), with anti-integrin α6 mAb (GoH3) and with secondary antibodies alone (controls). α7-transfected cells display similar α7 surface expression levels and reduced α6 surface expression levels, whereas mock-transfected cells express some α6 but no α7. B, Western blot analysis of α7 integrin expressed by untransfected (Mock), 293α7X2B, and 293α7Δcyt cells. Total cell lysates (10 μg) were separated by 10% SDS-PAGE under either nonreducing (lanes 1–6) or reducing conditions (lanes 7–9) and transferred to nitrocellulose. Blots were probed with an antiserum (242AE) against the extracellular portion of α7 (lanes 1–3) and with an antiserum (U4+) against the cytoplasmic domain of α7 (lanes 4–9). Lanes 1, 4, and 7, untransfected cells. Lanes 2, 5, and 8, 293α7X2B cells; lanes 3, 6, and 9, 293α7Δcyt cells. Molecular mass positions (kDa) are shown on the left. Positions of the unprocessed and unreduced α7 chain (complete) and C-terminal part of the processed chain (C-term) are indicated on the right. C, immunoprecipitation and LN-1/E8-Sepharose chromatography of surface-biotinylated cell lysates. Surface-biotinylated cell lysates (condition B) of mock-transfected 293 cells (lanes 1–3), 293α7X2B cells (lanes 4–7), and 293α7Δcyt cells (lanes 8–11) were subjected to immunoprecipitation with anti-integrin β1 mAb TS2/16 (lanes 3, 7, and 11), anti-α7 mAb 3C12 (lanes 6 and 10) or to chromatography with LN-1/E8-Sepharose Cl 4B (lanes 2, 5, and 9). Lanes 1, 4, and 8 represent 0.25% of the material used for immunoprecipitations and chromatography of 293, 293α7X2B, and 293α7Δcyt cell lysate. Protein complexes were separated by 12% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and probed with streptavidin-peroxidase. Molecular mass positions (kDa) are shown on the left. Positions of the β1 chain, the α7 N-terminal part, and the α7 C-terminal part are indicated on the right. Arrows indicate integrin α chains different from α7.
showed essentially the same behavior as the full-length receptor. Attachment of mock-transfected cells to the laminin-1 E8 fragment is mediated by integrin α6 and switches to α7 after overexpression of α7 due to down-regulation of α6 (see Fig. 1 and Ref. 38). A LN-1/E8 fragment coating concentration of 2 μg/ml was chosen for the following cell migration and biochemical experiments (Figs. 3–9) because wild-type and Δcyt cells attached similarly at these coating concentrations.

The Integrin α7 Cytoplasmic Domain Controls Integrin α7-mediated Cell Migration and Polarization—Although surface presentation of integrin α7 and cell adhesion to LN-1/E8 did occur independently of the α7 cytoplasmic domain, its deletion affected significantly cell motility on LN-1/E8, i.e. the mean migration speed (Fig. 3A). Deletion of the integrin α7 cytoplasmic domain reduced α7-dependent cell motility significantly to about 50% of the wild-type level (p < 0.01). Integrin α7-mediated cell migration required furthermore the presence of serum because serum-starved cells did not migrate under serum-free conditions (Fig. 3A).

The cell attachment assays presented in Fig. 2, showing equal attachment of 293α7X2B and 293α7X2Δcyt cells to LN-1/E8, had been carried out under serum-free conditions, in contrast to cell migration assays. It seemed possible that the different migration rates of 293α7X2B and 293α7X2Δcyt cells were due to different effects of serum upon their attachment cells. To rule this out, serum-starved cells were plated in the presence of serum or 1% BSA on LN-1/E8. 293α7X2B and 293α7Δcyt cells attached similarly under either condition (Fig. 3B), indicating that the reduced cell migration was not a consequence of differences in cell attachment in the presence of serum.

To test whether serum alone accounted for the observed migration, cells were plated on PLL in the presence of serum and video images were taken 1 h after plating the cells and after 12 h (Fig. 4, A and B). Cells did neither spread nor move under these conditions (see Fig. 4, arrows), thus confirming serum alone does not induce cell migration.

Analysis of the cell morphology of migrating cells by microscopy revealed that 293α7X2Δcyt cells displayed a different morphology on LN-1/E8 than cells expressing the wild-type receptor (Fig. 4). To analyze this in detail, we quantitated the percentage of spread cells plated on LN-1/E8 (Fig. 4). First, only about 40% of 293α7X2Δcyt cells spread after 30 min in contrast to about 80% of 293α7X2B cells. After 16.5 h of adhesion to LN-1/E8, 80% of 293α7X2B cells were still spread, whereas spreading 293α7X2Δcyt cells reached only a level of 60% (Fig. 4E). Second, despite spreading as compared with cells plated on PLL, 293α7X2Δcyt cells remained in a more or less round shape and extended filopodia, reflecting a different organization status of the actin cytoskeleton as compared with 293α7X2B cells. These elongated and polarized (see Fig. 4, C and D). To examine changes in the actin cytoskeleton cells were

| Cell type          | Antibody | Mean fluorescence |
|--------------------|----------|-------------------|
| 293α7X2B          | 3C12     | 22.53 ± 3.8*      |
| 293α7X2Δcyt       | 3C12     | 17.33 ± 2.5*      |
| 293α7X2B          | 5A6      | 6.0b              |
| 293α7X2Δcyt       | 5A6      | 6.12b             |

* Mean fluorescence ± S.D. of three measurements.

One experiment.

The Integrin α7 Cytoplasmic Domain Controls Integrin α7-initiated Tyrosine Phosphorylation—Activation of non-receptor protein tyrosine kinases like FAK and Src and subsequent protein-protein interactions are rapid responses of cells to attachment to ECM molecules (44) and are believed to regulate cell adhesion as well as cell migration (45). We thus examined tyrosine phosphorylation events specifically induced by integrin α7. Adhesion of 293α7X2B cells to the LN-1/E8 fragment induced tyrosine phosphorylation of 60–80- and 120–140-kDa proteins already after 10 min (data not shown). To identify the tyrosine-phosphorylated proteins, Triton X-100 extracts from serum-starved, suspended 293α7X2B cells and from serum-starved 293α7X2B cells plated on PLL and LN-1/E8 fragment were subjected to immunoprecipitation with antibodies against tensin, p130CAS, FAK, vinculin, paxillin, Erk2, and Shc or anti-phosphotyrosine-agarose (Fig. 6). Immunoprecipitation with anti-phosphotyrosine-agarose revealed three major tyrosine-phosphorylated bands of 60–70, 120, and 130 kDa on LN-1/E8 fragment, which were not seen when cells were kept in suspension or plated on PLL. Three of these proteins were identified as p130CAS, FAK, and paxillin (Fig. 6B, lanes 5, 6, and 8). Tensin and vinculin were not tyrosine-phosphorylated (Fig. 6B, lanes 4 and 7), nor were Erk or Shc (data not shown).

Integrin α7-initiated p130CAS Tyrosine Phosphorylation and p130CAS/Crk Coupling Are Dependent on the Cytoplasmic Domain of α7—Klemke and co-workers (27) reported an essential role for p130CAS in cell migration. The observation of integrin α7-dependent p130CAS tyrosine phosphorylation prompted us to examine the role of the integrin α7 cytoplasmic domain in this event. Starved 293α7X2B and 293α7X2Δcyt cells were plated on LN-1/E8 fragment in the absence or in the presence of serum for 30 min and lysed. Lysates were immunoprecipitated with anti-p130CAS and immunoprecipitates were subjected to anti-phosphotyrosine blotting (Fig. 7).

Fig. 7 shows that p130CAS was not phosphorylated on tyrosine residues when 293α7X2B cells were kept in suspension or plated on poly-l-lysine. Tyrosine phosphorylation of p130CAS was, however, induced by E8 fragment via integrin α7 and strongly enhanced after addition of serum, paralleling the effect of serum on cell migration. In contrast, 293α7X2Δcyt cells plated on LN-1/E8 in the presence of serum showed a markedly reduced p130CAS tyrosine phosphorylation with unaltered p130CAS protein levels. Thus, the presence of the α7 cytoplasmic domain is necessary for full p130CAS tyrosine phosphorylation in this system. Furthermore, the cytoplasmic domain of integrin α7 was essential for coprecipitation of a tyrosine-phosphorylated 60-kDa protein with p130CAS, which we could not identify so far. This protein coprecipitated with p130CAS from 293α7X2B cells but not from 293α7X2Δcyt cells. We also examined the effect of the deletion of the α7 cytoplasmic domain on FAK tyrosine phosphorylation (Fig. 8). According to the data obtained with CAS precipitates, we also found less tyrosine phosphorylation of FAK in 293α7Δcyt cells plated on LN-1/E8 as compared with 293α7X2B cells.

The reduced tyrosine phosphorylation of p130CAS in the 293α7X2Δcyt cells suggested that p130CAS/Crk coupling could be affected due to fewer Crk SH2-binding sites offered by p130CAS. For examination of p130CAS/Crk complexes, 293α7X2B and
The Integrin α7 Cytoplasmic Domain

DISCUSSION

In the present study we have investigated the function of the integrin α7X2 cytoplasmic domain by comparing 293EBNA cells expressing an integrin α7 wild-type receptor or an integrin α7 lacking the cytoplasmic domain. The cells were compared in terms of (i) α7 protein expression, (ii) α7 surface presentation, (iii) cell attachment and migration conferred by α7, and (iv) initiation of p130Cas/Crk signaling complexes. Both cell types assembled α7β1 heterodimers and attached equally well on LN-1/E8. We showed that the integrin α7B cytoplasmic domain is not required for heterodimer formation with β1 and not necessarily linked to α7 protein stability, surface expression, or receptor activation but contributes to cell spreading, migration, and intracellular signaling via p130Cas/Crk complex formation in a serum-dependent manner.

Role of the Integrin α7X2 Cytoplasmic Domain in Receptor Assembly and Activity—The function of the α7 cytoplasmic domains of various other β1 integrin receptors has been extensively studied by truncation and point mutation analysis. It has been reported that particularly the two phenylalanines in the conserved GFFKR motive regulate heterodimerization and surface transport, e.g., in the case of α6β1 (46), and protein stability in the case of α7β1 (47, 48). Moreover, deletion of the cytoplasmic domains of α1β1 and α7 including the GFFKR caused reduced surface expression and heterodimerization but, on the other hand, resulted also in a high integrin affinity state of

293α7X2Δcyt were plated on LN-1/E8 fragment for 1, 2, and 4 h in the presence of serum (conditions used for cell migration) or kept in suspension for 4 h in the presence of serum. Crk was immunoprecipitated from the standardized cell lysates and the immunoprecipitates were probed with anti-phosphotyrosine, anti-Crk antibodies (Fig. 9A). p130Cas coprecipitated with Crk in 293α7Δcyt cells only after cell adhesion to LN-1/E8 but not when cells were kept in suspension. However, p130Cas was absent in Crk immunoprecipitates obtained from 293α7X2Δcyt cells although it was present in each lysate used for Crk immunoprecipitation (Fig. 9B). Crk itself was not tyrosine-phosphorylated (data not shown). Thus, our results point to an essential role for the integrin α7 cytoplasmic domain in p130Cas/Crk coupling.

FIG. 2. Attachment of mock-transfected, 293α7X2B, and 293α7Δcyt cells to laminin-1 LN-1/E8 fragment. Experiments were performed with starved and cycloheximide-treated cells. A, dose-response curve. Cells were plated on laminin-1 LN-1/E8 fragment-coated 96-well plates under serum-free conditions. Symbols represent the average of three wells ± S.D. B, attachment of α7-transfected cells to LN-1/E8 fragment is mediated solely by integrin α7. Starved 293α7Δcyt cells were plated on 96-well plates coated with laminin-1 (10 μg/ml), laminin-1 LN-1/E8 fragment (2 μg/ml), or PLL (20 μg/ml) either without (white bars) or in the presence of a blocking anti-α7 mAb (6A11; 10 μg/ml) (gray bars). Bars represent the average of three wells ± S.D.

FIG. 3. Integrin α7X2-mediated cell migration depends on the α7 cytoplasmic domain and on serum. A, migration of integrin α7X2B- and α7X2Δcyt-transfected 293 cells on laminin-1 LN-1/E8 fragment (2 μg/ml). Bars, total cell tracks per 12 h expressed as average of five independent experiments ± S.D. The number of cells scored was 83 for 293α7X2B cells (gray bars) and 86 for α7X2Δcyt cells (white bars). α7X2Δcyt cells migrated 300 μm/12 h and α7X2Δcyt cells migrated 171 μm/12 h. The difference is significant (p < 0.01; *). B, the observed difference in cell migration between 293α7X2B and 293α7Δcyt cells is not due to differences in cell attachment. 293α7X2B cells (gray bars) and 293α7Δcyt cells (white bars) were allowed to attach to laminin-1 LN-1/E8 fragment (2 μg/ml) for 1 h either in the presence of serum or in the presence of 1% BSA. No significant difference in cell attachment was observed under either condition.
In marked contrast, we show here that deletion of the complete \(\alpha_7\)X2 cytoplasmic domain (including the conserved GFFKR motive) did not influence \(\alpha_7\)X2 heterodimerization with the integrin \(\beta_1\) chain, correct processing of \(\alpha_7\beta_1\), or cell attachment conferred by \(\alpha_7\beta_1\). This is, on the other hand, in agreement with other studies showing that the GFFKR sequence does not influence inside-out signaling or surface presentation of \(\alpha_7\) and \(\alpha_5\beta_1\) integrins (49, 51), as deletion of the GFFKR motive did not alter the activation state of the affected receptors. Thus, integrins differ in the requirement of the GFFKR motive for heterodimerization, surface transport, and inside-out activation. This may be due to different affinities of the \(\alpha_7\) chains for the \(\beta_1\) chain as proposed previously (47).

For ligand binding and signaling of integrins both cytoplasmic domains and extracellular domains are involved. Ziober et al. have shown that the \(\alpha_7\)X1 splice variant but not the X2 fragment in K562 and \(\alpha_7\) in LFA-1-deficient Jurkat cells (49, 50).

In FIG. 4, spreading and polarization of 293 cells depends on the cytoplasmic domain of integrin \(\alpha_7\)X2 and laminin-1 LN-1/E8 fragment in the presence of serum. Video images of 293 cells (A and B) were taken during cell migration experiments. Pictures in C and D were taken under a light microscope. A and B show \(\alpha_7\)X2 cells plated on PLL for 1 h (A) and 12 h (B). Cells did neither spread nor migrate on PLL but still divided (arrow; images show the same frame). C and D show 293aX2 (C) and 293aX2X2 (D) cells kept on laminin-1 LN-1/E8 fragment for 6.5 h. 293aX2X2 cells spread (D) but polarized less than 293aX2 cells (see closed arrows in C). Open arrow, nonpolarized 293aX2X2 cell. Bar, 100 m. E, quantification of spreading cells plated on LN-1/E8 fragment. Bars represent the average percentage of cell spreading obtained by counting cells from three fields. 293aX2X2 cells were less spread than 293aX2 cells. 150–230 cells per field were analyzed.

In FIG. 5, deletion of the \(\alpha_7\) cytoplasmic domain affects lamellipodia formation. 293aX2B and 293aX2X2xcyt cells were plated on LN-1/E8 (2 \(\mu\)g/ml) or PLL (20 \(\mu\)g/ml) in the presence of serum for 12 h, fixed, and stained with FITC-phalloidin. 293aX2B cells plated on LN-1/E8 develop lamellipodia and stress fibers, ruffle their membranes, and polarize (arrows). 293aX2X2xcyt cells plated on LN-1/E8 extend filopodia instead of forming lamellipodia (arrows) but can still spread, as compared with PLL. Bar, 10 \(\mu\).n.
splice variant, required activation with the β1 activating antibody TS2/16 to bind to laminin-1 in MCF7 cells (52), although both extracellular splice variants carried the same intracellular splice variant. In contrast, α7X2B-β1 complexes are constitutively active and bind to laminin-1 and -2 when expressed in HEK293-EBNA cells and MCF-7 cells, independently of the cytoplasmic domain (Refs. 37, 38, and 52; this report). Thus, our results are in support of a key role of both intra- and extracellular domains of integrin α7 in inside-out signaling and ligand binding.

**Effect of the Integrin α7Cytoplasmic Domain on Cell Spreading and Migration**—Cell motility on the extracellular matrix is largely dependent on integrin surface expression levels, integrin activation state, and matrix concentration (53). Therefore, quantitative biochemical analysis of the truncated receptor and analysis of cell attachment was necessary to allow a direct functional description of the role of the integrin α7 cytoplasmic domain in cell migration and signaling events. The results of these studies show that (a) α7 surface and total protein levels in wild-type and mutant transfected cells were similar, (b) processing and heterodimer formation were independent of the integrin α7 cytoplasmic domain, and (c) 293αX2B and 293αX2Δcyt cells attached similarly via integrin α7 to LN-1/E8 at coating concentrations of 2 μg/ml. Taking these parameters into account, we conclude that deletion of the α7 cytoplasmic domain significantly affected cell migration.

α7-mediated cell migration and cytoskeletal reorganization was specific for the LN-1/E8 fragment. 293αX2B and 293αX2Δcyt cells plated on LN-1/E8 did not display marked differences and did not migrate, even in the presence of serum. Thus, integrin α7-mediated cell migration of transfected 293 cells is not due to endogenous proteins deposited as ligands like such as fibronectin. However, serum factors are required for integrin α7-mediated continuous cell migration. This is consistent with the notion that sustained cell migration requires the presence of growth factors (54). Thus, integrin α7 alone is not sufficient to provide enough signals for cell migration but co-operates with soluble, so far by us unspecified serum factors.

In support of the results we obtained from our cell migration experiments we observed fewer lamellipodia, cell polarization, and stress fibers in 293αX2Δcyt cells plated on LN-1/E8 than in 293αX2B cells plated on LN-1/E8. In contrast, filopodial extensions were remarkably increased in 293αX2Δcyt cells. Deletion of the α7 cytoplasmic domain may lead to a block in transmission of signals from cdc42 to Rac, hence from Rac to Rho, and thus in accumulation of filopodia-inducing signals in 293 cells. This would explain the lack of membrane ruffling and stress fibers. Nobes and co-workers (55) reported that cdc42 activation, filopodia. Cells may not be able to polarize due to lack of lamellipodial formations, despite extending filopodia and attaching.

**Influence of the Integrin α7X2 Cytoplasmic Domain on p130Cas/Crk Coupling**—The role of the adaptor protein p130Cas and the p130Cas/Crk complex in α7β1 integrin-mediated cell migration on fibronectin has previously been demonstrated (27, 28). p130Cas becomes tyrosine-phosphorylated after cell adhesion to fibronectin, and this allows formation of a p130Cas/Crk complex (25). Serum factors like platelet-derived growth factor, lysophosphatidic acid, and bombesin induce p130Cas tyrosine phosphorylation as well, leading to formation of a p130Cas/Crk complex (56). We showed for the first time that not only fibronectin, but also laminin can pro-
enhanced by serum. It has been reported that p130CAS expression converges with those of soluble factors and their receptors like osteopontin as compared with the wild-type receptor. Accordingly, deletion of the C-terminal 23 kDa cytoplasmic domain may reduce Rac activation in comparison with the nonmutated p130CAS receptor as a consequence of reduced p130CAS tyrosine phosphorylation.

A mechanism explaining the failure of lamellipodia formation induced by 293α2X2Acyt cells on LN-1/E8 may finally be through reduction of p130CAS/Crk induced signaling to Rac; Klemke and coworkers (27) have demonstrated that p130CAS/Crk coupling acts in a Rac-dependent manner on cell migration. It seems possible from our morphological and biochemical data that deletion of the α7 cytoplasmic domain may reduce Rac activation in comparison with the nonmutated α7 receptor as a consequence of reduced p130CAS tyrosine phosphorylation.

To identify the 60-kDa phosphoprotein coprecipitating with p130CAS, we performed coimmunoprecipitation analyses of p130CAS with Src and Lyn, both of which being expressed at same protein levels in 293α2X2B and 293α2X2Acyt cells (data not shown). However, we could not detect complexes of p130CAS with Src or Lyn, nor with FAK (data not shown), suggesting that the 60-kDa protein is neither Src nor Lyn, and that CAS tyrosine phosphorylation induced by integrin α7 in 293 cells may occur in a FAK-independent manner, as is the case in p125FAK−/− cells. There, p130CAS can be phosphorylated on tyrosine by cell adhesion kinase β (64).

Acknowledgment—We thank Dr. Ulrike Mayer for providing the U.9 antibody, Helga Moch for providing 3C12 and 6A11 antibodies, and Drs. Guido Posern and Stefan Feller (University of Wurzburg) for helpful discussions. We greatly acknowledge Dr. Victor Wixler for critical reading of the manuscript.
The Integrin $\alpha_7$ Cytoplasmic Domain

(1996) Dev. Dyn. 207, 355–371
33. Pinkstaff, J. K., Detterich, J., Lynch, G., and Gall, C. (1999) J. Neurosci. 19, 1541–1556
34. Ziober, B. L., Vu, M. P., Waleh, N., Crawford, J., Lin, C. S., and Kramer, R. H. (1993) J. Biol. Chem. 268, 2671–2678
35. Cello, G., Barron, T., and Quanta, V. (1993) J. Biol. Chem. 268, 19019–19024
36. Song, W. K., Wang, W., Sato, H., Bielser, D. A., and Kaufman, S. J. (1993) J. Cell Sci. 106, 1139–1152
37. Echtermeyer, F., Scheler, S., Hoppe, S., Poschl, E., von der Mark, H., and von der Mark, K. (1996) J. Biol. Chem. 271, 2071–2075
38. Scheler, S., Mielenz, D., Echtermeyer, F., Hoppe, S., Poschl, E., von der Mark, H., Moeh, H., and von der Mark, K. (2000) Exp. Cell Res. 255, 303–313
39. Cohn, R. D., Mayer, U., Saher, G., Herrmann, R., van der Flier, A., Sonnenberg, A., Sorokin, L., and Voit, T. (1999) J. Neural. Sci. 163, 146–152
40. Belkin, A. M., Ornatsky, O. I., Glukhova, M. A., and Koteliansky, V. E. (1988) J. Cell Biol. 107, 545–553
41. Laemmli, U. K. (1970) Nature 227, 680–685
42. Landegren, U. (1984) J. Immunol. Methods 67, 379–388
43. Aumailley, M., Gerl, M., Sonnenberg, A., Deutzmann, R., and Timpl, R. (1990) FEBS Lett. 262, 82–86
44. Yamada, K. M., and Miyamoto, S. (1995) Carr. Opin. Cell Biol. 7, 681–689
45. Schoenwaelder, S. M., and Burridge, K. (1999) Carr. Opin. Cell Biol. 11, 274–286
46. De Melker, A. A., Kramer, D., Kuijken, I., and Sonnenberg, A. (1997) Biochem. J. 328, 529–537
47. De Melker, A. A., and Sonnenberg, A. (1996) Eur. J. Biochem. 241, 254–264
48. O'Toole, T. E., Katagiri, Y., Faull, R. J., Peter, K., Tamura, B., Quanta, V. L., Luftus, J. C., Shattil, S. J., and Ginsberg, M. H. (1994) J. Cell Biol. 124, 1047–1059
49. Briesewitz, R., Epstein, M. R., and Marcantonio, E. E. (1993) J. Biol. Chem. 268, 2989–2996
50. Lu, C. F., and Springer, T. A. (1997) J. Immunol. 159, 268–278
51. Bauer, J. S., Varner, J., Schreiner, C., Kornberg, L., Nicholas, R., and Juliano, R. L. (1993) J. Cell Biol. 122, 209–221
52. Ziober, B. L., Chen, Y., and Kramer, R. H. (1997) Mol. Biol. Cell 8, 1723–1734
53. Palecek, S. P., Lofts, J. C., Ginsberg, M. H., Lauffenburger, D. A., and Horwitz, A. F. (1997) Nature 385, 537–540
54. Ware, M. F., Wells, A., and Lauffenburger, D. A. (1998) J. Cell Sci. 111, 2423–2432
55. Noake, C. D., and Hall, A. (1999) J. Cell Biol. 144, 1235–1244
56. Casamassima, A., and Rozengurt, E. (1997) J. Biol. Chem. 272, 9363–9370
57. Moro, L., Venturino, M., Bozzo, C., Silengo, L., Altruda, F., Beguinot, L., Tarone, G., and Defilippi, P. (1998) EMBO J. 17, 6623–6632
58. Miyamoto, S., Teramoto, H., Gutkind, J. S., and Yamada, K. M. (1996) J. Cell Biol. 135, 1633–1642
59. Plopper, G. E., McNamee, H. P., Dike, L. E., Bojanowski, K., and Ingber, D. E. (1995) Mol. Biol. Cell 6, 1349–1365
60. Sastry, S. K., Lakonishok, M., Thomas, D. A., Muschler, J., and Horwitz, A. P. (1996) J. Cell Biol. 133, 169–184
61. Soldi, R., Mitola, S., Stras, M., Defilippi, P., Tarone, G., and Bussolino, F. (1999) EMBO J. 18, 882–892
62. Klemke, R. L., Cai, S., Giannini, A. L., Gallagher, P. J., de Landerolle, P., and Cheresh, D. A. (1997) J. Cell Biol. 137, 481–492
63. Chellaia, M., Fitzgerald, C., Filardo, E. J., Cheresh, D. A., and Hruska, K. A. (1996) Endocrinology 137, 2432–2440
64. Ueki, K., Mima, T., Nakamoto, T., Sasaki, T., Aizawa, S., Hirai, H., Yano, S., Naruse, T., and Nojima, T. (1998) FEBS Lett. 423, 192–201
