Distinct Involvement of β3 Integrin Cytoplasmic Domain Tyrosine Residues 747 and 759 in Integrin-mediated Cytoskeletal Assembly and Phosphotyrosine Signaling

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We have investigated the structural requirements of the β3 integrin subunit cytoplasmic domain necessary for tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin during αβ3-mediated cell spreading. Using CHO cells transfected with various β3 mutants, we demonstrate a close correlation between αβ3-mediated cell spreading and tyrosine phosphorylation of FAK and paxillin, and highlight a distinct involvement of the NPLY747 and NITY759 motifs in these signaling processes. Deletion of the NITY759 motif alone was sufficient to completely prevent αβ3-dependent focal contact formation, cell spreading, and FAK/paxillin phosphorylation. The single Y759A substitution induced a strong inhibitory phenotype, while the more conservative, but still phosphorylation-defective, Y759F mutation restored wild type receptor function. Alanine substitution of the highly conserved Tyr747 completely abolished αβ3-dependent formation of focal adhesion plaques, cell spreading, and FAK/paxillin phosphorylation, whereas a Y747F substitution only partially restored these events. As none of these mutations affected receptor-ligand interaction, our results suggest that the structural integrity of the NITY759 motif, rather than the phosphorylation status of Tyr747 is important for αβ3-mediated cytoskeleton reorganization and tyrosine phosphorylation of FAK and paxillin, while the presence of Tyr at residue 747 within the NPLY747 motif is required for optimal β3 post-ligand binding events.

Anchorage of cells to the extracellular matrix is mediated in part by integrins, a large family of heterodimeric cell surface receptors, that regulate numerous aspects of cell behavior, such as cell motility, proliferation, differentiation, and apoptosis (1). Cell engagement with extracellular matrix ligands induces integrin translocation to subcellular structures known as focal adhesion plaques that form at regions of close contact between the cell and its underlying substratum (2). Integrin clustering at focal contact sites in turn triggers major intracellular events, including cytoskeleton reorganization, intracellular ion transport, phosphoinositide turnover, kinase activation, and tyrosine phosphorylation of intracellular proteins (3). A large number of tyrosine-phosphorylated proteins have been identified within focal adhesion plaques. These include cytoskeletal proteins, kinases and adaptor proteins, growth factor receptors, and growth factor receptor-related signaling molecules, thus emphasizing the potential role of integrins as recruiting centers for molecules involved in various signaling pathways.

Although the link of integrins with focal adhesions is well established, the precise mechanism by which integrins associate with cytoskeletal proteins, regulate focal adhesion plaque assembly, and participate in the activation of intracellular signaling cascades is still unclear. There is convincing evidence that integrin β subunits are likely to play a major role in these processes: (i) truncation of the β subunit cytoplasmic domain impairs integrin recruitment to focal contacts (4–6), and (ii) information contained in β subunit cytoplasmic tails coupled to the transmembrane and extracellular domains of the interleukin-2 receptor is sufficient to target these chimeric receptors to focal contacts (7) and to activate the focal adhesion kinase (FAK)1 signaling pathway (8). Based on mutational analysis of the cytoplasmic domain of the β1 integrin, three motifs have been identified that are important for the recruitment of integrins to adhesion plaques; these motifs correspond to the highly conserved acidic membrane-proximal domain and to two C-terminal NPXY motifs (6, 9), which constitute typical recognition sites for tyrosine kinases and adaptor proteins (10). Subsequent complementary studies (based on a combination of deletion analysis, single amino acid substitution, and the use of cytoplasmic domain synthetic peptides) have provided evidence that these highly conserved cytoplasmic motifs in the various integrin β subunits have similar functional properties (11–16) and display overlapping binding sites for the structural cytoskeletal proteins α-actinin and talin, the adaptor protein paxillin, as well as regulatory proteins including FAK, integrin-linked kinase-1 (ILK-1) (17), β3-endonexin (18), Shc, Grb2 (19), and integrin cytoplasmic domain-associated protein-1 (ICAP-1) (20).

The importance of tyrosine phosphorylation of focal adhesion proteins during focal contact formation is well established as tyrosine kinase inhibitors prevent the organization of focal adhesion plaques and stress fibers (21), and treatment of cells with cytochalasin B or D, which block actin polymerization,
inhibits tyrosine phosphorylation of FAK and paxillin (22). In contrast, the precise mechanisms by which integrin β subunits trigger tyrosine phosphorylation of focal adhesion proteins during integrin-dependent cell attachment and spreading are less well understood. In an attempt to identify amino acids of the β3 cytoplasmic domain involved in the photophosphorylation signaling cascade induced by β3 integrins, Tahiliani et al. (23) have expressed various mutant β3 cytoplasmic domains as separate tails connected to an extracellular reporter protein. Using this approach, they deliberately excluded the role of upstream events, such as integrin-dependent ligand binding, cell adhesion, and cell spreading, in triggering the FAK signaling cascade (23). In the present study, we have used an alternative approach to investigate the structural requirements of the β3 subunit cytoplasmic domain necessary to stimulate intracellular tyrosine phosphorylation during cell spreading. By expressing various human β3 integrin cytoplasmic domain mutants, which either promote or inhibit αβ3-dependent CHO cell spreading, we demonstrate a close correlation between a structurally conserved β3 integrin cytoplasmic tail, cell spreading and FAK/paxillin phosphorylation, as all C-terminal truncation mutants unable to induce cell spreading, also failed to trigger tyrosine phosphorylation. Our data further highlight major differences in the involvement of the cytoplasmic domain tyrosine residues in β3-mediated post-ligand binding events. The presence of residue Tyr759 in the membrane-distal NITY759 sequence is not necessary for β3-mediated focal contact formation, cell spreading, and β3-triggered tyrosine phosphorylation of FAK or paxillin, whereas residue Tyr477 of the membrane-proximal NPLY747 motif is required for optimal αβ3 receptor function. And finally, both the NPLY747 and NITY759 motifs contribute in defining the appropriate β3 cytoplasmic domain conformation necessary for post-ligand binding signaling events.

EXPERIMENTAL PROCEDURES

Cell Culture

The Chinese hamster ovary (CHO) cell line CRL 9096, defective in the dihydrofolate reductase gene (CHO dhfr-), was purchased from the American Type Culture Collection (Rockville, MD). The cells were grown in Iscove’s modified Dulbeco’s medium (IMDM) (Life Technologies, Inc., Merelbeke, Belgium), supplemented with glutamine, penicillin, and streptomycin, 10% heat-inactivated fetal calf serum (complete IMDM), and, when required, hypoxanthine (100 μM) and thymidine (10 μM). The cells were routinely passaged with EDTA buffer, pH 7.4 (1 mM EDTA, 126 mM NaCl, 5 mM KCl, 50 mM Hepes).

Antibodies and Purified Adhesive Proteins

The following polyclonal or monoclonal antibodies were used: anti-α, from Life Technologies (24), anti-phosphotyrosine (PY-20), anti-paxillin and anti-FAK from Transduction Laboratories (Lexington, KY), anti-phosphotyrosine (PY-20), anti-paxillin and anti-FAK from Transduction Laboratories (Lexington, KY), and the blocking monoclonal antibody P37 (anti-human β3) by Dr. J. Gonzalez-Rodriguez (Instituto de Quimica Fisica, Madrid, Spain), and the blocking monoclonal antibody MA-16NTC2 (anti-human β3) by Dr. M. Hoyalerts (Centre for Molecular and Vascular Biology, University of Leuven, Leuven, Belgium). Purified human fibronectin and bovine serum albumin (BSA, fraction V) were purchased from Sigma (Bornem, Belgium).

Construction of Mutant β3 Integrin cDNA

The full-length cDNA encoding wild type β3 was inserted into the 5’-EcoRI/EcoRV-3’ site of the expression vector pB1 as described previously (25). The β3 Y747A and β3 Y747F mutations were introduced in the full-length β3 cDNA by site-directed mutagenesis using the Altered Sites™ in vitro mutagenesis kit (Promega, Lyon, France). Briefly, full-length cDNA encoding wild type β3 was cloned into the phagemid pALTER-1, and the mismatched primers 5’-GGCTAACACCCCGTGTAAAGGGCCAGCG3’ (β3 Y747A) and 5’-GGCTAACACCCCGTGTAAAGGGCCAGCG3’ (β3 Y747F) (Eurogentec, Seraing, Belgium) used for the generation of the mutant constructs. Primers β3 Y747F allowed the generation of a new BamHI restriction site (GTTGCA) in addition to the previously generated PstI restriction site of the expression vector pB1. Mutagenesis was performed according to the manufacturer’s instructions. The full-length mutant β3 cDNA was finally excised from the pALTER phagemid with 5’ XbaI/HindIII 3’ and inserted into the XbaI/HindIII site of the pBJ1 mammalian cell expression vector. The cDNAs encoding the mutant β3 Y759A, β3 Y759F, β3 Y747A/Y759F, β3 Δ754, β3 Δ744, and β3 Δ722 subunits were generated by excision of the 3’ end of the full-length β3 cDNA coding sequence, starting at the BamHI site at nucleotide position 1501 of the published β3 cDNA sequence for mutant β3 Δ722 and starting at the EcoRI site at nucleotide position 2274 for the other mutants. The mutations were followed by an insertion of a BamHI-EcoRV or an EcoRI-EcoRV cassette, obtained by oligonucleotide-directed polymerase chain reaction (PCR) mutagenesis. The nucleotides used to generate the cassette were purchased either from Genetix (Paris, France) or from Eurogentec. The upstream primer (sense) for the β3 Y759A, β3 Y759F, and β3 Y747A/Y759F mutant constructs was a 23-mer corresponding to the β3 nucleotide sequence 2023–2045: 5’-GTGAAAAAGCTTAAAGGACTGG-3’. The upstream primer (sense) for the β3 Δ754 and β3 Δ744 mutant constructs was a 28-mer corresponding to the β3 nucleotide sequence 2244–2271: 5’-CTCTAGAGAAGAAATTCGTGGGAAGGC-3’ comprising an EcoRI restriction site (GAATTCC). The upstream primer (sense) for the β3 Δ722 mutant construct was a 22-mer corresponding to the β3 nucleotide sequence 1497–1518: 5’-GGTCTGGACATCCATGTGG-3’. The following downstream primers were used: 5’-CTCTGGATGACCTGGCCTGCGC-3’, 5’-CTCTGGATGACCTGGCCTGCGC-3’ (β3 Y759A); 5’-CTTAAAGCTTGGCAATCTTGTTAGGCGGCGTGATATTGG-3’ (β3 Y759F and β3 Y747A/Y759F); 5’-CTCTAGGGTATGATCTTTATGTTGGCGGCGTGATATTGG-3’ (β3 Δ754); 5’-CTCTAGGGTATGATCTTTATGTTGGCGGCGTGATATTGG-3’ (β3 Δ744); 5’-CTCTAGGGTATGATCTTTATGTTGGCGGCGTGATATTGG-3’ (β3 Δ722). For β3 Y759A, β3 Y759F, β3 Δ754, β3 Δ744, and β3 Δ722 constructs, the pb1 β3 wt plasmid was used as a template for cDNA amplification, while the β3 Y747A/Y759F mutant was generated using the plasmid pb1 β3 Y747A. For the β3 Δ722 mutant construct, the PCR-amplified fragment was purified, digested with BamHI and EcoRV, and inserted into the pb1 β3 plasmid from which the wild type BamHI-EcoRV fragment had been removed. For all the other mutant constructs, the PCR-amplified fragments were digested with EcoRI and EcoRV after purification and inserted into the pb1 β3 plasmid from which the wild type EcoRI-EcoRV fragment had been removed. Each mutant β3 construct was verified by dyeo sequencing using the 26-mer corresponding to the β3 nucleotide sequence 2264–2290 as a 5’ primer.

Transfection and Selection of Stable Cell Clones

Full-length β3 cDNA in pb1 vector (20 μg) and 1 μg of dihydrofolate reductase plasmid (pMDR901) were mixed with 40 μl of LipofectAMINE (Life Technologies, Inc.) in a final volume of 200 μl of IMDM and added to CHO dhfr- cells grown to 60% confluence in 100-mm tissue culture plates. After 24 h, fetal calf serum was added to the culture medium and 48 h after transfection, the cells were grown in nucleoside-free α-minimal essential medium (Life Technologies, Inc.) used as selective medium. Positive transfectants were analyzed for cell surface expression of the recombinant human integrin β3 subunit using the anti-β3 monoclonal antibody P37 and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (Caltag Laboratories, Burlingame, CA). Stably transfected cells were subcloned by limiting dilution and controlled for cell surface expression of the transfection β3 integrin subunit.

Immunofluorescence and Flow Cytometry

Surface expression of the transfected human β3 integrin was analyzed by flow cytometry using the monoclonal antibodies P37 (anti-human β3), 13C2 (anti-human αv), and 23C6 (anti-αv). Selected transfectants were detached from culture plates with EDTA buffer, pH 7.4, and washed twice in phosphate-buffered saline (PBS) (136 mM NaCl, 2.7 mM KOH, 8 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4). The cells (5 × 10⁶) were then incubated for 30 min on ice with the primary antibody, washed with PBS, and further incubated for 30 min on ice with a FITC-conjugated goat anti-mouse secondary antibody. Cells were
washed and resuspended in PBS and then analyzed on an Epics Elite ESP flow cytometer (Coulter Corp., Hialeah, FL).

**Reverse Transcriptase-PCR of mRNA and cDNA Sequencing**

Total RNA was isolated from 5 × 10⁶ transfected cells according to the method of Chomczynski and Sacchi (26). First strand cDNA synthesis from 2 μg of total RNA was performed with the Perkin-Elmer RNA-PCR kit using oligo(dT) as a primer. The coding sequence, corresponding to the cytoplasmic domain of the β₃ integrin subunit was amplified using specific primers. The amplified products were analyzed by agarose gel electrophoresis and directly sequenced using the fmol™ DNA sequencing kit (Promega).

**Ligand Coating of Latex Beads and Cell-Bead Attachment Assay**

For cell-bead attachment assay, 200 μl of polystyrene 3-μm beads (Sigma) were washed twice in distilled H₂O, and resuspended in 1 ml of 0.1 M bicarbonate coating buffer. 0.9 mg of the monoclonal antibody to the 90-kDa α₁β₃ integrin (P37, Sigma) were washed twice in distilled H₂O, and resuspended in 1 ml of 0.1 M bicarbonate coating buffer, pH 9. Ligand coating was performed by adding fibrinogen or BSA to the beads to a final concentration of 100 μg/ml. The beads were rotated for 1 h at room temperature, washed once in PBS, and blocked with 0.1% BSA in IMDM for 2 h at room temperature. The beads were finally washed twice and resuspended in IMDM. For the cell-bead attachment assay, CHO cells were detached with EDTA buffer, washed twice, and resuspended in serum-free IMDM. After a preincubation of 45 min at room temperature in the presence or absence of either 500 nM echistatin or 1.5 mM EDTA. After a preincubation of 45 min at room temperature in the presence or absence of either 500 nM echistatin or 1.5 mM EDTA. The Slide glasses were examined with a Nikon inverted microscope equipped with phase contrast.

**Cell Adhesion Assay**

Adhesion assays were carried out as described previously with minor modifications (27). Briefly, cultured cells were detached with EDTA buffer, washed twice, and resuspended in serum-free IMDM. The cells (3 × 10⁶) were then added to individual wells of 96 well microtiter plates coated with fibrinogen at 20 μg/ml in serum-free IMDM overnight at 4 °C, and cell attachment was allowed to occur at 37 °C. For time-course experiments, the cells in the individual microtiter wells were microphotographed at different time points without prior washing of the plates or discharge of nonadherent cells. Quantitation of spread fibroblastoid cells versus non-spread round cells was performed on the micrographs according to cell morphology. For each time point, approximately 200 cells were counted and the data reported as mean percent of three independent experiments performed in triplicate.

**Cell Spreading and Immunofluorescence Staining of Focal Adhesion Plaques**

Intracellular immunofluorescence staining of adherent cells was performed using eight-well glass chamber slides (Lab-Tek, Nunc International, Naperville, IL) precoated overnight at 4 °C with 20 μg/ml of fibrinogen in serum-free IMDM. The cultured cells were detached with EDTA buffer, washed twice with IMDM, and incubated overnight in individual compartments of the chamber slides. The cells were fixed for 15 min at 4 °C with 3% paraformaldehyde, 2% sucrose in PBS, pH 7.4, rinsed twice with PBS, and permeabilized with labeling buffer (0.5% Triton X-100, 0.5% BSA in PBS, pH 7.4) for 15 min at room temperature. Immunofluorescent staining was performed by incubating the glass slides for 30 min with a primary mouse monoclonal antibody to human β₃ (P37) or to the α₁β₃ complex (23C6) diluted in labeling buffer. After three washing steps, the glass slides were incubated for another 30 min with FITC-conjugated goat anti-mouse IgG in the presence or absence of 0.5 μg/ml phalloidin conjugated to tetramethylrhodamine isothiocyanate (Molecular Probes, Eugene, OR). Negative controls were stained in the absence of the primary antibody. The slides were then washed three times in labeling buffer and mounted in Mowiol 40–88/DABCO (Sigma). The specimens were examined with a Leica-DMIIRB fluorescence microscope using a 63 × oil immersion objective. Microphotographs were taken using Ifford HPS Plus 400 films (Ilford, Mobberley, United Kingdom).

**Tyrosine Phosphorylation Assay**

Petri dishes (100 mm) were coated overnight at 4 °C with 100 μg/ml of purified human fibrinogen in serum-free IMDM. The dishes were then blocked with 5 mg/ml BSA in serum-free IMDM for 1 h at 37 °C and finally washed twice with serum-free IMDM. Cultured cells were detached with EDTA buffer, carefully washed twice with serum-free IMDM, and resuspended in IMDM, and either kept in suspension or added to the coated dishes in the presence or absence of 2 μM cytochalasin B (Sigma). After a 2-h incubation at 37 °C, nonadherent cells were sedimented at 1000 rpm for 10 min and lysed with the following lysis buffer: 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 10 mM NaF, 1 mM Na₃VO₄, 10 μg/ml pepstatin A, 3 mM phenylmethylsulfonfyl fluoride. Adherent cells were lysed in situ with the same lysis buffer. Lysates were clarified by centrifugation at 12,000 rpm for 10 min at 4 °C, and the protein content determined with the Bio-Rad protein assay reagent (Bio-Rad, Nazareth, Belgium).

**Immunoprecipitation and Western Blot Analysis**

**Preparation of Cell Lysates—**Cultured cells were detached with EDTA buffer, washed twice in cold PBS buffer, and lysed for 30 min in 300 μl of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM phenylmethylsulfonyl fluoride). Lysates were cleared by centrifugation at 12,000 rpm for 10 min at 4 °C, and the protein concentration determined according to the method of Markwell (28). 

**Immunoprecipitation—**For each cell clone, equal amounts of protein lysate (1–1.5 μg of protein) were incubated for 1 h at 4 °C with either monoclonal antibody P37 (to human β₃), or, for tyrosine phosphorylation assays, with polyclonal rabbit anti-FAK or monoclonal mouse anti-phosphotyrosine antibody. Immune complexes were precipitated by a 30-min incubation at 4 °C with protein A-Sepharose beads (75 μl of a 1:1 suspension in PBS). The beads were then washed three times with lysis buffer, and the precipitates recovered by boiling the beads in 30 μl of SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4.6% SDS, 20% glycerol, 0.5 mg/ml bromphenol blue) either in the presence or absence of 1.4 mM β-mercaptoethanol.

**Western Blot Analysis—**Immunoprecipitates or total cell lysates (50 μg of protein) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose using a semi-dry transblot apparatus (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). The membranes were blocked for 1 h in blocking buffer (Tris-buffered saline (TBS) (20 mM Tris-HCl, pH 7.4, 137 mM NaCl) containing 0.1% Tween and either 1% BSA for tyrosine phosphorylation assays or 5% nonfat dry milk) and incubated overnight with the primary antibody diluted in blocking buffer. After several 5 to 10 min washes in TBS-Tween (pH 7.4, 0.1% Tween), the membranes were incubated for 1 h with sheep anti-mouse IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) in TBS-Tween containing 5% nonfat dry milk at pH 7.4. The membranes were then washed in TBS and band visualization was performed using enhanced chemiluminescence (ECL) (Pierce) according to the manufacturer's instructions. After exposure to autoradiography films, the membranes prepared for tyrosine phosphorylation assays were treated with 0.3%H₂O₂, quenched in 0.1 M sulfuric acid, and bands were quantitated by scanning densitometry and the results expressed as the ratio of phosphorylated FAK versus total immunoprecipitated FAK. The data for each cell clone were normalized to the ratio obtained for CHO β₃wt cells adherent on fibrinogen (expressed as 100%).

**RESULTS**

In order to determine how the β₃ integrin cytoplasmic domain regulates integrin-dependent tyrosine phosphorylation during cell spreading, a series of β₃ integrin subunit mutants were generated that either promote or fail to promote β₃ integrin dependent cell spreading (Fig. 1). After stable transfection of wild type or mutant β₃ cDNA into CHO cells, cell clones were analyzed by flow cytometry for surface expression of the chimeric α₁β₃(hamster)β₃(human) receptor, using monoclonal antibodies specific to human α₁ (13C2), human β₃ (P37), and the α₁β₃ complex (23C6). As shown in Fig. 2, all the cell clones selected for the present study revealed similar levels of cell surface expression of the chimeric α₁β₃ Receptor, except mutant...
expression on their cell surface the recombinant protein. Together, these data demonstrate that the selected cell clones expressed the amplified segment sequenced (results not shown). Taken together, these data demonstrate that the selected cell clones expressed the expected mutation, and that an almost complete deletion of the cytoplasmic domain was amplified using specific primers, and the amplified segment sequenced (results not shown). Taken together, these data demonstrate that the selected cell clones express on their cell surface the recombinant protein. Together, these data demonstrate that the selected cell clones expressed the amplified segment sequenced (results not shown).

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\beta_3^{\Delta722}, \text{for which only weak labeling could be observed, despite several successive transfection attempts. Western blot analysis of the expressed recombinant } \beta_3 \text{ subunit in each cell clone essentially confirmed the immunofluorescence data (Figs. 3 and 4A). Interestingly however, despite the weak surface expression of deletion mutant } \beta_3^{\Delta722}, \text{ a band even stronger in intensity to that observed for wild type } \beta_3 \text{ could be demonstrated in CHO } \beta_3^{\Delta722} \text{ cells. The slightly increased electrophoretic mobility of deletion mutants } \beta_3^{\Delta744} \text{ and } \beta_3^{\Delta722} \text{ as compared with recombinant wild type } \beta_3 \text{ confirmed their smaller molecular size. Correct heterodimerization of endogenous } \alpha_v \text{ with the human } \beta_3 \text{ subunit was demonstrated for each deletion mutant by immunoprecipitation experiments using the anti-human } \beta_3 \text{ antibody P37. As shown in Fig. 4B, two bands corresponding to } \alpha_v \text{ and } \beta_3 \text{ were coprecipitated with similar intensities for all deletion mutants, including } \beta_3^{\Delta722}. \text{ Finally, to confirm that each selected cell clone expressed the human } \beta_3 \text{ integrin subunit with the expected cytoplasmic mutation, mRNA was isolated from the transfected cell clones and transcribed into cDNA. The cDNA segment encoding the cytoplasmic domain was amplified using } \beta_3 \text{ specific primers, and the amplified segment sequenced (results not shown). Taken together, these data demonstrate that the selected cell clones express on their cell surface the recombinant } \beta_3 \text{ subunit with the expected mutation, and that an almost complete deletion of the cytoplasmic domain of the integrin } \beta_3 \text{ subunit (} \beta_3^{\Delta722}) \text{ interferes with surface exposure of the preformed heterodimer } \alpha_v \beta_3^{\Delta722} \text{ integrin complex.}
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**Fig. 1.** Amino acid sequence of the cytoplasmic domain of wild type and mutant } \beta_3 \text{ integrin subunits. The cytoplasmic amino acid sequence of } \beta_3 \text{, beginning with Lys}^{716} \text{ of the published sequence is shown (48). Residues of the highly conserved NPLY\textsuperscript{747} and NITY\textsuperscript{759} motifs are indicated in bold letters. Mutants are named according to the position of their amino acid substitution or stop codon. For the deletion mutants, the position of the stop codon is indicated by the number of the corresponding amino acid. For the substitution mutants, the modified amino acid residue(s) are underlined.}

**Fig. 2.** Flow cytometry analysis of chimeric } \alpha_v \beta_3 \text{ expression in CHO cells transfected with recombinant human } \beta_3 \text{. CHO cells, stably transfected with the } \beta_3 \text{ subunits listed in Fig. 1, were grown to confluence in complete IMDM, detached with EDTA buffer, washed twice, and resuspended in serum-free IMDM. The suspended cells were then labeled with saturating amounts of a primary monoclonal antibody to human } \alpha_3 \text{ (13C2), human } \beta_3 \text{ (P37), or to the } \alpha_3 \beta_3 \text{ complex (23C6) and stained with FITC-conjugated goat anti-mouse IgG. The ordinate depicts the number of cells per channel and the abscissa the relative fluorescence intensity in arbitrary units (log scale).}

NPLY\textsuperscript{747} and membrane-distal NITY\textsuperscript{759} sequence in } \alpha_v \beta_3 \text{-mediated cell spreading on fibrinogen, adherence of CHO cells expressing the } \beta_3 \text{ mutants indicated in Fig. 1 was performed using a steady state adhesion assay. The quantitative analysis of cell spreading is shown in Fig. 6 (A and B). Spreading of CHO cells expressing wild type } \beta_3 \text{ was essentially complete after a 2-h incubation at } 37 \text{ °C, in contrast to mock-transfected CHO cells that lacked the } \alpha_3 \beta_3 \text{-dependent adhesive phenotype on fibrinogen, demonstrating that CHO cell spreading on fibrinogen completely relies on the transfected human } \beta_3 \text{ subunit. None of the three deletion mutants (} \beta_3^{\Delta754}, \beta_3^{\Delta744}, \text{ and } \beta_3^{\Delta722}) \text{ underwent shape change on fibrinogen, demonstrating that a minimal deletion of 9 C-terminal amino acids comprising the membrane-distal NITY\textsuperscript{759} motif was already sufficient to completely prevent } \beta_3 \text{ integrin-dependent cell spreading. When}

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\begin{align*}
\beta_3^{\Delta722} & : \text{KLLITHDRKEFSAEERARAKWDTANPPLYKEATSTFTNYVRGT} \\
\beta_3^{\Delta744} & : \text{KLLITHDRKEFSAEERARAKWDTANPPLYKEATST} \\
\beta_3^{\Delta754} & : \text{KLLITHDRKEFSAEERARAKWDTANPPLYKEATSTFTNYVRGT} \\
\beta_3^{Y747A} & : \text{KLLITHDRKEFSAEERARAKWDTANPPLYKEATSTFTNYVRGT} \\
\beta_3^{Y747F} & : \text{KLLITHDRKEFSAEERARAKWDTANPPLYKEATSTFTNYVRGT} \\
\beta_3^{Y754A} & : \text{KLLITHDRKEFSAEERARAKWDTANPPLYKEATSTFTNYVRGT} \\
\beta_3^{Y754F} & : \text{KLLITHDRKEFSAEERARAKWDTANPPLYKEATSTFTNYVRGT} \\
\beta_3^{Y759A} & : \text{KLLITHDRKEFSAEERARAKWDTANPPLYKEATSTFTNYVRGT} \\
\beta_3^{Y759F} & : \text{KLLITHDRKEFSAEERARAKWDTANPPLYKEATSTFTNYVRGT} \\
\end{align*}
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the single tyrosine residues 747 or 759 were mutated into alanine, a complete inhibition of cell spreading on fibrinogen was observed with mutant \( \beta_3\)Y747A and a strong inhibition was observed with mutant \( \beta_3\)Y759A. Similarly, the double mutant \( \beta_3\)Y747A/Y759F exhibited the same defective cell spreading phenotype as mutant \( \beta_3\)Y747A. On the other hand, when the more conservative, but still phosphorylation-defective substitutions of tyrosine by phenylalanine were tested (Y747F and Y759F), almost complete restoration of cell spreading was observed with mutant \( \beta_3\)Y747A, and a complete inhibition of cell spreading on fibrinogen was observed with mutant \( \beta_3\)Y759A. The double mutant \( \beta_3\)Y747A/Y759F revealed the round morphology of firmly attached but unspread cells, and the complete absence of stress fibers in selected CHO cell clones. The wild type human \( \beta_3\) subunit was localized in focal contacts at the tips of well organized actin stress fibers. In contrast, immunostaining of the transfected cells expressing the point mutants \( \beta_3\)Y747A or \( \beta_3\)Y747A/Y759F revealed the round morphology of firmly attached but unspread cells, and the complete absence of \( \beta_3\) integrin-induced focal adhesions or stress fibers, as visualized by the diffuse staining of the cells with the anti-\( \beta_3\) antibody and phalloidin. An identical result was obtained with the deletion mutants \( \beta_3\)Δ754, \( \beta_3\)Δ744, and \( \beta_3\)Δ722 (data not shown). The cell clone expressing mutant \( \beta_3\)Y759A exhibited strongly reduced stress fiber formation and \( \beta_3\) focal contact recruitment in those cells that were able to spread on fibrinogen, whereas cells expressing mutant \( \beta_3\)Y759F had a wild type phenotype. Interestingly, with mutant \( \beta_3\)Y747F, an intermediate phenotype...
was observed; in the cells that had undergone shape change, \( \beta_3 \) integrin was detectable in focal adhesion plaques, but the number of focal adhesion plaques was reduced and the few actin stress fibers were located predominantly at the cell periphery. Altogether, these results essentially confirm the data described for cell spreading experiments.

**Correlation between \( \beta_3 \) Integrin-mediated Cell Spreading and \( \beta_3 \)-triggered Tyrosine Phosphorylation**—In an effort to determine how \( \beta_3 \) integrin-dependent cell spreading correlated with tyrosine kinase activation and intracellular phosphoryrosine signaling, we investigated the effect of the cytoplasmic domain mutations on \( \beta_3 \) integrin-triggered postreceptor occupancy events, namely tyrosine phosphorylation of the intracellular proteins FAK and paxillin. As tyrosine phosphorylation of FAK and paxillin is not only an integrin-mediated response, but can also be stimulated by growth factors, the transfected cells were carefully washed before plating, in order to eliminate all traces of fetal calf serum. After a 2-h incubation at 37 °C on immobilized fibrinogen, attached cells were lysed in situ, and the lysate used for FAK or paxillin immunoprecipitation. Immunoblots of the precipitates were first probed with a monoclonal anti-phosphotyrosine antibody (PY20), then stripped and reprobed with a monoclonal anti-FAK or anti-paxillin antibody. In a control experiment shown in Fig. 9, stimulation of tyrosine phosphorylation of FAK was observed when transfected CHO cells expressing wild type \( \beta_3 \) integrin were allowed to spread on immobilized fibrinogen, whereas only background tyrosine phosphorylation was observed when the same cells were kept in suspension for 2 h or when mock-transfected CHO cells were plated on fibrinogen, demonstrating that the observed increase in FAK tyrosine phosphorylation could be specifically attributed to \( \beta_3 \) integrin-triggered outside-in signaling. When the mutant cell clones were tested, a strong correlation between \( \beta_3 \)-mediated cell spreading and \( \beta_3 \)-triggered FAK phosphorylation was observed (Fig. 10). All the cell clones that were unable to spread on fibrinogen were also unable to trigger FAK phosphorylation above background levels (CHO \( \beta_3\Delta754 \), CHO \( \beta_3\Delta744 \), CHO \( \beta_3\Delta722 \), as well as CHO \( \beta_3Y747A \) and CHO \( \beta_3Y747A/Y759F \)). The \( \beta_3\)Y759A mutant failed to signal tyrosine phosphorylation of FAK, consistent with the strongly reduced spreading phenotype of CHO \( \beta_3\)Y759A cells. In contrast, the more conservative, but still phosphorylation-defective phenylalanine substitution of Tyr779 restored FAK tyrosine phosphorylation, while the Y747F substitution gave an intermediate phenotype, suggesting that the presence of Tyr779, and hence phosphorylation of this residue, is not strictly required to signal FAK tyrosine phosphorylation. These data further indicate that \( \beta_3 \) integrins with a structural modification of the cytoplasmic tail, due to an alanine substitution of Tyr747 or Tyr779, fail to trigger FAK tyrosine phosphorylation.

In order to determine the specificity of paxillin phosphorylation during \( \beta_3 \) integrin-stimulated cell spreading, CHO \( \beta_3 \)wt cells were incubated on fibrinogen in the presence or absence of cytochalasin B, known to prevent cell spreading by inhibiting actin polymerization and subsequent stress fiber formation. As shown in Fig. 11, in the absence of cytochalasin B, CHO \( \beta_3 \)wt cell spreading was complete after 2 h of incubation on fibrinogen, and a band corresponding to the 68-kDa protein paxillin was identified with the anti-phosphotyrosine antibody PY20, indicating that paxillin was phosphorylated to a modest, but significant and consistently reproducible level. In contrast, cytochalasin B abolished \( \beta_3 \)-mediated cell spreading and tyrosine phosphorylation of paxillin. The amount of paxillin immunoprecipitated from cells incubated in the absence or presence of cytochalasin B was roughly the same. When the \( \beta_3 \) mutants were tested, the results correlated essentially with those observed for FAK phosphorylation; the C-terminal deletion mutants \( \Delta754 \), \( \Delta744 \), and \( \Delta722 \) consistently abolished \( \beta_3 \)-triggered paxillin phosphorylation (Fig. 12). Concerning the substitution mutants, only the point mutant \( \beta_3\)Y759F was reproducibly able to signal paxillin phosphorylation to wild type levels. For the other point mutants, \( \beta_3Y747A \), \( \beta_3Y747F \), \( \beta_3Y759A \), and \( \beta_3Y747A/Y759F \), the results were less clear, as the band corresponding to phosphorylated paxillin was of variable intensity depending on the experiment. The amount of immunoprecipitated paxillin from each transfected cell clone was approximately the same as shown after stripping and rehybridization of the anti-phosphotyrosine blot with an anti-paxillin antibody. These results confirm that the presence of the conserved amino acid Tyr779 of the membrane-distal NITY779 sequence within the \( \beta_3 \) cytoplasmic domain is not required for \( \beta_3 \)-triggered phosphotyrosine signaling.

**DISCUSSION**

Integrin cytoplasmic domains are key effectors in regulating integrin-receptor function. In many cell types, both the \( \alpha \) and \( \beta \) subunit cytoplasmic domains modulate integrin affinity for extracellular ligands, and hence play a role in inside-out signaling (14, 30–32). Integrin-mediated cell spreading, in contrast, appears to rely essentially on integrin \( \beta \) subunits, as the \( \beta \) subunit cytoplasmic tail by itself contains sufficient information to target integrins to focal adhesions (7) and to trigger tyrosine phosphorylation of intracellular proteins (8). Following the initial identification of three regions within the cytoplasmic tail of the \( \beta_1 \) integrin subunit necessary for focal contact recruitment of integrins (6), numerous studies on \( \beta_1 \) and \( \beta_3 \) subunits have focused on the functional role of two of these

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**Fig. 6. Effect of \( \beta_3 \) mutations on transfected CHO cell spreading onto fibrinogen.** \( \beta_3 \)-transfected CHO cells were grown to confluence in complete IMDM, detached with EDTA buffer, washed twice, resuspended in serum-free IMDM, and allowed to adhere to microtiter plates precoated with 20 mg/ml fibrinogen overnight at 4 °C. A, after a 2-h incubation at 37 °C, the cells were microphotographed and the percentage of spread cells was determined by correlating the number of spread cells versus the total cell number on the photograph. B, time course of cell spreading onto immobilized fibrinogen. Microphotographs were taken over a period of 12 h at the indicated time points, and cell spreading was quantified as described above. The means ± S.D. of three independent experiments performed in triplicate are reported.
highly conserved sequences, NPXY and NXXY, which constitute typical recognition sites for tyrosine kinases and are encoded by a single exon known to undergo alternative splicing (33). Both of these tandem domains appear to be crucial for integrin receptor function, although studies of various recombinant mutant β3 integrin subunits expressed as heterodimers with either αIIb as a fibrinogen receptor, or αv as a major vitronectin receptor, have generated divergent results: β3 mutants with a deletion of the membrane-distal NITY759 sequence up to amino acid 756 completely prevented αIIbβ3 integrin-dependent cell spreading on immobilized fibrinogen (13), while deletion of the same C-terminal domain up to amino acid 751 allowed normal αvβ3-dependent cell spreading on vitronectin (12). Furthermore, by using cell-permeable peptides carrying different linear β3 cytoplasmic domain sequences, Liu and co-workers (15) identified the β3 C-terminal segment (residues 747–762) as a major cell adhesion regulatory domain capable of inhibiting the interaction of αIIbβ3-expressing HEL cells or αvβ3-expressing endothelial cells with immobilized fibrinogen, while peptides with a Y759F substitution were unable to induce this inhibitory effect. Differences concerning the involvement of the membrane-proximal NPLY747 sequence in signal transduction have also been reported; mutations in the β3 cytoplasmic domain that eliminate or disrupt the membrane-proximal NPLY747 motif prevented αvβ3-mediated cell attachment to immobilized vitronectin, but did not perturb the ability of αvβ3 to interact with soluble vitronectin (12), while mutations in the NPLY747 sequence abolished inside-out signaling of αIVβ3 (14).

The observations that the NPLY747 and NITY759 motifs in the β3 integrin subunit might differently regulate αIIbβ3 and αvβ3 receptor function prompted us to investigate the effect of β3 cytoplasmic domain mutations, that either promote or inhibit cell spreading, on αvβ3-mediated tyrosine phosphorylation of two major focal adhesion proteins, the focal adhesion kinase FAK (34), and the cytoskeleton-related “bridging” protein paxillin (35). Our results demonstrate a close correlation between αvβ3-mediated cell spreading and tyrosine phosphorylation of FAK and paxillin, and highlight a distinct involvement of the NPLY747 and NITY759 sequences in these post-

FIG. 7. Immunofluorescence analysis of the intracellular localization of recombinant human β3 integrins in CHO cells adherent on fibrinogen. Glass coverslips were coated with 20 μg/ml fibrinogen at 4 °C for 24 h. Transfected CHO cells, grown to confluence in complete IMDM, were detached with EDTA buffer, washed twice, and resuspended in serum-free IMDM. Cells were allowed to adhere overnight at 37 °C to the coverslips, fixed, permeabilized, labeled with a primary monoclonal antibody to human β3 (P37) or to αvβ3 (23C6), and stained with FITC-conjugated goat anti-mouse IgG. Negative controls were performed by staining the cells in the absence of a primary antibody. Scale bar, 10 μm.
considering the membrane-distal NITY759 motif, deletion of this motif was sufficient to completely prevent αβ3-dependent focal contact formation, cell spreading, as well as FAK/paxillin tyrosine phosphorylation. A Y759A substitution also resulted in a strong inhibitory phenotype. In contrast, the more conservative, but still phosphorylation-defective Y759F mutation was able to restore wild type receptor function. These data suggest that the structural integrity of the NITY759 motif, rather than the phosphorylation status of Tyr759, is important for β3-mediated cytoskeletal reorganization or tyrosine phosphorylation of FAK and paxillin. Concerning the membrane-proximal NPLY747 sequence, our mutagenesis studies demonstrate that an alanine substitution of the highly conserved tyrosyl residue at 747 completely abolished αβ3-dependent formation of focal adhesion plaques and cell spreading, and prevented FAK and paxillin tyrosine phosphorylation, while a Y747F substitution, compared with the Y759F substitution, only partially restored these receptor functions, suggesting that phosphorylation of residue Tyr747 might be required for optimal β3-mediated post-receptor signaling events. These data could explain why the non-phosphorylated NPLY747-containing peptides used by Liu et al. were unable to impair αβ3 integrin-mediated cell adhesion (15). Our findings, together with previously reported results on the effect of substitutions of Tyr747 on cell adhesion and spreading (12, 13), strengthen the notion that the conformational organization of the β3 cytoplasmic domain defined by the NPLY747 and NITY759 motifs is essential for β3-mediated cytoskeletal organization and FAK/paxillin phosphorylation. These data are in accordance with the results of Tahiliani et al. (23) and with the findings obtained with the alternative spliced variants β1B (36) and β3B (8), previously shown to be defective in triggering FAK tyrosine phosphorylation. In contrast, our data seem to differ from a previous report, showing that the membrane-distal β3 tail is not necessary for α1Bβ3 integrin-triggered tyrosine phosphorylation of FAK (37). Concerning cell spreading, our results are also in good agreement with data on α1Bβ3-mediated cell spreading (13), and further demonstrate that the structural requirements of the β3 cytoplasmic domain necessary for α1Bβ3- or αβ3-mediated cell spreading are essentially the same. This conclusion is supported by the fact that the naturally occurring S752P mutation, which is closely located to the NITY759 motif and responsible for a variant Glanzmann's thrombasthenia phenotype, renders α1Bβ3 defective in both inside-out and outside-in signaling, while a S752A mutation restores wild type receptor-mediated cell spreading for α1Bβ3 (13) as well as αβ3 (25).

Recently, several novel β subunit cytoplasmic domain-specific binding proteins have been identified, which selectively interact with the C-terminal region of β1 (ICAP-1), β3 (cytohesin-1), and β5 (β3-endonexin) integrin cytoplasmic tails (18, 20, 23).
It is quite interesting that both β3-endonexin and ICAP-1, which display restricted binding to the β3 and the β1 cytoplasmic domain, respectively, rely on a structurally intact membrane-distal NITY (β3) or NPKY (β1) motif for integrin binding, as a Tyr → Ala substitution has been shown to completely prevent these protein-protein interactions, while a Tyr → Phe substitution has only minimal inhibitory effect (20, 39). ICAP-1, which is a phosphoprotein and whose phosphorylation is regulated by cell-matrix interaction, appears to play a major role during β1 integrin outside-in signaling processes, and could represent the missing adaptor protein necessary for linking the β1 integrin cytoplasmic tail to downstream signaling events. In contrast, the functional role of β3-endonexin appears to be restricted to inside-out signaling, as no strong colocalization of β3-endonexin with αvβ3 has been observed in β3-triggered focal adhesion plaques (40). Since β3-endonexin modulates the affinity state of αvβ3, it has been suggested that this protein might participate in integrin activation, and dissociate during later stages of cell adhesion, allowing a β3-endonexin-independent interaction of the integrin cytoplasmic tail with cytoskeletal proteins (41), suggesting that transient posttranslational modifications of the β3 subunit might be involved in modulating distinct β3 receptor functions.

Tyrosine phosphorylation of integrin β subunits has been documented in a number of different cell types. In Rous sarcoma virus-transformed fibroblasts, tyrosine phosphorylation of the β3 subunit of the fibronectin receptor resulted in defective cytoskeletal organization (41). Using an antiserum reacting specifically with the phosphorylated cytoplasmic tail of β1, Johanson et al. (42) were able to demonstrate a distinct subcellular localization of tyrosine-phosphorylated β1 as compared with non-phosphorylated β1. In vivo tyrosine phosphorylation of the β3 subunit of αIIbβ3 has been shown to occur during thrombin-stimulated platelet aggregation (19), or after ligand-, antibody- or Mn2⁺- stimulated clustering of αvβ3 in erythroleukemic K562 cells and ovarian carcinoma cells (43). Interestingly however, in the K562 cell model coexpressing αvβ3, αvβ5, and αIIbβ3, Mn2⁺ stimulation of the cells in suspension only stimulated tyrosine phosphorylation of the β3 integrin subunit associated with αv, suggesting that inducible tyrosine phosphorylation of the β3 integrin requires the αv integrin cytoplasmic tail. Data by Law et al. (19) have further shown that in vitro tyrosine-phosphorylated β3 peptides associate with Grb2 as well as Shc, a phosphotyrosine-binding adaptor protein interacting through its PTB (phosphotyrosine binding) domain with phosphorylated NPXY motifs (44). A similar direct in vitro association of αvβ3 with Grb2 has also been reported by Blystone and co-workers (43). More recently, the same group has provided evidence that the presence of residue Tyr747 of the membrane-proximal NPLY747 motif is required for β3 tyrosine phosphorylation and for stimulated αvβ3-mediated adhesion in K562 cells (45). Our data are in good agreement with these findings, and further underline the distinct involvement of the NPLY747 and NITY759 sequences in triggering FAK/paxillin phosphorylation, as they clearly demonstrate that Tyr759 is not required for this process.

In summary, the results of this work provide evidence that modification of the overall conformation of the β3 cytoplasmic domain, due to deletion of the 9 C-terminal amino acids or to a...
structural change within the membrane-proximal NPLY\(^{747}\) and to a lesser extent within the membrane-distal NITY\(^{759}\) sequence, impairs \(\beta_3\)-mediated cell spreading and actin stress fiber formation as well as \(\beta_3\)-triggered paxillin or FAK tyrosine phosphorylation. Phosphorylation of residue Tyr\(^{759}\) of the membrane-distal NITY\(^{759}\) sequence is apparently not necessary for the investigated \(\beta_3\) integrin receptor functions, while phosphorylation of Tyr\(^{747}\) might be required to optimize these functions. The presently described stable CHO cell clones, expressing various \(\beta_3\) mutants, should provide a valuable tool to further investigate interactions of the \(\beta_3\) subunit cytoplasmic tail with structural, regulatory or signaling proteins, and to dissect the involvement of distinct \(\beta_3\) cytoplasmic sequences in various \(\beta_3\) integrin-mediated signaling pathways.

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