Mathew Fig_3

A

WT

YY/AA

YY/FF

B

C

D

E

WT

YY/AA

YY/FF

pAKT

AKT

pERK

ERK

GDNF (min) 0 15 30 0 15 30 0 15 30

0

20

40

60

80

100

Migration (number of cells/hpf)

Adhesion (%)
β1 Integrin NPxY Motifs Regulate Kidney Collecting Duct Development and Maintenance by Induced-Fit Interactions with Cytosolic Proteins

Sijo Mathew1, Zhenwei Lu2, Riya J. Palamuttam1, Glenda Mernaugh1, Arina Hadziselimovic2, Jiang Chen2, Nada Bulus1, Leslie S. Gewin7,1, Markus Voehler7, Alexander Meves8,10, Christoph Ballestrem9, Reinhard Fässler8, Ambra Pozzi1,3,7, Charles R. Sanders2,5,6, Roy Zent1,3,4,5,7

1Division of Nephrology and Hypertension, Department of Medicine, 2Department of Biochemistry, 3Department of Cancer Biology, 4Department of Cell and Developmental Biology, 5Center for Matrix Biology, 6Center for Structural Biology, Vanderbilt University School of Medicine, Nashville, 7Veterans Affairs Hospital, Nashville, Tennessee 37232, United States, 8Department of Molecular Medicine, Max Planck Institute of Biochemistry, 82152, Martinsried, Germany, Department of Biochemistry Department of Medicine, Division of Nephrology, 9Faculty of Life Sciences, University of Manchester, Manchester, M13 9PT, England, 10Current Address Department of Dermatology, Mayo Clinic, Rochester, MN, 55905, United States.

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Address Correspondence to
Roy Zent or Charles R. Sanders
Room C3210 MCN
Vanderbilt Medical Center
Tel: 615-322-4632 or 615-936-3756
E-mail: roy.zent@vanderbilt.edu or chuck.sanders@vanderbilt.edu
Abstract

Loss of β1 integrin expression inhibits renal collecting system development. Two highly conserved NPxY motifs in the distal β1 tail regulate integrin function by associating with PTB proteins such as talin and kindlin. Here we define the role of these two tyrosines in collecting system development and delineate the structural determinants of the distal β1 tail using NMR. Mice carrying alanine mutations have moderate renal collecting system developmental abnormalities relative to β1-null mice. Phenylalanine mutations did not affect renal collecting system development but increased susceptibility to renal injury. NMR spectra in bicelles showed the distal β1 tail is disordered and does not interact with the model membrane surface. Alanine or phenylalanine mutations did not alter β1 structure or interactions between α and β1 subunit transmembrane/cytoplasmic domains; however they did decrease talin and kindlin binding. Thus these studies highlight that the functional roles of the NPxY motifs is organ dependent. Moreover, the β1 cytoplasmic tail, in the context of the adjacent transmembrane domain in bicelles, is significantly different from the more ordered, membrane associated β3 integrin tail. Finally, tyrosine mutations of β1 NPxY motifs induce phenotypes by disrupting their interactions with critical integrin binding proteins like talins and kindlins.
Introduction

The multi-branched kidney collecting system develops from the ureteric bud (UB), which undergoes iterative branching morphogenesis following its interactions with the metanephric mesenchyme (MM). This process depends on growth factor-mediated cell signaling and integrin-dependent cell-extracellular matrix (ECM) interactions. β1, the most abundantly expressed β integrin subunit, is a component of at least 12 integrin heterodimers. These include the laminin-binding integrins, α3β1 and α6β1, and the collagen-binding integrins α1β1 and α2β1, which are the principal integrins expressed by the kidney (24). The importance of β1 integrins in UB development was verified in mice where the β1 integrin gene was selectively deleted in the UB at day E10.5 when it invades the MM. These mice develop a severe UB branching morphogenesis defect with decreased nephron formation, a major proliferation defect, and decreased activation of multiple integrin-activated signaling pathways (43, 46).

The β1 integrin cytoplasmic tail plays key roles in integrin functions, many of which are mediated by two well-defined NPxY motifs found in the distal region of the tail (29). Mutating Y783/Y795 to F783/F795 (YY/FF) in keratinocytes and fibroblasts moderately impaired cell adhesion, spreading, chemotaxis and integrin signaling (11, 32-35), while Y783/Y795 to A783/A795 mutations (YY/AA) in keratinocytes resulted in abnormalities indistinguishable from β1-null keratinocytes (11). Consistent with the in vitro data, mice with the YY/AA mutation constitutively or selectively knocked into keratinocytes resembled β1 null phenotypes (8, 11). Surprisingly, mice with the YY/FF knock-in mutation displayed no developmental abnormalities (8, 11), although they developed fewer tumors than wild type (WT) mice in a skin carcinogenesis model (26). The YY/AA and YY/FF mice phenotypes were shown to be caused by decreased affinity for the integrin tail binding proteins talin and kindlin. Consistent with the difference in severity between the genotypes, the affinity of talin and kindlin is higher for β1 with NPxF
motifs than those with NPxA motifs. Besides skin, no other organ specific studies have been conducted to define the role of the NPxY motifs in development or injury models. In addition it is unclear whether these mutations induce the phenotypes by altering binding affinities of integrin binding proteins or by inducing a major structural change in the β1 integrin tail.

The NPxY motifs of the β1 tail are critical for the binding of cytosolic proteins, including talins, kindlins and integrin cytoplasmic domain–associated protein-1 (ICAP-1) (3, 4, 21, 29), which are required for integrin affinity regulation and outside-in signaling. YY/AA mutations inhibit binding of and abrogate signaling by these proteins (6, 27, 30). Although developmental studies in YY/FF mice suggests that these tyrosine residues (or at least the phenolic –OH) are not critical in the mechanisms regulating integrin function (8, 11), there is evidence suggesting that β integrin tyrosine phosphorylation is a conserved mechanism for turning off talin-induced integrin activation (2). Indeed, it was shown that Y/F783 or Y/A783 mutations in the membrane proximal NPxY motif of the β1 tail results in a dramatic reduction in the binding affinity of both talin1 and talin2 for the two major splice variant forms (β1A and β1D) of the cytosolic domain of this integrin (3). These data suggested an essential role for unphosphorylated Tyr783 in activation of β1 by talin-induced disruption of the interface between the transmembrane (TM) and cytosolic domains (CTD) of β1 with its α integrin subunit partners, while phosphorylation of Y783 favors the inactive state by both reducing the affinity of β1 for talin and enhancing the affinity of the CTD with Dok1. Similarly kindlins have been shown to bind to the membrane distal NPxY motif of integrins (14, 27) and this binding is disrupted by a Y/A793mutation. Phosphorylation of the corresponding membrane-distal NITY759 motif in the β3 CTD disrupts kindlin-2 recognition and the ability of kindlin-2 to co-activate the αIIβ3 integrin (14).

The TM and CTD of the β3 integrin have been subjected to numerous structural studies (1, 12, 13, 16, 18, 19, 22, 23, 25, 40, 41, 44). However, only recently have experimental
advances for β1 (predominantly the β1D isoform) been made, and have focused on analyzing its CTD free in solution or in complex with talin (2, 4). These studies showed that the isolated β1 CTD is structurally disordered in the absence of a binding partner. Upon complex formation with the talin F3 domain, the membrane proximal portion of the CTD (residues 752-773) adopts an α-helical structure that makes several important contacts with talin. Part of the membrane distal portion of the CTD in the complex adopts ordered (but irregular) structure in which the NPIY motif adopts a β turn conformation and Y_783 makes the initial contacts with talin. Interestingly, the local conformation and orientation of the corresponding NPLY_{747} motif of the β3 CTD in complex with talin differs considerably, although the orientation of the Tyr side chain in the talin contact interface is essentially the same. The more membrane-distal NPKY_{795} motif of β1 appears to be in a disordered segment both before and after binding of talin, indicating that its roles in integrin recognition by cytosolic effectors must involve proteins other than talin. This motif is the primary binding site for the kindlins (14, 27, 28, 39). Taken together, these studies of the β1 CTD indicate an induced fit or conformational selection mechanism whereby the disordered domain becomes ordered upon binding with talin. However, it could be that the conformational properties of the CTD are different when they are associated with the intact β1 TM domain within the plasma membrane.

To address this question we conducted structural studies of the intact β1 TM/CTD domains under conditions in which the protein is embedded in bilayered model membranes (i.e., bicelles). Furthermore, we also defined the effect of YY/AA and YY/FF mutations of the β1 integrin NPxY motifs on the collecting system of the kidney and examined whether the double mutant forms exhibit any perturbations in the ability of their TM/CTD domain to heterodimerize with the TM/CTD domain of the α2 integrin. We demonstrate that unlike the constitutive and skin-specific YY/AA β1 integrin knockins, which resembled the β1 loss-of-function phenotype...
(8, 11), the YY/AA mutation resulted in a significantly less severe UB branching phenotype, and that inducing the YY/FF mutations rendered mice highly susceptible to stress injury by unilateral ureteric obstruction. Mechanistically, YY/AA and to a lesser extent YY/FF mutations induced adhesion defects and signaling abnormalities in response to growth factors. These defects and the structural findings are discussed in the paper.

Results

YY/AA mutations of β1 integrin cytoplasmic tails cause moderate developmental defects of the kidney collecting system. To define the role of the two NPxY motifs of the β1 integrin cytoplasmic domain in UB development we studied the phenotypes of mice constitutively expressing Y_{783}Y_{795}/F_{783}F_{795} (YY/FF) mutations or selectively expressing Y_{783}Y_{795}/A_{783}A_{795} (YY/AA) mutations in the UB. The generation of the YY/FF mice was previously described (11) and they survive as homozygous animals. Due to the embryonic lethality of the YY/AA mice, we generated mice with YY/AA mutations only in the UB by intercrossing heterozygous YY/AA mutant mice with floxed β1 integrin mice and transgenic mice expressing Cre recombinase under the hoxB7 promoter (17) resulting in Cre-mediated deletion of the floxed β1 allele in the UB and expression of only the YY/AA mutant allele.

Both the YY/FF and YY/AA mice were born in the normal Mendelian ratio. The YY/FF animals lived a normal lifespan and their kidneys were normal at all ages (Figure 1A-D). By contrast, the YY/AA mice died between 4 and 6 months of age due to renal failure and the kidneys were on average 50% smaller than WT mice at their time of death. The principal lesion seen in the kidneys of the YY/AA mice between 1 month of age death was papilla hypoplasia, which resulted in obstruction and ultimately destruction of the cortex and the medulla (Figure 1A, B, E-H). The number of tubules found within the renal papilla of the
YY/AA mice was moderately decreased compared to WT controls suggesting the mice had a branching morphogenesis defect (Figure 1A vs. 1E). When the collecting ducts in both the medulla and cortex were observed under higher power, severe abnormalities of the tubular architecture, characterized by increased luminal cellularity and tubular obstruction, were noted (Figure 1G and H). There was also evidence of tubulointerstitial fibrosis. Interestingly, by performing Ki67 staining we found continued cellular proliferation in the collecting ducts of YY/AA mutants in mice older than 3 weeks when renal development should be complete, while there was very little proliferation in the WT controls (Figure 1I-J), suggesting the increased luminal cellularity in the YY/AA mice was due to continued collecting duct proliferation.

We previously published (46) and verified in this study (data not shown) that the key feature observed upon deletion of β1 integrin from the developing UB was an early and severe branching morphogenesis defect with the formation of severely hypoplastic kidneys. As this feature was not prominent in the YY/AA kidneys post-development, we performed histological analysis of kidneys from embryos at different time points. Consistent with our observations in the adult mice, there was only a mild to moderate branching defect in the YY/AA mice at E15.5 (Figure 2A-B) and this was confirmed at P1 (Figure 2C-D). The kidneys of the YY/AA mutant were only slightly smaller than controls at both time points. At P10 the kidneys still demonstrated a moderate branching phenotype, however the mutant kidneys were approximately 40% smaller than WT mice (3.5+/− 0.5 cm vs 5+/− 0.2 cm) (Figure 2E-F). Consistent with the smaller size of the YY/AA kidneys there was decreased papillary tubular cell proliferation in mice from E15 to P1, which are the developmental stages characterized by high proliferation; however the difference was less obvious at P10 (Figure 2I). By P10 the intratubular cellularity in the papilla of the YY/AA mice became evident (Figure 2G-H).
Taken together, these data demonstrate that introducing the $\beta$1YY/AA mutation in the UB of the kidney resulted in a moderate branching UB defect and decreased cell proliferation during the highly proliferative stages of renal development, which is followed by abnormal tubular architecture post-development, characterized by intratubular cellularity and tubulointerstitial fibrosis.

$\beta$1 YY/FF and YY/AA mutations differentially regulate collecting duct cell function and signaling. To define the mechanisms whereby the NPxY mutations induced the in vivo phenotypes, we expressed either the human $\beta$1 integrin (WT), YY/AA or YY/FF mutants in $\beta$1-null collecting duct (CD) cells (46). CD cells were sorted for equal levels of expression by flow cytometry (data not shown). Tubulogenesis in 3-dimensional collagen I and Matrigel gels revealed that WT-CD cells were able to make well defined tubules (Figure 3A), however the YY/AA- and YY/FF-CD cells made cysts that were indistinguishable from each other.

Since cell adhesion, migration and proliferation are key components of tubule formation, we tested the effect of the mutations on these cell functions on collagen I. The YY/AA- and YY/FF-CD cells adhered to and migrated on collagen I approximately 25% and 60% as well as WT-CD cells, respectively (Figure 3B-C). Similar results were seen with laminin-511 (data not shown), however all cells adhered equally well to and migrated on the $\beta$3 integrin-dependent ligand vitronectin. The YY/AA- and YY/FF-CD cells proliferated significantly less on collagen I than WT-CD cells (Figure 3D). As with adhesion and migration, similar results were seen with laminin 511 (data not shown), while all cells proliferated equally well on vitronectin (Figure 3D).

Thus, consistent with the severe phenotype in the YY/AA mouse there were major abnormalities in CD cells expressing this mutation. More interestingly, despite the lack of a developmental
phenotype in the YY/FF mice, this mutation resulted in abnormalities in functional assays of CD cells in vitro, although always to a lesser extent than the YY/AA mutations.

The growth factors GDNF and the FGFs play a critical role in UB branching morphogenesis and β1 integrin expression is required for GDNF- and FGF-dependent signaling both in vivo and in vitro (46). We therefore tested whether mutating the β1 NPxY motifs alters the ability of these growth factors to mediate well defined cell signaling pathways in CD cells. When the YY/AA-CD cells were plated on collagen I matrices in the presence of either GDNF or FGF-10, there was a marked attenuation of AKT and ERK MAPK activation (Figure 3E and Supporting Figure 1); however this was not as severe as that seen in β1-null CD cells (46). By contrast the YY/FF mutants transduced these signals to the same extent as the WT cells. No differences in p38MAPK activation between the WT and either mutant in response to these growth factors were seen (Data not shown). Thus, the YY/AA mutant demonstrated markedly decreased GDNF and FGF-mediated AKT and ERK MAPK signaling, while signaling was normal in the YY/FF mutant.

YY/FF mutations of β1 integrin tail render mice more susceptible to renal injury. Although the YY/FF mice developed normally, it is clear that YY/FF-CD cells had abnormal cell adhesion, migration and proliferation. These in vitro data led us to hypothesize that the YY/FF mice are more susceptible to stress-induced injury. Therefore, we tested stress-induced injury by subjecting 6 week old YY/FF and WT mice to unilateral ureter obstruction (UUO), a well-established model of tubular injury. There was markedly increased tubular dilatation and flattening of tubular epithelial cells in the YY/FF mice compared to WT controls at 5 (Figure 4A-B) and 10 (data not shown) days following injury. The increased renal injury was verified by scoring at day 5 (4.7±0.5 versus 1.9±0.4 P<0.01). Although there was no increase in matrix
production in the contralateral kidney of either genotype (Supporting figure 2), the YY/FF mice had more matrix deposition as shown by Trichome Blue staining (Figure 4C-D) and excessive collagen I production as verified by immunoblotting of the obstructed kidneys (Figure 4H,I).

More tubular apoptosis, as shown by TUNEL staining, was present in the injured YY/FF mice (Figure 4E-G). Thus, although YY/FF mice developed normally, they were more susceptible to obstructive renal injury.

β1 integrin is a fundamental regulator of EGF receptor signaling, which is a key controller of renal tubule responses to injury (45). We therefore investigated whether the YY/FF mutations affected EGF-dependent signaling and CD function. When we analyzed the ability of WT- and YY/FF-CD cells to migrate towards EGF on collagen-I coated transwells, there was a significant increase in WT cell migration towards this ligand which was not seen with the YY/FF-CD cells (Figure 4J). When proliferation of cells plated on collagen I in response to EGF was determined, both the WT and YY/FF-CD cells increased proliferation significantly, but the effect of EGF was more profound on WT-CD cells (Figure 4K). Consistent with these abnormalities in migration and proliferation, the YY/FF-CD cells plated on collagen I activated AKT and ERK MAPK less robustly than WT cells following EGF treatment (Figure 4L). Thus, in contrast to GDNF and FGF-10 where YY/FF-CD cells signaled like WT-CD cells with respect to AKT and ERK MAPK pathways, YY/FF-CD cells had reduced signaling following EGF stimulation and YY/FF cells migrated less towards EGF than WT cells plated on a collagen I matrix.

The β1 integrin tyrosine mutations did not alter the membrane interactions of the distal cytoplasmic tail region. In an effort to define a structural basis for the differences in the in vivo and in vitro phenotypes of the YY/AA and YY/FF mutants, we undertook studies using NMR
spectroscopy of the combined β1 transmembrane/cytoplasmic tail domain (TM/CTD) in bicelles (residues 719-798). We initially investigated the interactions between the WT, YY/AA and YY/FF CTD with the adjacent membrane (i.e. bicelles) surface. The proteins investigated correspond to the TM/CTD of integrin β1 (residues 719 to 798) (Figure 5A). Hexa-His-tagged WT, YY/AA and YY/FF mutant proteins were expressed in E. coli as uniformly $^{15}$N-labeled proteins and purified, with final NMR samples being composed of the TM/CTD in bicelles comprised of 20% dimyristoylphosphatidylcholine/diheptanoylphosphatidylcholine (DMPC/D7PC) at 1:3.3 mol-to-mol, pH 6.5 and 298K. Backbone NMR resonance assignment of the protein spectrum was carried out by using 3D experiments with uniformly-labeled $^{13}$C and $^{15}$N protein (Supporting Figure 3 and Supporting Table 1). Resonance assignments revealed that the majority of the observable peaks and all of the ones that could be assigned using the 3-D data were from the membrane-distal cytosolic domain (residues 777-798, Figure 5B-D). The vast majority of the peaks from the TM domain and membrane-proximal CTD (residues 719-776) are too broad to observe at 298K. The visible distal CTD peaks correspond to a segment that further analysis (below) indicates to be disordered. To gain insight into the conformations of the spectroscopically invisible TM and membrane proximal CTD a far-UV circular dichroism spectrum of the protein was collected under the same sample conditions as used for NMR. The spectrum (Supporting Figure 4) exhibited a typical pattern for a highly helical protein. The analysis of this spectrum indicated 85% α-helical content, suggesting that virtually all of the TM/CTD is helical and that the TMD and the membrane proximal CTD very likely form a single extended helix. The absence of NMR resonances for most of this helix at 298K is likely due to the high molecular weight and slow tumbling of the bicelle/protein complex. This hypothesis is supported by observation of TMD peaks at 313K, where tumbling is more rapid (data not shown).
When TROSY-HSQC spectra were obtained from the WT, YY/AA and YY/FF mutant proteins (Figure 5B-D) and overlaid (Supporting Figure 5) we observed that both mutants exhibited similar spectra as WT, with the only major changes being for peaks from residues at or near the mutation sites (Figure 5 and Supporting Figure 5). This suggests that the three peptides maintain similar structures, despite the mutations. To determine whether the mutations change the association of the CTD of the protein with the bicelle surface, these $^{15}$N-labeled proteins were titrated with a water soluble paramagnetic compound Gd(III)DTPA. The addition of this compound led to a $>50\%$ decrease in intensity for all peaks (Figure 6A), consistent with the above notion that all three peptides were largely unstructured and water-exposed. The residue-by-residue intensity reductions were very similar for the WT protein and both mutants, pointing to very similar structures and a lack of avid interactions with the membrane surface (such that residue-specific Gd(III)DTPA accessibility patterns are similar). Titration of the WT and mutant proteins with the hydrophobic 16-DSA failed to induce significant reductions in peak intensities and thus corroborated the absence of a significant interaction of the cytosolic domain of the integrin $\beta$1-TM/CTD protein with the bicellar surface. Together these results indicate that most residues in the membrane-distal CTD are located in the aqueous phase with no observable surface association of this domain either in the WT or mutant protein. Furthermore, the paramagnetic probe experiments failed to provide strong evidence for more than transient higher order structure, although the modest variations in residue-to-residue accessibility (Figure 5A) are supportive of some preferences in the ensemble of interconverting conformations. These preferences, however, were very similar for the WT and mutant proteins.

**Substitutions of the $\beta$1 tail tyrosines maintain the structure of the cytosolic domain.** The measured backbone chemical shifts for the CTD of bicelle-associated $\beta$1-TM/CTD (Supporting
Table 1) can be interpreted in terms of protein secondary structure using Chemical Shift Index (CSI) analysis and in terms of backbone torsion angles using Talos+. Deviations of observed backbone shifts from the corresponding amino acid-specific random coil values are summarized in Figure 6B-D. Comparison of the data patterns of the WT with the two mutant proteins revealed high similarity, indicating the WT structure remained unperturbed, or at most little perturbed by the YY/AA or YY/FF mutations, respectively. Moreover, both CSI and Talos+ analysis were consistent with the distal CTD of the β1-TM/CT being primarily random coil with a single extended strand that spans residues T788 to N792. Neither of the tyrosine residues appeared to be part of a segment containing regular secondary structure or a beta turn. This was further confirmed by measuring backbone amide-amide $^1$H-$^1$H NOEs (data not shown), which did not show the expected patterns for helices or for well-defined turns associated with the tyrosines.

The β1 tail tyrosine substitutions maintain the avidity with the α2 TM/CTDs. Correct integrin function requires association of the α and β subunits in the resting state, which involves adhesive contacts between the TM and membrane proximal CTD. To determine whether the tyrosine substitutions in the β1 tail alter the heterodimerization propensity of the β1 TM/CTD with the corresponding α2 TM/CTD, we carried out titrations of $^{15}$N-labeled WT, YY/AA, and YY/FF proteins with unlabeled α2 TM/CTD in bicelles (Figure 7) and monitored formation of the heterodimeric complex formation by NMR spectroscopy. The TROSY NMR spectra for the WT titration are shown in Figure 7A. At least two peaks (labeled 1 and 2) shifted significantly in response to the titration of the α2 subunit. When the shifts of these two peaks were plotted vs. the α2-to-β1 mol:mol ratio, the data for both peaks fitted well with a 1:1 binding model in which the dissociation constant of heterodimers was in the range of 0.5 mM (Figs. 7B-D). Titration of the
YY/AA and YY/FF peptides produced similar titration spectra (Supporting Figure 5). The chemical shift changes for the same two peaks observed to shift for the WT case exhibited very similar magnitudes of shifts and dependency on the α2-to-β1 mol:mol ratio (Figs 7C-D). This result indicates that the tyrosine substitutions do not significantly affect the avidity between the α2 and β1 TM/CTDs.

**β1 YY/FF and YY/AA mutations alter binding of cytosolic proteins and integrin affinity.**

The structural data shows that the YY/AA and the YY/FF mutations do not affect the β1 secondary structure, its interactions with the membrane or heterodimerization with the α2-TM/CTD. We therefore investigated whether the mutants exhibited differential binding to cytosolic proteins and whether they altered integrin activation. To assess the impact of YY/FF and YY/AA mutations on talin and kindlin binding, which are known to bind to the membrane proximal and membrane distal NPxY motifs respectively (29), we synthesized peptides corresponding to amino acids 758-798 of wild type, YY/FF, and YYAA forms of the β1 integrin. Binding of talin and kindlin to these peptides was assessed by a SILAC-based quantitative proteomics approach (26) and SILAC ratios were used to calculate the binding strength as a percentage of WT (Figure 8A). While YY/FF mutated integrin tails showed only a moderate reduction in binding to talins and kindlins, binding was almost completely inhibited by the less conservative YY/AA mutations. We next defined the consequences of these mutations on β1 integrin activation in CD cells by defining the affinity of β1 integrin for 12G10 monoclonal antibody (Gift from Dr. Martin Humphries), which only binds to the active integrin. This is done using flow cytometry and is expressed as an activation index, (percentage of 12G10 monoclonal antibody binding relative to total β1 integrin expression). CD cells expressing WT β1 exhibited an activation index of 80%, while the YY/FF and YY/AA mutants had activation indices of
approximately 20% and 5%, respectively (Figure 8B). Thus, both pairs of mutations severely attenuated integrin activation; however this effect was markedly more severe for the YY/AA mutant. In addition, the activation index directly correlated with the binding of kindlins and talins to the β1 cytoplasmic tail, consistent with the notion that NPxY motifs regulate integrin function by altering the CTD binding affinities for critical integrin binding proteins.

Discussion

UB development requires both regulated growth factor signaling and cell-ECM interactions and loss of β1 integrin expression in the UB causes hypoplastic and dysplastic kidneys due to abnormalities in both integrin adhesive functions and growth factor-dependent signaling (46). In this manuscript we defined the role of the two tyrosines in the canonical integrin β1 cytoplasmic tail NPxY motifs on UB development. YY/AA mutations cause a moderate branching and proliferation phenotype during development; followed by postnatal dysregulated epithelial cell proliferation that results in intratubular obstruction and renal fibrosis. By contrast, although YY/FF kidneys developed normally they were more susceptible than WT mice to UUO-mediated injury. These in vivo data suggest that divergent signals transduced by the β1 integrin tail NPxY motifs differentially regulate UB development and response of the collecting system of the kidney to injury.

We further demonstrated that both YY/AA and YY/FF CD cells had adhesion, migration and proliferation abnormalities, indicating a crucial role for either Tyr phosphorylation and/or for the phenolic –OH moiety in supporting these integrin-dependent functions. A similar conclusion could be reached for EGF-responsiveness as YY/FF CD cells were unable to respond to EGF, one of the mediators of renal repair following injury (45). By contrast, only YY/AA CD cells had signaling deficiencies in response to growth factors important for UB development,
suggesting that both Tyr phosphorylation and the phenolic –OH groups are dispensable for the integrin/growth factor cross-talk involved in normal UB development. Utilizing solution NMR we confirmed that the distal β1 integrin tail is mainly unstructured and showed that the YY/AA and YY/FF mutations did not alter α2β1 heterodimerization, membrane association or the secondary structure of the β1 integrin tail. Together these data suggest that NPxY motif-dependent actions are not determined by their intrinsic structural conformation but rather by their interactions with critical integrin binding proteins. Furthermore, specific (and often divergent) functions are likely regulated in a cell type-specific manner by different combinations of NPxY-binding proteins, which might be impacted by the phosphorylation status of each NPxY motif.

The YY/FF kidneys were normal, and the YY/AA kidneys were very different than the β1-null kidneys (46). Normal development in the YY/FF kidneys was the same as that seen in the constitutive (8) and skin-specific knockins (11), and indicates that neither tyrosine phosphorylation nor the phenolic –OH of Tyr is required for the β1 integrin to support development. The β1-null mice died significantly earlier than the YY/AA mice (12 weeks vs. 24 weeks) due to renal failure caused by a severe branching abnormality (46), while the YY/AA mice had a moderate branching morphogenesis phenotype and died from obstruction and/or severe renal fibrosis. This major discrepancy in phenotype between β1-null and YY/AA mice was unexpected because constitutive (8) or skin-specific (11) expression of the YY/AA mutation resulted in phenotypes indistinguishable from β1-null mice. An interesting feature in the YY/AA mice not present in the β1-null mice was dysregulated proliferation after development resulting in cell accumulation within the tubules causing destructive obstruction of the collecting system. This characteristic was similar to that of ILK-null mice where it was ascribed to a defect in P38 MAPK-dependent contact inhibition of CD cells (37); however this was not the case in the YY/AA mutants as they undergo contact inhibition and have no defect in P38 MAPK signaling.
Another possible explanation for the increased luminal cell number in the lumens of the adult YY/AA mice is that defective cell adhesion leads to a failure of newly divided cells to reconnect to extracellular matrix. This potential mechanism has not been experimentally tested.

We utilized CD cells to better define the mechanisms underlying the YY/AA kidney phenotype. Like β1-null CD cells, YY/AA cells had a serious adhesion and migration defect; however the proliferation defect was less severe (46). Interestingly, although there was decreased activation of GDNF- and FGF-10-dependent signaling pathways in the YY/AA CD cells compared to WT, this was not as marked as in β1-null CD cells. As the phenotype of the β1-null kidneys was previously attributed to both adhesion- and growth factor-dependent signaling abnormalities (46), the increased growth factor-dependent signaling could explain the less severe developmental phenotype in the YY/AA relative to the β1-null kidneys. In this regard the ERK pathway regulates UB branching morphogenesis (31).

Although the YY/FF mice developed normally and the YY/FF CD cells signaled like WT CD cells in response to GDNF and FGF-10, YY/FF CD cells exhibit abnormalities in tubulogenesis, adhesion, migration and proliferation. In addition they have a decreased ability to migrate towards EGF and an attenuated signaling response to EGF. Furthermore YY/FF mice developed significantly worse stress induced injury than WT controls. Thus YY/FF mutations inhibited some integrin β1-dependent functions but not others. These functions, which are dependent on normal interactions with crucial binding partners, are evidently dependent on Tyr phosphorylation and/or the side chain phenolic –OH group. The conservative Y/F mutations, which are sufficient to decrease but not totally abolish the binding of integrin binding proteins (Figure 8) to β1 tails, result in alterations in inside-out and outside integrin signaling that only cause abnormalities when the mice are placed in stress situations such as the UUO model.
Consistent with our data, YY/FF mice developed fewer tumors than WT in a skin cancer model (26). Although our structural studies do not offer direct insight into whether Tyr phosphorylation plays a role in supporting native functions, it is known that Y₇₈₃phosphorylation reduces the affinity of β1 for talin and enhances affinity for Dok1 (2). In addition, Y/A₇₉₅ mutations of β₁ integrin abrogate kindlin binding (14, 27) and it is known that phosphorylation of the corresponding membrane-distal NITY(759) motif in the β3 CTD disrupts kindlin-2 recognition and the ability of kindlin-2 to coactivate the αIIβ3 integrin (14). Although specific mechanisms whereby the YY/AA and YY/FF mutations differentially altered signaling pathways in UB development and after injury were not defined, our structural and binding studies suggest it is due to the altered ability of the mutant tails to bind critical cytoplasmic integrin binding proteins which are spatially and temporally expressed in a cell type specific manner.

Our NMR spectra of the β₁ TM/CTD are the first for any intact integrin TM/CTD in a bicellar model membrane environment. Bicelles represent a more native-like membrane environment than conventional detergent micelles or organic solvent mixtures and have previously been used in structural studies of integrin TM domains (16, 19, 20). Previous studies of the β1 and other integrin CTDs did not investigate the possibility that interactions of the CTD with the model membrane surface could perturb the structure. We found that the distal CTD of the β1 TM/CTD domain was disordered even when inserted into membranes, confirming previous reports that the membrane distal CTD domain of β1 is disordered until it binds talin, at which point its structure becomes ordered (although irregular in terms of secondary structure) (3). Moreover, we showed that this domain does not interact with the bicelle surface. On the other hand, while the membrane proximal domain of the CTD in the absence of an intact TM domain was shown to be mostly disordered (3), we observed that in the context of an intact TM domain in a bicelle environment this domain is helical. Similarly, interactions between the α2
and β1 TM/CTDs were not altered by YY/FF or YY/AA mutations of the β1 tail. Our results contrast with NMR studies of the full length β3 integrin CTD in micelles (25), where a disulfide-stabilized complex consisting of portions of the TM domains and the full CTDs of αIIbβ3 was studied. In that work the β3 CTD was shown to consist of a stable membrane proximal helix contiguous with the TM helix and two distal amphiphilic helices. The two distal helices of β3 were shown to associate with lipid bilayers but undergo fluctuations that would allow rapid binding of cytoplasmic proteins regulating integrin activation. Two other NMR studies performed in aqueous (38) or detergent micelle conditions (44) verified that the membrane proximal NPxY motif of the β3 CT domain is structured. In addition, phosphorylation of the NxxY motifs was shown to affect the structure and association of the β3 CTD with the membrane and suggested that the bi-phosphorylation of the NxxY motifs of the β3 CTD favors disruption of the inter-subunit clasp that keeps αIIbβ3 integrin in a low affinity state (12). By contrast, the isolated β2 cytoplasmic tail in aqueous or micelles was previously shown to also be largely unstructured except for the N-terminal residues Leu⁶-Arg¹⁴ (5, 9). Thus it is clear that major differences in the structures of the diverse β integrin tails exist.

We also demonstrated that YY/FF and YY/AA mutations result in decreased talin and kindlin binding to the β1 tails and this correlates with decreased integrin activation, despite the lack of alteration in integrin structure. In the case of Tyr⁷⁸³, this mutational affect appears to be unrelated to Tyr phosphorylation, since Tyr⁷⁸³ phosphorylation was previously shown to reduce binding to talin and direct interactions of the Y⁷⁸³ side chain with talin being were shown to be critical for avidity of this complex (2). Similarly, phosphorylation of the membrane-distal NITY⁷⁵⁹ motif in the β3 CTD disrupts kindlin-2 recognition, binding to the integrin and its consequent activation (14).
In conclusion we show that mutations in the integrin β1 tail NPxY motifs in the kidney give phenotypes different from those seen in constitutive and skin-specific knockins, demonstrating that these highly conserved motifs differentially regulate integrin-dependent processes in a cell specific manner. Our data also confirm that the structure and membrane association of the distal integrin tails that contain these highly conserved domains are different in β1 and β3 integrins. Thus our studies highlight the importance of defining the binding partners and phosphorylation patterns for the highly conserved NPxY motifs of integrin β subunits in different organs because they likely regulate markedly different integrin-dependent functions. In addition, they highlight the importance of studying the structure of the integrin CTDs in complex with their effector proteins, since induced fit and/or conformational selection is clearly a major factor in the binding of some CTD/effector protein complexes.

Materials and Methods

**Generation of YY/AA and YY/FF mice.** All experiments were approved by the Vanderbilt University Institutional Animal Use and Care Committee. The YY/FF mice, which were generated as previously described (11), were a F7-F10 generation toward the C56/Black6 background. Aged-matched WT and homozygote littermates were obtained from heterozygote by heterozygote crosses. The YY/AA mice were generated as previously described (11). All mice were a F7-F10 generation toward the C56/Black6 background. Because YY/AA mutants were embryonic lethal, they were intercrossed with mice carrying a floxed β1 integrin gene and mice carrying the hox B7 promoter-driven Cre recombinase transgene (17). Littermates carrying the floxed and the YY/AA mutated β1 integrin allele without the Cre recombinase transgene served as controls for experiments performed with the YY/AA mutant.
Generation of integrin β1 cell lines. β1-null CD cells described previously (46) were transfected with either full length human integrin β1 or β1 integrins carrying YY/AA and YY/FF mutations. To insure equal surface expression of the WT or mutant β1 integrin subunits, they were selected by FACS sorting using antibody AIIB2, a mononclonal antibody directed against the extracellular domain of human integrin β1 (primary) and an anti-rat PE (secondary).

Cell adhesion. Cell adhesion assays were performed in 96 well plates as previously described (7). 1X10^5 cells were seeded in serum free media onto plates containing different concentrations of ECM for 60 minutes. Adherent cells were fixed, stained with crystal violet, solubilized and the optical density of the cell lysates were read at 570nm.

Cell migration. Cell migration was assayed as previously described (7). Transwells with 8uM pores were coated with different ECM components and 1x10^5 cells were added to the upper well in serum free media. Cells that migrated through the filter after 4 hours were counted.

Cell proliferation. Cell proliferation was determined on 96 well plates coated with different ECM proteins as previously described (46). 5x10^3 cells were seeded in each well and maintained in 10% serum for 4 hours. Cells were then incubated in 1% serum media for 12 hours, pulsed with 1uCi/well thymidine (3H) for 12hours and solubilized. Radioactivity was measured using a scintillation counter.

Tubule formation. CD cells were grown in collagen/matrix gels as previously described (7). 5x10^3 CD cells were seeded into the gels, which were overlaid with 100uL of medium and
allowed to grow for 5-7 days. Gels were stained with rhodamine phalloidin and the tubules were photographed using a Zeiss Axio 510 confocal microscope (400X).

**Growth factor-dependent cell signaling** was analyzed as previously described (46). The CD cells were seeded to collagen coated plates (10µg/mL) in serum free media and allowed to attach for 1 hour. Cells were exposed to GDNF, FGF-10 or EGF for different time periods and signaling examined by immunoblotting.

**Unilateral Ureteric Obstruction.** The right ureters of 6-week-old WT and YY/FF mice were ligated and mice were sacrificed 5 and 10 days after the surgery as described (46). Tubular injury was scored by calculating the percentage of tubules with cell necrosis, loss of brush border, cast formation or tubular dilatation as follows. Degree of injury was scored as 0 (none), 1 (1-10%), 2 (11-25%), 3 (26-45%), 4 (46-75%) and 5 (76-100%). Degree of interstitial fibrosis and inflammatory infiltrate were judged using the same scale. At least 10 fields were analyzed for each slide.

**Statistics.** Student's *t*-test was used for comparisons between two groups, and analysis of variance using Sigma Stat software was used for statistical differences between multiple groups. *P*<0.05 was considered statistically significant.

**Expression purification of integrin β1 TM/CTD proteins.** The N-terminally 6xHis-tagged integrin β1-TM/CTD (residues 719 to 798 of the full length protein) was cloned into a pET16b vector, which was then transformed into BL21(DE3) CodonPlus-RP cell. The N-terminally 6xHis-tagged integrin β1-TM/CTD (residues 719 to 798 of the full length protein) was cloned into a pET16b vector, which was then transformed into BL21(DE3) CodonPlus-RP cell. These bacteria were grown in 1L of M9 medium at room temperature, which was rotary shaken until
OD$_{600}$ reached 0.8, when protein expression was induced by 1mM IPTG followed by continued rotary shaking for ~16h at room temperature. $^{15}$N-$\text{NH}_4\text{Cl}$ M9 media was used for $^{15}$N labeling. $^{13}$C-glucose and D$_2$O were used for $^{13}$C and $^2$H labeling. Cells were harvested by centrifugation and the His$_6$-tagged protein was purified as follows. Cells were suspended in lysis buffer (75mM Tris-HCl pH 7.7, 300mM NaCl, 0.2mM EDTA, 0.2 mg/ml PMSF, Lysozyme/DNAase/RNAase at 0.2/0.02/0.02 mg/mL, and 5 mM magnesium acetate) and tumbled at room temperature for ~30 minutes. The cells were probe-sonicated for 5 minutes with cycles of 5 sec on and 5 sec off.

Empigen (30% solution) was added to the cell lysate (1mL per 10mL lysate) to a final concentration of 3% (v/v) and mixed at 4°C for 30min. Solubilized cell lysate was centrifuged at 20,000xg for 20 minutes. The supernatant was mixed with Ni(II)-NTA resin for 45 min at 4°C. The resin was then collected by centrifugation at 3400xg for 10min. The Ni resin was washed with 5 X 1 column volumes of ice cold Emp/A (3%Empigen in 40mM HEPES, 300mM NaCl, pH7.5) and further washed with wash buffer (1.5% Empigen, 40mM imidazole, 40mM HEPES, 300mM NaCl pH7.8) until all non-His tagged proteins were eluted. The resin was re-equilibrated using 8 X 1 column volumes of pre-equilibration buffer (20 mM imidazole plus 100 mM NaCl, pH 6.5, plus 1% D7PC and 0.5 mM DTT) and further with 2 X 1 column volumes of column equilibration buffer (20 mM imidazole, 100 mM NaCl, pH 6.5, 2% bicelles 0.5 mM DTT, 10% D2O). The bicelles were composed of 3.3:1 mol:mol D7PC:DMPC, q = 0.3, where q is the lipid-to-detergent mole ratio). The proteins were then eluted using elution buffer (250 mM imidazole, plus 2% bicelles, 0.5 mM DTT, and 10% D2O, pH 7.4). The pH of the eluted protein solution was adjusted to 6.5 after addition of 1mM EDTA and concentrated 10 times using centrifugal ultrafiltration using 10 kDa molecular weight cut-off filters. The sample was then used for NMR spectroscopy.
Backbone NMR resonance assignments. The NMR sample used for backbone assignment contained 0.7mM uniformly $^2$H,${}^{13}$C,${}^{15}$N-labeled $\beta_1$ TM/CTD in bicelle solution, which was comprised of 20% (w/v) DMPC/D7PC (1:3.3 mol:mol), 250mM imidazole, pH 4.0 and 10% D$_2$O. A set of TROSY-based triple resonance NMR experiments with deuterium decoupling, including HNCA, HNCACB, HN(CO)CA, HN(CO)CACB and HNCO, were carried out on a Bruker 800MHz spectrometer equipped with cryoprobe at 25°C. NMR data were processed using NMRpipe and analyzed by NMRview. Peaks were initially assigned at pH 4.0 and then used to assign the pH 6.5 amide $^1$H-$^15$N TROSY peaks by collecting a series of spectra between pH 4.0 and pH 6.5, allowing correlation of the assigned pH 4.0 peaks with their pH 6.5 counterparts.

Protein secondary structure and predicted backbone torsion angles were estimated from the backbone chemical shift data using chemical shift index (CSI) analysis (42) and the Talos+ program (36).

Probing membrane topology using paramagnet-induced relaxation enhancement. 1.0 mM U-$^{15}$N-$\beta_1$-TM/CTD in bicelles was titrated with a lipophilic paramagnetic probe, 16-doxyl stearic acid (16-DSA), or with a hydrophilic paramagnetic probe, Gd(III)DTPA. The impact of the paramagnet on the NMR spectrum of $\beta_1$-TM/CTD was monitored using the $^{15}$N TROSY-HSQC experiment, which was carried out on a 600MHz Bruker spectrometer equipped with a cryoprobe. Gd(III)DTPA was titrated over 0-10mM from a stock concentration of 250mM Gd(III)DTPA, 250mM imidazole, 250mM EDTA pH 6.5. 16-DSA was titrated over a concentration range of 0-0.8mM. During the course of the titration the samples were diluted by a factor of 4%. Peak intensities were measured as an indicator of line broadening by proximal paramagnetic probes.
**SILAC-based peptide pull downs.** Pull downs were performed as described previously (10). Briefly, desthiobiotinylated peptides corresponding to amino acids 758-798 of the β1 integrin were synthesized and immobilized on Dynabeads MyOne Streptavidine C1 (10 mg per mL, Invitrogen). Cell lysates were generated from immortalized WT mouse keratinocytes cultured in the presence of natural (light) or heavy labeled arginine (L-$^{13}$C$_6$$^{15}$N$_4$-arginine) and lysine (L-$^{13}$C$_6$$^{15}$N$_2$-lysine). Lysates were sonicated briefly and cleared by centrifugation. 1 to 2 mg of light and heavy-labeled supernatant was incubated with either control or experimental peptide overnight at 4°C. After washing with lysis buffer beads of corresponding peptide pairs were combined. For the cross-over experiment the labeling was reversed. Protein was eluted by incubating the beads in 16 mM biotin (Sigma) subsequently precipitated by acetone. Protein was separated by SDS-PAGE and bands were analyzed by LC-MS. LC-MS and data analysis was performed as previously described (26).

**Integrin activation assay.** β1 integrin activation was determined using a state-specific antibody that specifically binds to the active conformation of integrin β1 (15). The amount of bound antibody was determined by flow cytometry. The total surface expression of the β1 integrin was determined using the AIIB2 antibody. The activation index of the β1 integrin was calculated as the percentage of cells that bind to the 12G10 antibody relative to the total surface expression of β1 integrin.

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References


Figure Legends

Figure 1. YY/AA, but not YY/FF mice develop end-stage renal failure. (A, B) Microscopy of PAS-stained kidney slides showing the medulla (A) and cortex (B) of 6-week-old WT (β1^floc/flox) kidneys (40X). (C, D) Microscopy of PAS-stained kidney slides showing the medulla (C) (100X) and cortex (D) (100X) of 6-week-old YY/FF kidneys. (E-H) There is a mild to moderate branching defect in some YY/AA kidneys (E) (40X) and destruction of the medulla and corticomedullary junction in others (F) (40X). The collecting ducts in the papilla (G) as well as in the medulla and cortex (200X) (H) of the YY/AA mice are dilated, disorganized and fibrosed (200X). (I-J) Ki67 staining of 3 week old WT papillae does not show any cell proliferation while there is proliferation in YY/AA mice papillae (400X) (J). The proliferation was noted to be within some tubules (depicted by the arrow) as shown in the highly magnified inset.

Figure 2. YY/AA kidneys have a moderate branching morphogenesis defect and intraluminal hypercellularity. (A-H) Kidneys were isolated from embryos of WT and YY/AA mice at E15 (A,B), P1 (C,D) and P10 (E,F). A moderate UB branching phenotype with decreased size was observed in YY/AA mice (100X). Intraluminal cellularity was noted in the YY/AA but not the WT mice at P10 (400X) (H). (I) Ki67 staining was performed on at least three WT (WT) and YY/AA kidneys. The number of Ki67-positive cells in the UB (E15.5) or collecting ducts (P1, P10) of the mice was quantified and expressed as the mean ± s.d. of five high power fields of five different mice. * indicates statistically significant differences (P<0.05) between WT and YY/AA mutant mice.
Figure 3. NPxY mutations in the β1 integrin tail results in severe defects of cell function. (A) WT and NPxY mutant CD cells placed in 3D collagen and Matrigel gels were allowed to undergo tubulogenesis over 7 days in the presence of 5% FBS. They were stained with Rhodamine Phalloidin and visualized by confocal microscopy. (B) CD cell populations were allowed to adhere to collagen I or vitronectin and cell adhesion was evaluated 1 hour after plating. Values are the mean ± s.d. of three experiments performed in triplicate. * denotes a statistically significant difference (P<0.05) between cells expressing WT and mutant integrins. (C) CD cells were plated on transwells coated with collagen I (10 μg/ml) or vitronectin and migration was evaluated after 4 hours. Values are the mean ± s.d. of three experiments performed in triplicates. * denotes a statistically significant difference (P<0.05) between cells expressing WT and mutant integrins. (D) The CD cell populations were plated on collagen I or vitronectin. After 24 hours, cells were treated with 3H-thymidine and incubated for a further 24 hours. 3H-thymidine incorporation was then determined as described in the Materials and Methods. Values are the mean ± s.d. of three experiments performed in triplicates. * indicates a statistically significant difference (P<0.05) between cells expressing WT and mutant integrins. (E) WT and mutant CD cells were allowed to adhere to collagen I for 1 hour, after which they were treated with GDNF for various times. The cells were then lysed and 20 μg of total cell lysates were analyzed by Western blot for levels of pAKT and pERK. Immunoblots of total AKT and ERK are shown to verify equal protein loading. A representative blot from 5 independent experiments is shown. The increases in intensity of phosphorylated proteins were quantified using image J software and expressed as fold increase relative to untreated cells.

Figure 4. YY/FF mice develop severe injury following unilateral ureteric obstruction. (A, B) Kidneys of 6-week-old YY/FF mice show more severe tubular dilatation and injury 5 days
after unilateral ureteric obstruction when compared with the WT mice (200X). (C, D) More intense and abundant Trichrome Blue staining was evident in 5 day injured YY/FF than WT mice. (E-G) Increased TUNEL staining was evident in 5 day injured YY/FF compared to WT mice. Apoptosis was quantified and expressed as the mean of apoptotic cells/microscopic field ± s.d. (10 fields of 10 kidneys from either genotype were analyzed). Differences between YY/FF and WT mice (*) were significant, with $P<0.05$. (H, I) Immunoblots with an antibody directed against collagen I was performed on individual medullas of 5 day injured YY/FF and WT mice (H). Intensities of collagen bands were quantified using Image-J software. Collagen expression in YY/FF mice was expressed as fold changes relative to that seen in WT controls (5 animals per group). # indicate the differences between YY/FF and WT mice were significant, with $P<0.001$ (I). (J) YY/FF and WT CD cells were plated on transwells coated with collagen I (10 μg/ml) and migration was evaluated after 4 hours. Values are the mean ± s.d. of three experiments performed in triplicates. * denotes a statistically significant difference ($P<0.01$) between WT and YY/FF mutants. # indicate a statistically significant difference ($P<0.01$) between EGF treated and untreated cells. (K) YY/FF and WT CD cells were plated on collagen I. After 24 hours cells were treated with EGF and 3H-thymidine and incubated for a further 24 hours. 3H-Thymidine incorporation was then determined as described in the Materials and Methods. Values are the mean ± s.d. of three experiments performed in triplicate. * denotes a statistically significant difference ($P<0.01$) between WT and YY/FF mutant proteins. # indicates a statistically significant difference ($P<0.01$) between EGF-treated and untreated cells. (L) WT and mutant CD cells were allowed to adhere to collagen I for 1 hour, after which they were treated with EGF at various times. The cells were then lysed analyzed by Western blot for levels of pAKT and pERK. Immunoblots of total AKT and ERK are shown to verify equal protein loading. A representative blot from 3 independent experiments is shown. The intensities of phosphorylated
proteins were quantified using image J software and expressed as fold increase relative to untreated cells.

Figure 5. TM/CTD of integrin \(\beta_1\) recombinant shows differences in TROSY NMR spectra due to mutation to the tyrosine residues. (A) Sequence of the TM/CTD of integrin WT and YY/AA, YY/FF mutant proteins expressed and purified in E.coli. (B-D) \(^1\)H,\(^{15}\)N-TROSY spectra of the integrin U-\(^{15}\)N-TM/CTD integrin were collected using a 600 MHz Bruker NMR Avance-III spectrometer at 298K, pH 6.5 in DMPC/D7PC bicelles (q=0.3), 20% total amphiphile and protein concentration of 0.6 to 0.8mM. (B) WT, (C) YY/AA, and (D) YY/FF. The spectra in B-D exhibit only modest differences.

Figure 6. YY/AA and YY/FF mutations did not alter membrane interactions or the secondary structure of the integrin \(\beta_1\) distal cytoplasmic domain. (A) Membrane interactions of the \(\beta_1\) integrin CTD were examined using \(^1\)H,\(^{15}\)N-TROSY NMR titration where \(^{15}\)N-labeled WT and mutant recombinant proteins (1.0mM) in bicelles were titrated with a water soluble paramagnetic probe, Gd(III) DTPA (5mM). Paramagnetic-induced decreases in peak intensity relative to untreated samples were measured. The uncertainty associated with the intensities of values was 5%. (B-D) The secondary structure of \(\beta_1\) tail region was assessed from the observed backbone chemical shifts using Chemical Shift Index analysis. (B) WT, (C) YY/FF and (D) YY/AA all showed similar chemical shifts and subsequent predicted secondary structural patterns.
Figure 7. YY/AA and YY/FF mutations of the β1 integrin TM/CTD did not alter the avidity of complex formation with the α2 integrin TM/CTD. (A) Titration of the U-15N-β1 TM/CTD with the unlabeled α2 TM/CTD was carried out in DMPC/D7PC bicelles (q=0.3, pH 6.5, 298K) and monitored using 1H,15N-TROSY NMR. The protein concentrations were 1.0mM for WT and mutants. Changes in the chemical shifts of peaks were interpreted as reporting on complex formation. (B) Two of the WT β1 peaks that were observed to shift in response to α2 titration were selected and the changes in chemical shifts of these two peaks were plotted vs. the α2-to-β1 mol:mol ratio. Corresponding plots are presented for titrations involving the two β1 mutants: (C) YY/AA and (D) YY/FF. It can be qualitatively seen that the mutations result in little change in avidity between the TM/CTD domains of the two integrin subunits.

Figure 8. YY/AA and YY/FF mutations of the β1 CTD alter talin and kindlin binding and activation of integrin β1. (A) The binding affinities of β1 integrin with cytoplasmic proteins talin and kindlin were determined using the SILAC assay. This was performed with WT, YY/AA, YY/FF and scrambled (scr) β1 integrin peptides. (B) The activation of the β1 integrin was determined by quantifying the amount of antibody 12G10 that binds to the active conformation of human β1 integrin using a FACS assay. The percentage of binding was in each case normalized with the total surface expression of integrin and expressed as an activation index. Total surface expression was quantified by using the AIIB2 antibody in the FACS assay.