PDZ Interaction Sites in Integrin α Subunits

TIP-2/GIPC BINDS TO A TYPE I RECOGNITION SEQUENCE IN α6β5 AND A NOVEL SEQUENCE IN α6β6

Received for publication, June 21, 2001, and in revised form, July 23, 2001
Published, JBC Papers in Press, July 30, 2001, DOI 10.1074/jbc.M105785200

Taneli T. Tani and Arthur M. Mercurio‡

From the Division of Cancer Biology and Angiogenesis, Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215

We used published peptide library data to identify PDZ recognition sequences in integrin α subunit cytoplasmic domains and found that the α6A and α5 subunits contain a type I PDZ binding site (TSDA*; asterisk indicates the stop codon). The α6A cytoplasmic domain was used for screening a two-hybrid library to find interacting proteins. The bulk of the captured cDNAs (60%) coded for TIP-2/GIPC, a cytoplasmic protein with one PDZ domain. The interaction of TIP-2/GIPC with different integrin subunits was tested in two-hybrid and in vitro binding assays. Surprisingly, TIP-2/GIPC bound strongly to the C terminus of both α6A and α6B, although the α6B sequence (ESYS*) is not suggestive of a PDZ binding site because of its polar C-terminal residue. For high affinity interaction with TIP-2/GIPC, at least one of the residues at positions –1 and –3 must be negatively charged. An aliphatic residue at position 0 increases the affinity of but is not required for this interaction. The α5 integrin subunit also bound to TIP-2/GIPC. The α5 integrin and TIP-2/GIPC co-localize in retraction fibers in carcinoma cells plated on laminin, a finding suggesting a functional interaction in vivo. Our results demonstrate that both splice variants of α5 integrin contain a conserved PDZ binding site that enables interaction with TIP-2/GIPC. The binding site in α5B defines a new subclass of type I PDZ interaction site, characterized by a non-aliphatic residue at position 0.

Integrins are heterodimeric transmembrane receptors composed of one α and one β subunit. The extracellular domains of integrins dictate the specificity toward extracellular ligands (1), whereas their highly diverse cytoplasmic domains have specific functions in cell signaling and in mediating interactions with the cytoskeleton (2). Integrin α subunit cytoplasmic domains are short, comprised of only tens of amino acids, and they are devoid of enzymatic activities. Despite their small size, these domains are important for integrin function. Studies with chimeric integrin constructs suggested that the conserved, membrane-proximal part of both the α and β subunit cytoplasmic tail is necessary to maintain integrins in a low activation state (3). Although many of the known interactions of integrins with cytoplasmic proteins are mediated by β subunits (4), integrin α subunits play important roles in regulating integrin function (5, 6).

The diversity of integrin α cytoplasmic domains derives from the large number of α subunit gene products, as well as alternative mRNA splicing of these genes (7). Comparative studies of specific cytoplasmic domain splice variants, as well as studies with chimeric integrin constructs have linked individual α-subunit sequences to specific cellular responses (8–13). The mechanisms by which cytoplasmic domain sequences dictate function, however, are largely unknown. Surprisingly, few direct interactions between integrin α subunit tails and intracellular proteins have been described so far (see recent review in Ref. 4). Paxillin binds to a conserved motif in the α5 cytoplasmic tail (14), suggesting that α5 is exceptional among α-subunits in interacting with a focal adhesion protein directly. Also, the focal adhesion protein talin has been reported to bind to the αIIb integrin subunit (15). Nischarin, a recently characterized cytoplasmic protein, binds to the α6 integrin cytoplasmic tail but not to several other α subunits (16). Nischarin has little homology to proteins with well characterized function, but initial studies suggested that nischarin inhibits cell motility on fibronectin and seems to modulate small G protein function.

PDZ domains are found in numerous cytoplasmic proteins, and they interact specifically with either C termini of other proteins or short recognition sequences in a β-hairpin structure somewhere else in the protein (17). Crystal structures of PDZ domains have explained the need for the C-terminal or β-hairpin location for the recognition sequence. A hydrophobic GLGF-motif acts as a steric block that efficiently prevents longer peptide sequences from interacting with the PDZ domain (18). This motif also provides the critical amino acids for coordinating the terminal COOH-group in PDZ interaction with C-terminal sequences (19). The recognition sequence is only a few amino acids in length, and the preferred recognition sequences of several different PDZ proteins have been analyzed by the peptide library approach. These studies divided PDZ recognition sequences into two major groups. In class I interactions, the –2 position in the recognition sequence is occupied by a hydroxyl group-containing amino acid (Ser, Thr, or Tyr), whereas in class II interactions, there is a hydrophobic residue in the –2 position. The C-terminal residue (position 0) is hydrophobic (20).

The peptide library studies make it possible to predict potential PDZ binding sites in the C termini of different proteins. In the current literature, there is no analysis of possible PDZ binding sites in integrin subunits, and no PDZ interactions have been described between integrin cytoplasmic domains and cytoplasmic proteins. For this reason, we analyzed the amino acid sequences of different integrin α subunits and identified two subunits, α6A and α6B, with a potential PDZ recognition...
motif. We used this information for a yeast two-hybrid screen to identify PDZ proteins that interact with the α₂A integrin subunit. Surprisingly, our results demonstrate the presence of PDZ interaction site in both splice variants of α₂A integrin. The PDZ binding site present in α₂B splice variant was not predicted by any previously published models of PDZ interactions, and it defines a new type of PDZ recognition sequence that may be present in many other proteins, too.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The pRC/CMV expression vectors containing the αₐA and α₂B cDNAs have been described previously (21). A new α₂A construct lacking the last three amino acids was created by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA). The three nucleotides that encode the serine in the −2 position in the C terminus were changed to a stop codon. cDNAs coding for integrin cytoplasmic domains were designed using a common strategy. These constructs started with the first residue of the conserved, membrane-proximal amino acid sequence. The primers used were as follows: αₐA-sense, AAAGAATTCCATGATGATCATTTTCAG; αₐA-antisense, TTGTGCACTTCTAGTGGGCGAGAG; α₂B-sense, TTGAATTCATGATGATCATTTTCAG; α₂B-antisense, TTGAATTCATGATGATCATTTTCAG. The PCR products were cloned into the SmaI site of the pACTII vector. 2-antisense, TTGTCGACCTATGAGTAAGTCACTCAGCAGAAGTAGCTTCTCCTAGTCTTCTTCGAG. An alternative construct was made by using modified antisense primers: αₐA-sense, AAAGAATTCAAGTGTGACTTCTTTCTTCATTTTGGACTTTTGAGTC; α₂B-sense, TTGAATTCAGGATGGGCTTTTTTGGACTTTTGAGTC. Two other constructs containing various parts of TIP-2/GIPC sequence were created by using standard PCR using the cDNAs retrieved from the yeast two-hybrid screen as a template. The antisense primers designed to introduce a stop codon immediately after the last included residue of TIP-2/GIPC. The primers were as follows: TIP-2/GIPC (115–217) sense, AAGAAATCTGCGAGGCTTGGGAG; antisense, AACTCGAATGGGCCTCTTGCGACGACGACTCAGGAGCT. TIP-2/GIPC (123–217) sense, AAAAGATTCTCATCTCGCCCATATGCGGAGGAC; antisense, AACTCGAATGGGCCTCTTGCGACGACGACTCAGGAGCT. TIP-2/GIPC (218–333) sense, AAGAAATCTGCGAGGCTTGGGAG; antisense, AACTCGAATGGGCCTCTTGCGACGACGACTCAGGAGCT. TIP-2/GIPC (387–504) sense, AAGAAATCTGCGAGGCTTGGGAG; antisense, AACTCGAATGGGCCTCTTGCGACGACGACTCAGGAGCT. TIP-2/GIPC (585–702) sense, AAGAAATCTGCGAGGCTTGGGAG; antisense, AACTCGAATGGGCCTCTTGCGACGACGACTCAGGAGCT. The pRC/CMV expression vectors containing the α₂A cytoplasmic domain in PAS2–1 vector (22) were constructed by cloning the PAS2–1 sequence into the BamHI site of the pActII vector. Bacterial clones were grown on Luria-Bertani (LB) agar plates containing 50 μg/ml of ampicillin and 34 μg/ml of chloramphenicol (Cm). Primers were designed (22), using the following oligonucleotides: sense, CAATGTCGGAGATGCGGGTGCTCGTG; antisense, AAGAAATCTCATCTCGCCCATATGCGGAGGAC. TIP-2/GIPC (387–504) sense, AAGAAATCTGCGAGGCTTGGGAG; antisense, AACTCGAATGGGCCTCTTGCGACGACGACTCAGGAGCT. Yeast Two-Hybrid System—Components of the Matchmaker II two-hybrid system were purchased from CLONTECH (Palo Alto, CA). The cDNA coding for the entire α₂B cytoplasmic domain in PAS2–1 vector was PCR amplified and cloned into the multiple cloning site of pActII vector. Bacterial clones were grown on Luria-Bertani (LB) agar plates containing 50 μg/ml of ampicillin and 34 μg/ml of chloramphenicol (Cm). The bacterial colonies were tested positive in initial screening and were further characterized by two-hybrid tests using several different bait proteins in the EcoRI/Sall site of the pAS2–1 vector.

**Northern Hybridization**—Total cellular RNA was isolated by Tri reagent (Sigma) method. 20 μg of total cellular RNA was run in a 1% agarose gel and transferred on Hybond-N nylon membranes. A fla-gellant reagent (Roche Molecular Biochemicals) was used for the 3 h ofRNAse A treatment at 37 °C. The filters were then hybridized with the 32P-labeled cDNA probe. The filters were then washed in 60 °C, and exposed on autoradiographic film. The PDZ full-length cDNA was used as a template. The other sequences were cloned by using the regular 6A-7aa-antisense, TTGTCGACCTATGCAGCAGAAGTAAGCCTCTCCTTTGCGAG. An alternative construct was created by using modified antisense primers: 6A-sense, AAGAATTCAAGTGTGACTTCTTTCTTCATTTTGGACTTTTGAGTC; 6A-7aa-sense, TTGTCGACCTATGCAGCAGAAGTAAGCCTCTCCTTTGCGAG. Three prey vector (pAC-T2) constructs containing various parts of TIP-2/GIPC sequence were created by using standard PCR using the cDNAs retrieved from the yeast two-hybrid screen as a template. The antisense primers designed to introduce a stop codon immediately after the last included residue of TIP-2/GIPC. The primers were as follows: TIP-2/GIPC (115–217) sense, AAGAAATCTGCGAGGCTTGGGAG; antisense, AACTCGAATGGGCCTCTTGCGACGACGACTCAGGAGCT. TIP-2/GIPC (123–217) sense, AAAAGATTCTCATCTCGCCCATATGCGGAGGAC; antisense, AACTCGAATGGGCCTCTTGCGACGACGACTCAGGAGCT. TIP-2/GIPC (218–333) sense, AAGAAATCTGCGAGGCTTGGGAG; antisense, AACTCGAATGGGCCTCTTGCGACGACGACTCAGGAGCT. Northern Hybridization—Total cellular RNA was isolated by Tri reagent (Sigma) method. 20 μg of total cellular RNA was run in a 1% agarose gel and transferred on Hybond-N nylon membranes (Amersham Pharmacia Biotech) by capillary transference using standard protocols. ApaI fragment of TIP-2/GIPC was used as the template for the probe. Labeling was done by Rediprime II random prime labeling kit (Amersham Pharmacia Biotech) using 50 μCi of [α-32P]dCTP (PerkinElmer Life Sciences). Hybridization was done at 68 °C in QuikHyb hybridization solution (Stratagene), and washes were done up to the stringency of 68 °C 0.1× SSC.

**In Vitro Binding Assays**—GST fusion proteins of integrin cytoplasmic tails were produced using pGEX-4T1 derived plasmids described above and BL21 bacterial strain (Stratagene). Cultures in late logarithmic growth phase were induced with 0.2% L-arabinose (Sigma) for 3.5 h at 37 °C, and the recombinant protein was isolated from the bacterial pellets with NiNTA agarose (Qiagen). The protein was eluted from the agarose with 250 mM imidazole and dialyzed against 100 mM KCl/20 mM Tris, pH 7.2. GST fusion proteins of integrin cytoplasmic tails were produced using pGEX-4T1 derived plasmids described above and BL21 bacterial strain (Stratagene). Cultures in late logarithmic growth phase were induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 30 °C for 3 h. Bacterial pellets were frozen on dry ice, thawed once, and then resuspended in PBS containing 1% Triton X-100, 10 mM dithiothreitol, 10 mM EDTA and a protease inhibitor mixture (Sigma). Bacterial homogenates were then sonicated, and recombinant proteins were captured from cleared lysates by glutathione-Sepharose beads (Amersham Pharmacia Biotech), followed by washes and elution by 5 mM glutathione in 100 mM KCl/50 mM Tris, pH 8.0. All recombinant proteins were stored as glycerol stocks at −80 °C.

**In vitro Binding Assays**—500 pmol of GST fusion constructs and 20 pmol of recombinant His6/GIPC protein were used in each in vitro pull-down assay. Recombinant proteins were diluted in 500 μl of buffer and stirred with glutathione-Sepharose beads at 4 °C for 2 h. Beads were blocked with 5% milk before use. RIPA binding buffer contained 50 mM Tris, pH 7.2, 100 mM KCl, 10 mM MgCl2, 10% glycerol, 0.1% BSA, 1% Triton-X, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate. Binding buffer “1×NP” contained 50 mM Tris, pH 7.2, 100 mM KCl, 10 mM MgCl2, 10% glycerol, 0.1% BSA, 1% Triton-X, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate. Binding buffer “1×NP” contained 50 mM Tris, pH 7.2, 100 mM KCl, 10 mM MgCl2, 10% glycerol, 0.1% BSA, 1% Triton-X, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate.
mm MgCl₂, 10% glycerol, 0.1% BSA, and 1% Nonidet P-40 (Roche Molecular Biochemicals). After the binding, beads were washed three times with a buffer identical to the binding buffer but lacking BSA. Samples were analyzed by standard Western blotting using INDIA His-Probe (Pierce). Signal was detected by West Pico chemiluminescence system (Pierce). The experiments were performed in quadruple, and the results were quantified from the film by image analyzing techniques. Binding to GST was used as the measure of background binding, and this was subtracted from the experimental data before analysis. Mutant constructs were compared with the wild type integrin construct, and the results were expressed as a percentage ± S.D.

**Cell Culture**—MDA-MB-435 and MDA-MB-231 breast carcinoma cell lines were obtained from Lombardi Breast Cancer Depository at Georgetown University. They were grown in low glucose DMEM (Gibco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. Clone A colon carcinoma cells were grown in RPMI-1680 medium supplemented with 10% fetal calf serum and antibiotics.

**Cell Adhesion Experiments and Confocal Microscopy**—Coverslips were coated with trypsin-EDTA and washed three times with RPMI 1680 medium containing 0.2% BSA. Cells were plated on coverslips and incubated at 37 °C in the presence of 5% CO₂ for the time periods described. In some experiments, the cells were plated on collagen for 50 min and then treated with 100 mM phorbol 12-myristate 13-acetate (Sigma) for 10 min. In another set of experiments, the cells were plated on laminin for 30 min and then treated with 100 nM staurosporine (Calbiochem, San Diego, CA) for 15 min. After the experiment the cells were washed once with PBS and fixed with paraformaldehyde for 5 min as previously described (23). After fixation, the cells were permeobilized with 0.05% Triton X-100 in PBS for 5 min and then washed several times with PBS. Immunohistochemical stainings with anti-GIPC antisera (kind gift of Dr. Marilyn Gist Farquhar) and anti-α6 integrin antibody (GoH3; Immunotech, Marseilles, France) were followed by fluorescein isothiocyanate- or Cy3-coupled antibodies (Jackson ImmunoResearch, West Grove, PA). After the staining and washing, the samples were treated with 1% paraformaldehyde for 3 min and then washed and embedded in medium containing 50 mM Tris, pH 8.5, 150 mM NaCl, 90% glycerol, and 1% n-propyl gallate. The samples were analyzed by confocal microscopy.

**Analysis of Expression of α6 Integrin Splice Variants in Clone A Cells**—The expression pattern of the αA and αB integrin splice variants was studied by RT-PCR. The primer pair was designed so that both upper and lower primer hybridized with the sequence common to both splice variants, and the reaction product spanned the differentially spliced region. The expected product sizes were 347 (αA) and 216 base pairs (αB). The primers were as follows: αA-sense, TCATCCTAGTG-CATATTCTC; αA-antisense, CTTACATCGTGGTTCCATCT. DNA-treated total RNA (0.2 μg) was used for the reaction, and the reaction products were analyzed by electrophoresis in 1% agarose gel. A 100-base pair ladder (New England Biolabs, Beverly, MA) was used as the molecular weight standard.

**RESULTS**

**Prediction of PDZ Recognition Sequences from Sequence Data**—Analysis of the sequences of human integrin α subunits (Fig. 1) revealed that two human integrin α chains have a typical class I PDZ binding sequence in their C terminus: αA and αA (last three residues SDA*). This motif constitutes a classical Class I PDZ binding site, with a hydroxyl group-containing residue at −2 and a hydrophobic residue at 0. Although most PDZ domains seem to favor isoleucine or valine at 0, the SDA* sequence is an almost perfect match with the peptide library data (20) and the structural model of critical interaction sites (19). Although some other integrin α subunits (αV, αB, and αC) have a serine residue at −2, they fail to comply with the requirement of an aliphatic residue at position 0. We did not recognize type II PDZ binding sites in any of the integrin sequences presented in Fig. 1. It should be noted that the αA sequences are of rat origin, because only the human αA isoform is currently available in data bases. Integrin cytoplasmic domains are highly conserved between species, and therefore the use of rat αA sequences in the analysis is unlikely to affect the results.

**Yeast Two-hybrid Screen**—Based on our sequence analysis, we sought to identify proteins that interact with the type I PDZ interaction site found in the αA and αB integrins. The entire αA cytoplasmic domain was cloned in the pAS2–1 bait vector and used for screening a human mammary gland yeast two-hybrid library. Most tissues and cell lines express both isoforms of the αA integrin, whereas the ducal and alveolar epithelia of the mammary gland are known to express the αA isoform solely (7). Therefore, a mammary gland library was considered particularly suitable for identifying potential binding partners of the αA integrin. Nineteen of the most rapidly growing clones that were positive in the β-galactosidase test were selected for analysis by automated sequencing. Of these clones, 11 of 19 contained varying lengths of the same cDNA sequence. The sequence data obtained was identical to AF208824 and AK022585; the former sequence is the partial reading frame of TIP-2 (Tax interaction protein clone 2) (24), and the latter sequence is the complete coding sequence of TIP-2 from an unrelated sequencing project. Except for some conservative mutations our sequences were also identical to the published sequence of human GIPC (AF089816) (25). One of our clones contained the entire reading frame, and we sequenced this clone to verify the identity of our sequence. Based on our analysis, we conclude that the amino acid sequences of TIP-2 and GIPC are 100% identical, and therefore we refer to the protein as TIP-2/GIPC. For functional studies we cloned the open reading frame of TIP-2/GIPC from MDA-MB-231 cells by RT-PCR, and also this sequence was identical to the published TIP-2 sequence (AF208824/AK022585).

**Verification of the Identity and mRNA Size by Northern Blotting**—The published sequences of TIP-2/GIPC contain very little sequence upstream of the proposed translation start site. Also, the clone that we isolated from the library started only a few nucleotides in the 5′ direction of the start codon. To exclude the possibility that there was any unpublished coding sequence, we performed Northern hybridization of total RNA isolated from two breast carcinoma cell lines, MDA-MB-231 and MDA-MB-435 (Fig. 2). Northern hybridization cannot dif-
is clearly a type I PDZ interaction, we reasoned that integrin sequence might represent a subtype of PDZ interaction, as established TIP-2 and GIPC sequences make no difference for functional studies.

**Confirmatory Two-hybrid Tests**—The specificity of the interaction between αA integrin and TIP-2/GIPC was verified by two-hybrid tests using wild type and mutated integrin sequences. Because the αA integrin has the same putative PDZ recognition sequence (TSDA*) as αA, this subunit subunit was also used in these tests. Mutant constructs of both αA and α5 with the last three amino acids deleted were created (named αAΔpdz and α5Δpdz). Because mutation of the −2 position serine should be sufficient for abolishing type I PDZ interaction, we also made constructs with the −2 position serine mutated to alanine. These constructs, as well as all other integrin cytoplasmic domain point mutants used in this study, were named by listing the last four residues using the standard one-letter code for amino acids (αAΔTADA* and α5ΔTADA*). The αB cytoplasmic domain was selected as a negative control.

The results of these experiments are shown in Table I. A TIP-2/GIPC clone containing residues 102–333 was used as a bait, and for clarity, the results obtained are summarized in Table II. Deletion of the last three amino acids (αBΔpdz) or point mutation of −2 position serine (αB/EAYS*) completely abolished interaction, suggesting that αB C terminus actually is a PDZ recognition sequence. The PDZ interaction hypothesis was further supported by the fact that the last seven amino acids of αA and αB were sufficient for interaction and actually resulted in higher level of reporter gene activation than the construct containing the entire cytoplasmic tail. Neither the αV subunit nor the α2 and α3/αB subunits interacted with TIP-2/GIPC.

**Demonstration of PDZ Interaction by Mutation of the GLGF Loop**—The fact that the point mutation of −2 position serine of αA, αB, and αC termini completely abolished the interaction with TIP-2/GIPC was highly suggestive of a PDZ interaction. To prove this definitively, we made more yeast two-hybrid tests using various TIP-2/GIPC constructs as a prey. The GLGF-loop is critically important for PDZ interactions, and point mutations in this sequence should abolish the interaction. We used a previously described point mutation strategy (22) to destroy the atypical GLGF-loop of TIP-2/GIPC (ALGL mutated to AAEL). The results of these experiments are summarized in Table I. Table II. The amino acid sequence of the PDZ domain and some of the sequence surrounding it are shown at the bottom of the figure to clarify how the constructs were designed. One of the plasmids captured by the original two-hybrid library screen, coding for residues 102–333 of TIP-2/GIPC, was used as a positive control and for creating the GLGF-loop mutation construct. The results show that point mutation of the GLGF-loop (ALGL mutated to AAEL) is sufficient to abolish the interaction with all three integrins (αA, αB, and αC).

**Recognition of the Minimal Sequence in TIP-2/GIPC Required for Binding to Integrins**—We next decided to test whether the PDZ homology domain alone is sufficient for interaction. Homology searches suggested that the PDZ domain of TIP-2/GIPC is formed by residues 133–216. The TIP-2/GIPC clones captured by the original library screen all contained the PDZ domain and the sequence on its C-terminal side, whereas the amount of sequence N-terminal to the PDZ domain varied.
The last seven amino acids of the α5 integrin subunit, 325–331, was used as a positive control, and it was also used for creating the GLGF-loop mutant construct. In the GLGF-loop mutant construct, the sequence ALGL was replaced by ALHL as previously described (19). The other constructs shown in this figure contain various parts of the TIP-2/GIPC sequence, and the position numbers of the first and last residues included are shown for each construct. The PDZ homology region is formed by residues 133–216, and the amino acid sequence of the entire PDZ domain and some of the sequence around it is shown at the bottom. Residues forming the PDZ domain are in boldface, and the atypical GLGF-loop (ALGL) is underlined. The threonine residue corresponding to the second residue of the second β pleated sheet (βB) of some more thoroughly characterized PDZ domains (28, 32) is highlighted by a shaded box. The β-galactosidase reaction was assessed as very strong (++) when apparent within 1 h, strong (+) if visible after 2 h, weak (+) if visible within 2–6 h, and negative if not detectable after 6 h.

Considerably. The yeast two-hybrid library was oligo-dT primed, and therefore it is impossible to make any conclusions of the importance of the C-terminal sequence for interaction on the basis of the original library screen. The shortest sequence obtained from the library screen coded for residues 115–217, starting at the same residue as the minimal sequence obtained from the library screen but discontinuing immediately after the end of the PDZ homology region. Another construct was designed to start closer to the PDZ homology region but to stop at the same residue as the previous one (residues 123–217). To exclude the possibility that the sequence on the C-terminal side of the PDZ domain binds to itself to the integrin cytoplasmic domains, we designed a construct consisting of residues 218–331. Our results (summarized in Fig. 3) show that residues 115–217 are sufficient for interaction with all integrins concerning α5A, α5B, and α5s, whereas the construct containing residues 123–217 failed to interact with any of them. The C-terminal sequence (residues 218–331) did not interact with any of the integrin cytoplasmic domains. We conclude that the interaction between the integrin α subunits and TIP-2/GIPC is mediated entirely by the PDZ domain of TIP-2/GIPC. The minimal sequence required for interaction contains a short stretch of amino acids on the N-terminal side of the PDZ domain. It should be noted, however, that the localization of PDZ domain to residues 133–216 is based entirely on the sequence homology data. The structural requirements for the formation of the PDZ domain may include the presence of these residues outside the actual PDZ homology region.

In Vitro Pull-down Assays—The interactions found in the yeast two-hybrid assays were confirmed in vitro. His-tagged bacterial recombinant TIP-2/GIPC and GST fusion proteins of integrin cytoplasmic domains were used in stringent RIPA conditions. Fig. 4A shows the binding of wild type α5A/B and to the constructs either lacking the last three residues (Δpdz) or having the point mutation of –2 serine to alanine (α5/5TADAΔ* and α5B/EAYSΔ*). The left lane in Fig. 4A is the positive control, containing 20% of the amount of TIP-2/GIPC used for the pull-down assays. α5A and α5B show strong interaction with TIP-2/GIPC in vitro, and the point mutation of the –2 serine and the deletion of the last three amino acids abolished the interaction equally well. Fig. 4B shows the corresponding data for the α5 integrin subunit. The binding to α5 was relatively weak in RIPA conditions, but nevertheless the point mutation of the –2 serine (α5/5TADAΔ*) and deletion of the last three residues (α5/5Δpdz) both abolished the interaction in vitro. We repeated the experiment for the α5 constructs using Nonidet P-40 detergent (Fig. 4B, right panel). There was a
The wild type sequences are TSDA* (αA) and ESYS* (αB). The experiment was performed in quadruplicate, and bindings of αA and αB mutants were compared separately to wild type αA and αB, respectively. The binding is expressed as the percentage of wild type ± S.D.

slightly higher background binding to GST protein itself, but even in less stringent conditions the deletion of the last three residues or mutation of the –2 position serine abolished the interaction. These results clearly demonstrate that α6 integrin C terminus binds to TIP-2/GIPC in vitro. We conclude that all three integrin subunits (αA, αB and β3) have a PDZ interaction site for binding to TIP-2/GIPC.

In Vitro Binding Assays with αA Cytoplasmic Domain Mutants—We next decided to investigate in detail the residues in αA and αB integrin that are critical for interaction with TIP-2/GIPC. The specific PDZ interaction sites are usually composed of the 4–5 most C-terminal residues of the protein. Therefore, we focused on the last five amino acids of the αA and αB integrins. Both subunits contain a serine in the –2 position, which is required by definition for a type I PDZ interaction site. Otherwise, these peptide sequences have little in common. αB has a negatively charged residue (Glu) at the –3 position, and αA has threonine in the same place. At the –1 position, the situation is reversed: αA has a negatively charged aspartate residue, whereas αB has a hydroxyl group-containing tyrosine. We hypothesized that these two positions might act cooperatively and that the ability to bind to TIP-2/GIPC would be dictated by the combined action of the two. Therefore, we continued the in vitro binding studies with mutational analysis of integrin cytoplasmic tails. The constructs used in these pull-down experiments contained the entire cytoplasmic domain of the integrins, with one of the last four residues being mutated. The constructs were named by listing the last four amino acids of the mutant using the standard one-letter code for amino acids. Each experiment was performed in quadruplicate, and the binding was quantified from Western blots by image analysis. The αA mutants were compared separately to wild type αA, and the αB mutants were compared with wild type αB. The binding efficiency was expressed as a percentage compared with the wild type integrin. The constructs lacking the last three residues were used as a negative control. The results using αA mutants are shown in Fig. 5A. Mutation of the –3 position threonine to alanine (αA/ASDA*) reduced the binding about 60%, whereas mutation of the –1 position aspartate to alanine (αA/TSAA*) abolished the interaction almost completely. The αA/ESDA* construct showed higher affinity toward TIP-2/GIPC than the wild type integrin, suggesting that glutamate (as in αB) was preferred at this position.

In Vitro Binding Assays with αB Mutant Constructs—The αB mutants were designed to study in detail how this atypical sequence could function as a PDZ interaction site. The results are shown in Fig. 5B. Mutation of the –3 position glutamate to alanine (αB/ASYS*) reduced the binding by 70%, and even the mutation to threonine (αB/TSYS*) reduced binding by almost 50%. Glutamate is therefore the preferred residue at –3, which is in line with the data obtained with the αA mutants. Replacement of the –1 position tyrosine with phenylalanine (αB/ESYS*) reduced the binding affinity only modestly, suggesting that the –3 position glutamate is the most critical residue for high affinity binding in αB integrin. The hydroxyl group-containing residues, however, can substitute to some extent for the lack of a negative charge, as shown by the comparison of wild type αA (Thr at –3) and αB (Tyr at –1) to mutants having an aliphatic residue at these positions.

Analysis of the Atypical C-terminal Residue in αB PDZ Recognition Sequence—Although the αB C terminus clearly functions as a PDZ interaction site based on our results, the previously published peptide library data do not predict this sequence to be such. We tested the effect of mutating the last residue in αB to alanine (αB/ESYA*), which fits the model of classical type I PDZ interaction site better (Fig. 5B). This mutant showed increased affinity toward TIP-2/GIPC, suggesting that even for TIP-2/GIPC, the preferred residue at the end of the recognition sequence is aliphatic. However, the presence of an aliphatic residue at the C terminus of the target protein is not absolutely required for binding if the residues farther upstream match the binding specificity of TIP-2/GIPC.

Localization of α6 Integrin and TIP-2/GIPC in Clone A Cells Plated on Laminin—Based on previous studies that linked the α6 integrins to the migration of carcinoma cells, we assessed the possibility that α6 integrins and TIP-2/GIPC co-localize in structures involved in migration. For this purpose, we used clone A colon carcinoma cells because the α6-dependent adhesion of these cells to laminin induces the formation of lamellae and retraction fibers and it stimulates their motility (23). The α6 integrin subunit can form heterodimers with both β1 and β4 integrin subunits. Clone A cells, however, express solely α6β4 (23, 26, 27). We also analyzed the expression pattern of α6 splice variants in clone A cells by RT-PCR, and our results show that clone A cells express both splice variants of α6 integrin subunit, although αA splice variant is expressed at a higher level (Fig. 6, top right). In clone A cells plated on laminin, a distinct co-localization of α6β4 and TIP-2/GIPC was most apparent in retraction fibers (Fig. 6, A and B). Indeed, there are several areas in which both the α6 integrin subunit and TIP-2/GIPC are concentrated (arrows). A brief exposure of these to 100 nM staurosporine increases the formation of retraction fibers dramatically and, under this condition, the co-localization of α6β4 and TIP-2/GIPC was striking (Fig. 6, C and D). A co-localization of these two proteins was much less apparent in lamellae (data not shown). The formation of lamellae can also be induced in clone A cells independently of α6β4 engagement by phorbol 12-myristate 13-acetate stimulation of cells adherent to collagen. Interestingly, an intense localization of TIP-2/GIPC was apparent at the edge of the lamellae in these cells, although no co-localization with α6β4 was apparent (Fig. 6, E and F), nor was any co-localization seen in the retraction fibers in these cells (data not shown). Based on these results, we conclude that the engagement of the α6β4 integrin by laminin promotes the co-localization of this integrin and TIP-2/GIPC in retraction fibers.

DISCUSSION

This is the first report describing and analyzing PDZ interactions with integrin α subunits. We demonstrate that the α6A
and α5 integrin subunits contain a conserved PDZ recognition sequence that enables these integrins to interact with TIP-2/GIPC, a PDZ domain-containing, cytoplasmic protein. Most likely, this conserved motif mediates interactions with other cytoplasmic PDZ proteins as well, possibly in a cell type-specific manner. In addition, our study revealed the presence of a novel PDZ interaction site in the α6 integrin subunit, the C terminus (ESYS*) of which is not consistent with the current peptide library based models of PDZ interaction sites (20). These findings indicate that other PDZ recognition sequences exist, and the number of PDZ interactions in vitro may be much higher than can be predicted on the basis of current assumptions about PDZ interaction sites. Moreover, our results have important implications for the mechanism by which specific integrins interact with cytoplasmic proteins.

The yeast two-hybrid screen with α6A integrin as a bait suggested a strong interaction with TIP-2/GIPC. Because α6A and α5 subunits have exactly the same last four residues (TSDA*), it was reasonable to assume that they would interact with the same PDZ protein. Although the interaction of both α6A and α5 with TIP-2/GIPC could be verified in vitro, the interaction with α5 was weak under the experimental conditions we used for α6A/B integrins. However, the interaction became clearly apparent under less stringent conditions, and we conclude that α6 integrin specifically binds to TIP-2/GIPC. There are many examples in the literature in which the sequence upstream of the actual PDZ recognition motif changes the affinity of the interaction (28, 29). The sequences of α6A and α5 are completely different upstream of the TSDA* motif, which probably explains why efficient binding of α5 to TIP-2/GIPC in vitro required the use of different detergents.

A surprising finding was the presence of a PDZ recognition sequence in the α6B integrin subunit, even though the C-terminal sequence (ESYS*) is not suggestive of one. The preference for an aliphatic residue at the very end of the sequence was so strong in the peptide library screen that it was considered mandatory for the PDZ interaction (20). The mutational analysis we performed may explain some discrepancies between peptide library work and studies with actual proteins. Mutation of the last residue of α6B to alanine (α6B/ESYS*) increased the binding affinity considerably, suggesting that the wild type sequence is sufficient but not optimal for binding to TIP-2/GIPC. TIP-2/GIPC seems to allow more flexibility at the 0 position than the PDZ proteins used in crystal structure studies. In this direction, recent studies have provided insight into how PDZ domains select for a particular C-terminal residue. The Na+/H+ exchanger regulatory factor PDZ domain shows a preference for C-terminal leucine. This binding specificity derives from the larger dimensions of the binding pocket compared with the PSD-95 PDZ3 domain that favors the smaller side chain of valine (30, 31). The critical structure that coordinates the terminal carboxyl group is GLGF motif inside the PDZ homology domain (19). In TIP-2/GIPC, even the GLGF-loop is atypical: Ala-Leu-Gly-Leu (22). This unusual GLGF-motif, together with other structural features yet to be identified, probably explains the promiscuity in preferences for C-terminal residue.

The fact that TIP-2/GIPC can bind to two seemingly very different sequences, α6A (TSDA*) and α6B (ESYS*), suggests that the current models of PDZ interaction are too restricted. It also shows that the binding site acts as a functional unit, and the relative importance of each residue may differ in different binding sequences even for the same PDZ protein. Apart from the −2 serine that is critical for all type I PDZ interactions and that is found in both the α6A and α6B isoforms, the −1 and −3 residues appear to be the most critical for the interaction between these integrins and TIP-2/GIPC. Constructs that did not have a negatively charged residue at either position (α6A/TSA* and α6B/ASYS*) showed the lowest affinity toward TIP-2/GIPC, whereas the α6A construct with a negatively charged residue at both positions (α6A/B/ESDA*) was superior to the wild type integrin in binding to TIP-2/GIPC. Thus, our data suggest that at least one carboxyl group-containing residue in these positions is needed for efficient binding. The preference for negatively charged residues at both −1 and −3 positions has been previously described for the first two PDZ domains of PSD-95 (28). Our studies on TIP-2/GIPC revealed an even more complicated pattern, because hydroxyl group containing residues were able to compensate for the lack of a negatively charged amino acid to some extent. This was shown by the weak but detectable binding to α6B/TSYS* construct and the slight difference in binding between the wild type α6B (ESYS*) and α6B/EFIS* mutant.

For wild type α5A, the −1 Asp is critically important for the interaction with TIP-2/GIPC. The mutation of −1 Asp to alan-
nine completely abolished interaction. This finding is somewhat contradictory to the published structural models of PDZ interactions. In the third PDZ domain of PSD-95, the side chain of the –1 residue is pointed outwards and does not participate in interactions with the PDZ domain (19). However, more recent reports have recognized PDZ interactions in which there is a preference for either a negatively charged or Ser/Thr residue at the –1 position. The critical residue for these preferences is the second residue of the second β-sheet pleated sheet (βB) in the PDZ homology domain. If this βB2 residue is serine, the PDZ domain selects for peptides with a negatively charged residue at –1, whereas if it is asparagine, the PDZ domain prefers peptides with –1 Ser/Thr (28, 32). Interestingly, the βB2 residue in TIP-2/GIPC PDZ homology domain is threonine (Fig. 3), which is probably also able to convert the preference toward negatively charged residues at –1.

TIP-2/GIPC contains one PDZ domain in the central part of the molecule. The C-terminal part shows weak homology to acyl carrier protein domain (25), but so far it has not been shown to have enzymatic activity. Most functions suggested for TIP-2/GIPC are those of a scaffold protein, as is typical for other PDZ proteins. An obvious problem for scaffold functions is the fact that TIP-2/GIPC has only one PDZ domain. However, TIP-2/GIPC self-associates (33, 34), which may enable it to cross-link two proteins with C-terminal PDZ recognition sequences. In addition, the interactions of TIP-2/GIPC with molecules such as myosin VI (33) and TrkA/B (22) are not PDZ interactions. Thus, a single TIP-2/GIPC molecule can simultaneously interact with a PDZ recognition sequence of one protein and an unrelated recognition sequence of another. The sequence outside the PDZ domain shows little homology to other proteins, and except for the weak match with the acyl carrier protein domain there, are no signs of the existence of other characterized protein domains. At the moment, the function of these unique N- and C-terminal peptide sequences is poorly understood.

Our identification of PDZ recognition sequences in integrin cytoplasmic tails has important implications for the mechanisms by which integrins interact with intracellular proteins and mediate function. Indeed, the interaction of α6A and α6B with TIP-2/GIPC may be significant for the functions of the α6 integrins based on the affinity of this PDZ interaction, the adhesion-dependent co-localization of these proteins in motile cells and the known functions of TIP-2/GIPC. Interestingly, TIP-2/GIPC has been shown to mediate the interaction of the glucose transporter 1 with the cytoskeleton (33) and cross-talk between receptor tyrosine kinases and G protein-coupled receptors (22). Most likely, this protein functions as a scaffold protein for the assembly of protein complexes. It is interesting to note that the TIP-2/GIPC interaction is specific for the α6 integrins because these integrins have been implicated in distinct functions including cell migration (9, 10, 23). Based on our finding that the co-localization of α6 and TIP-2/GIPC was most evident in retraction fibers, the speculation could be made that one function of TIP-2/GIPC is to detach these integrins from the matrix. Nonetheless, the presence of PDZ recognition sequence in both splice variants of α6 integrin is striking and suggestive of the need for a common structural motif in the assembly of α6 integrins in adhesion and signaling complexes. Clearly, other PDZ proteins may also interact with these integrins and influence their functions.

Acknowledgments—We thank Dr. Olivier Kocher for technical advice and for sharing reagents and Dr. Marilyn Gist Farquhar for the TIP-2/GIPC antiserum.

REFERENCES
1. Plow, E. F., Haas, T. A., Zhang, L., Loftus, J., and Smith, J. W. (2000) J. Biol. Chem. 275, 21785–21788
2. Calderwood, D. A., Shattil, S. J., and Ginsberg, M. H. (2000) J. Biol. Chem. 275, 22607–22610
3. Hughes, P. E., O’Toole, T. E., Ylanne, J., Shattil, S. J., and Ginsberg, M. H. (1995) J. Biol. Chem. 270, 12411–1247
4. Liu, S., Calderwood, D. A., and Ginsberg, M. H. (2000) J. Cell Sci. 113, 3563–3571
5. Ylanne, J., Chen, Y., O’Toole, T. E., Loftus, J. C., Takada, Y., and Ginsberg, M. H. (1993) J. Cell Biol. 122, 223–233
6. Kawaguchi, S., Bergelson, J. M., Finberg, R. W., and Hemler, M. E. (1994) Mol. Cell. Biol. 5, 977–988
7. de Meler, A. A., and Sonnenberg, A. (1999) Bioessays 21, 499–509
8. Chan, B. M., Kassner, P. D., Schiro, J. A., Byers, H. R., Kupper, T. S., and Hemler, M. E. (1992) Cell 68, 1051–1060
9. Shaw, L. M., and Mercurio, A. M. (1994) Mol. Cell. Biol. 5, 679–690
10. Gimond, C., Baudoin, C., van der Neut, R., Kramer, D., Calafat, J., and Sonnenberg, A. (1998) J. Cell Biol. 143, 253–266
11. Wei, J., Shaw, L. M., and Mercurio, A. M. (1998) J. Biol. Chem. 273, 5903–5907
12. Ivaska, J., Reunanen, H., Westermarck, J., Koivistio, L., Kahari, V. M., and Heino, J. (1999) J. Cell Biol. 147, 401–416
13. Kleckотka, P. A., Santoro, S. A., and Zutter, M. M. (2000) J. Biol. Chem. 276, 9503–9511
14. Liu, S., and Ginsberg, M. H. (2000) J. Biol. Chem. 275, 22736–22742
15. Knezevic, I., Leisner, T. M., and Lam, S. C. (1996) J. Biol. Chem. 271, 16416–16421
16. Ahahari, S. K., Lee, J. W., and Juliano, R. L. (2000) J. Cell Biol. 151, 1141–1154
17. Fanning, A. S., and Anderson, J. M. (1999) J. Clin. Invest. 103, 767–772
18. Hillier, B. J., Christopherson, K. S., Prehoda, K. E., Bredt, D. S., and Lim, W. A. (1999) Science 284, 812–815
19. Doyle, D. A., Lee, A., Lewis, J., Kim, E., Sheng, M., and MacKinnon, R. (1996) Cell 85, 1067–1076
20. Songyang, Z., Fanning, A. S., Fu, C., Xu, J., Marfatia, S. M., Chistihi, A. H., Crompton, A., Chan, A. C., Anderson, J. M., and Cantley, L. C. (1997) Science 275, 73–77
21. Shaw, L. M., Lota, M. M., and Mercurio, A. M. (1993) J. Biol. Chem. 268, 11401–11408
22. Lou, X., Yano, H., Lee, F., Chao, M. V., and Farquhar, M. G. (2001) Mol. Biol. Cell 12, 615–625
23. Rahibinovitz, I., and Mercurio, A. M. (1997) J. Cell Biol. 139, 1873–1884
24. Rousset, R., Fabre, S., Desbois, C., Bantignies, F., and Jalino, P. (1998) Oncogene 16, 643–654
25. De Vriese, L., Lou, X., Zhao, G., Zheng, B., and Farquhar, M. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12340–12345
26. Lee, E. C., Lota, M. M., Steele, G. D., Jr., and Mercurio, A. M. (1992) J. Cell Biol. 117, 671–678
27. Chao, C., Lota, M. M., Clarke, A. C., and Mercurio, A. M. (1996) Cancer Res. 56, 4811–4819
28. Niethammer, M., Valschesonff, J. G., Kapoor, T. M., Allison, D. W., Weinberg, T. M., Craig, A. M., and Sheng, M. (1998) Neuron 20, 693–707
29. Gee, S. H., Quenneville, S., Lombardo, C. R., and Chabot, J. (2000) J. Biol. Chem. 275, 21785–21788
30. Karthikeyan, S., Leung, T., and Ladias, J. A. (2001) J. Mol. Biol. 308, 963–973
31. Karthikeyan, S., Leung, T., Birrane, G., Webster, G., and Ladias, J. A. (2001) J. Mol. Biol. 305, 225–237
32. Bunn, R. C., Jensen, M. A., and Reed, B. C. (1999) Mol. Biol. Cell 10, 819–832
33. Gao, Y., Li, M., Chen, W., and Simons, M. (2000) J. Cell. Physiol. 184, 373–379