In vivo β1 integrin function requires phosphorylation-independent regulation by cytoplasmic tyrosines

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Integrins are heterodimeric adhesion receptors associated with bidirectional signaling. In vitro studies support a role for the binding of evolutionarily conserved tyrosine motifs (NPxY) in the β integrin cytoplasmic tail to phosphotyrosine-binding (PTB) domain-containing proteins, an interaction proposed to be dynamically regulated by tyrosine phosphorylation. Here we show that replacement of both β1 integrin cytoplasmic tyrosines with alanines, resulting in the loss of all PTB domain interaction, causes complete loss of β1 integrin function in vivo. In contrast, replacement of β1 integrin cytoplasmic tyrosines with phenylalanines, a mutation that prevents tyrosine phosphorylation, conserves in vivo integrin function. These results have important implications for the molecular mechanism and regulation of integrin function.

Supplemental material is available at http://www.genesdev.org.

Received January 9, 2006; revised version accepted February 17, 2006.

Integrin receptors bridge extracellular matrix proteins and intracellular cytoskeletal and signaling proteins and are essential regulators of cellular behavior and function (for review, see Hynes 2002). Since the cytoplasmic tails of integrin receptors are relatively short and lack intrinsic enzymatic activity, integrin signals are believed to be mediated by protein–protein interactions. Biochemical studies have identified a large number of intracellular cytoskeletal and signaling proteins that are capable of binding integrin cytoplasmic tails, but a clear picture of how these many potential protein–protein interactions result in bidirectional integrin signals has not yet emerged.

A highly recognizable motif in the integrin cytoplasmic tail is NPxY, two of which are found in the cytoplasmic tails of β integrin subunits. Biochemical and structural studies have demonstrated that NPxY motifs bind phosphotyrosine-binding (PTB) domains and that the specificity of NPxY–PTB domain interactions is conferred by both the NPxY motif and the PTB domain (for review, see Uhlik et al. 2005). Some PTB domains (e.g., the Shc [Src homology 2 domain-containing] transforming protein 1 PTB domain) require phosphotyrosines for high-affinity interaction with NPxY motifs, while others (e.g., the talin PTB domain) bind through hydrophobic interaction with nonphosphorylated tyrosine or phenylalanine residues. The specificity and diversity of NPxY–PTB domain interactions have recently been proposed as a molecular mechanism by which integrin cytoplasmic tails transmit a variety of bidirectional signals (Caldewood et al. 2003). Biochemical studies performed in vitro suggest that distinct β integrin cytoplasmic tails bind distinct PTB domain-containing proteins, and that tyrosine phosphorylation can alter these interactions through addition of a charged phosphate group to the hydrophobic tyrosine aromatic ring (Caldewood et al. 2003; Garcia-Alvarez et al. 2003). Dynamic regulation of NPxY–PTB domain binding by tyrosine phosphorylation is therefore a potentially elegant means of explaining how short integrin cytoplasmic tails transmit diverse bidirectional signals.

Despite the wealth of biochemical and structural data available, the in vivo role of β integrin NPxY motifs is not yet defined. β3-integrins in platelets are critical for hemostasis, and substitution of the two β3 NPxY motifs with NPxF to block phosphorylation-dependent integrin signaling results in a hemostatic defect (Law et al. 1999). Whether this phenotype reflects a broad role for β integrin tyrosine phosphorylation or a more restricted role for β3 integrins in platelets is unknown. Furthermore, a Y-F mutation is conservative and predicted to not disrupt many NPxY–PTB domain interactions. Thus the absolute requirement for β integrin cytoplasmic tyrosines in vivo is untested. In the present study we have used in vivo mutagenesis and conditional in vivo mutagenesis to address the role of NPxY motifs in β1 integrins. Since β1 integrins are the most utilized β integrin subunits in mammals and are highly evolutionarily conserved, mediating adhesion to similar matrix proteins in organisms as diverse as nematodes, flies, and mammals, they provide a means of stringently testing the role of cytoplasmic tyrosines for integrin function in vivo.

Results and Discussion

To address the role of β integrin subunit cytoplasmic tyrosines in vivo mice were generated in which the cytoplasmic tyrosines [Y783 and Y795] encoded by the Itgb1 gene were replaced with either alanines or phenylalanines [Fig. 1A]. The phenylalanine [YF] mutation blocks interactions requiring tyrosine phosphorylation but retains those requiring the hydrophobic aromatic ring of the amino acid, while the alanine [YA] mutation ablates both. Itgb1YA/− and Itgb1YF/− mice were healthy and fertile [data not shown] and were intercrossed to generate β1YA [Itgb1YAYA] and β1YF [Itgb1YFYF] animals. Analysis of the progeny of heterozygous matings revealed expected numbers of Itgb1YA/− mice but failed to reveal live-born β1YA animals [Supplementary Table 1], indicating that β1YA animals die during embryonic life. Timed Itgb1YA/− matings identified resorptions that

[Keywords: Integrin, tyrosine phosphorylation, phospho-tyrosine-binding domain, inside-out signaling, outside-in signaling, conditional knock-in]

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GENES & DEVELOPMENT 20:927–932 © 2006 by Cold Spring Harbor Laboratory Press ISSN 0890-9369/06; www.genesdev.org
could be genotyped as Itgb1\textsuperscript{YA/YA} animals but no live \(\beta1\)YA embryos as early as embryonic day 6.5 [\(E6.5\)] (Fig. 1B,C, Supplementary Table 1). Histologic examination of uteri collected at \(E6.5\) revealed that presumptive \(\beta1\)YA embryos were resorbed with only a residual population of trophoblast cells visible, a phenotype similar to that reported for \(\beta1\) integrin-deficient embryos [Fig. 1C, Fassler and Meyer 1995; Stephens et al. 1995]. Direct histologic comparison with \(E6.5\) embryos from Itgb1\textsuperscript{YA} intercrosses revealed identical findings in presumptive Itgb1\textsuperscript{YA} embryos [Fig. 1C], demonstrating that \(\beta1\) cytoplasmic tyrosines are required for integrin function in early mouse embryonic development.

In contrast to \(\beta1\)YA animals, Itgb1\textsuperscript{YF} intercrosses generated normal numbers of live-born \(\beta1\)YF animals that were healthy and fertile (Supplementary Table 1; data not shown). Consistent with these genetic findings, \(\beta1\) integrin tyrosine phosphorylation could not be detected in phosphotyrosine immunoblots of \(\beta1\) integrin immunoprecipitated from wild-type E12.5 or E16.5 embryos [data not shown]. Thus the requisite role for the \(\beta1\) integrin subunit in the development and function of tissues as varied as the skin, blood, neural crest, and cartilage is independent of \(\beta1\) integrin cytoplasmic tyrosine phosphorylation.

Essential roles for \(\beta1\) integrins in the development and function of specialized tissues that develop after the blastocyst stage of embryogenesis have been demonstrated through analysis of chimeric animals generated by injecting \(\beta1\)-deficient embryonic stem (ES) cells into wild-type blastocysts. These studies utilized lineage tracing to demonstrate that \(\beta1\) integrins are cell autonomously required to form liver and blood cells but not skin or skeletal muscle cells [Fassler and Meyer 1995; Hirsch et al. 1996]. To determine if the requirement for \(\beta1\) cytoplasmic tyrosines in vivo was restricted to early embryogenesis or if these residues were more broadly required for \(\beta1\) integrin function during development we injected \(\beta1\)YA (Itgb1\textsuperscript{YA/YA}) and \(\beta1\)YF (Itgb1\textsuperscript{YF/YF}) ES cells that were tagged with GFP using a lentivirus that is ubiquitously expressed in vivo into wild-type blastocysts (Fig. 2A,B). Chimeric animals with high levels of ES cell contribution to the dermis of the skin (>90% based on analysis of agouti coat color, which is derived exclusively from the SV/129 ES cells following injection into C57Bl/6 blastocysts) were obtained using both \(\beta1\)YA ES cells and control \(\beta1\)YF ES cells [Fig. 2C; data not shown]. \(\beta1\)YA and \(\beta1\)YF contribution to hematopoietic cells was determined using flow cytometry to detect GFP+ bone marrow cells and contribution to other tissues measured by immunodetection of GFP protein in tissue lysates. As expected, \(\beta1\)YF cells contributed robustly to all tissues, but \(\beta1\)YA cells failed to contribute to hematopoietic cells in the bone marrow or spleen [Fig. 2D,E] or to liver despite contribution to skeletal muscle [where fusion with wild-type cells can rescue mutant cell function], brain, and dermis [Fig. 2C,E; Supplementary Table 2]. Thus \(\beta1\)YA embryos and \(\beta1\)YA chimeras phenocopy those lacking \(\beta1\) entirely, indicating that \(\beta1\) cytoplasmic tyrosines are universally required for integrin function during development.

Loss of \(\beta1\) integrin function in \(\beta1\)YA embryos and \(\beta1\)YA chimeras could result from the disruption of interactions with intracellular proteins required for integrin function or could reflect loss of stable surface expression of the mutant receptor. The latter possibility is suggested by biochemical studies of \(\beta\) integrin cytoplasmic tails demonstrating that the Y-A mutation disrupts interaction with the cytoskeletal protein talin [Calderwood et al. 2002] and the observation that talin-deficient ES cells lose surface expression of \(\beta1\) integrins [Priddle et al. 1998]. To address this question, mutant \(\beta1\) integrin expression and function were next studied in homozy-
The existing model of bidirectional integrin signaling has been developed primarily on the basis of studies performed in platelets where integrin αIIbβ3, a fibronectin receptor, is maintained in a strictly inactive conformation until activated by inside-out signals generated by other receptors on the platelet surface (O’Toole et al. 1991). Following inside-out activation and fibrinogen binding, the cytoplasmic tail of the β3 subunit is tyrosine phosphorylated, an event that initiates a secondary wave of integrin signaling referred to as outside-in. In vivo replacement of the β3 cytoplasmic tyrosines with phenylalanine does not interrupt inside-out activation of platelet integrins, but results in defective hemostasis due to the loss of outside-in integrin signals [Law et al. 1999]. Mouse and human platelets express the α2β1 integrin, which functions as a receptor for vessel wall collagen. The α2β1 integrin is essential for the adhesion of flowing platelets to exposed collagen under arterial levels of hemodynamic shear [Sarratt et al. 2005] and is believed to require inside-out activation similar to that described for αIIbβ3 [Jung and Moroi 2000]. To address the role of β1 cytoplasmic tyrosines in the context of defined inside-out and outside-in integrin signaling, we...
therefore studied platelet collagen responses mediated by β1YA integrins. β1YA integrins were generated using conditional β1cYA knock-in (β1cYA) animals that grew to adulthood without phenotypic abnormalities (Fig. 4; data not shown). Cre-mediated excision of the endogenous Itgb1 terminal exon in the β1cYA allele resulted in splicing to the mutant terminal exon and expression of the β1YA mRNA (Fig. 4B). Cre expression was induced in the hematopoietic cells of β1cYA animals carrying the MX1-Cre transgene. Cre-induced β1cYA animals had circulating platelets with normal surface levels of β1 integrin (Fig. 4C). Following ADP stimulation, β1 integrins on the surface of wild-type but not β1YA platelets became 9EG7 positive (Fig. 4D), however, consistent with an inability to activate the β1YA integrin. Inside-out activation of α2β1 was directly measured using the α2β1 ligand soluble collagen. In contrast to wild-type and β1YF platelets, β1YA platelets failed to bind soluble collagen after ADP stimulation, although binding of the αIIbβ3 ligand fibrinogen was preserved in β1YA platelets (Fig. 4E; Supplementary Fig. 1). Thus cytoplasmic tyrosines are required for inside-out activation of β1 integrins in platelets.

Figure 4. Inside-out and outside-in signaling defects in platelets expressing β1YA and β1YF integrins. (A) Itgb1 gene targeting to generate β1-conditional YA β1cYA) animals. Gene targeting inserts loxP sites 5’ and 3’ of Itgb1 exon 15, as well as a mutant exon 15 encoding the Y-A mutation of the floxed wild-type exon 15. Cre-mediated recombination excises the wild-type exon 15 and drives splicing to the mutant exon 15 [indicated by dashed lines]. β1YA is expressed from the β1cYA allele in the presence of Cre recombinate. RT-PCR was performed to amplify β1 intein cDNA between exons 13 and 15 as shown from the spleen of wild-type (+/+), Itgb1+/YA (+/YA), Itgb1+/cYA (+/cYA), Itgb1cYA/cYA (cYA/cYA), and Itgb1+/cYA; CMV-Cre (+/YA; Cre) mice. The β1YA allele can be distinguished from the wild-type β1 allele by the presence of a 282-bp band following MwoI digestion. Note the absence of β1YA transcript in β1cYA animals lacking Cre expression and the expression of β1YA in β1cYA mice carrying the Cre transgene. (C) Expression of β1YA on the surface of platelets from induced β1cYA; MX1-Cre transgenic mice. Surface β1 was measured with the activation-state-insensitive antibody HMb1-1. (D) β1 integrins in wild-type but not β1YA platelets undergo extracellular conformational changes following ADP stimulation. Surface β1 was measured with the activation-state-sensitive antibody 9EG7 following platelet exposure to 50 µM ADP. N = 5; mean and standard deviation are shown. (E) β1YA integrins confer defective platelet adhesion to collagen under flow. Heparinized blood derived from wild-type (WT), β1YF (YF), β1YA (YA), and α2β1-deficient (α2 KO) mice was flowed over a collagen-coated slide at the shear rates indicated and the percentage surface coverage measured at 4 min. N = 6; mean and standard error of the mean are shown. (*) P = 0.03 by Student’s t-test.
Platelet adhesion to collagen under flow conditions requires ligand binding by α2β1, which mediates firm attachment of the growing thrombus to exposed collagen, an interaction that is likely to require stabilizing outside-in signals analogous to those required for αβ3 to stabilize platelet-fibrinogen binding within the thrombus (Law et al. 1999). To further test the role of β1 integrin tyrosine residues, we next tested the ability of β1YA and β1YF platelets to mediate collagen adhesion under flow. Consistent with a complete loss of integrin function, β1YA platelets exhibited severe defects in collagen adhesion under flow similar to those of platelets lacking α2β1 entirely [Fig. 4F; Supplementary Fig. 1]. Despite normal inside-out responses, however, β1YF platelets also exhibited mildly reduced adhesion to collagen under flow [Fig. 4E], a result most consistent with an outside-in signaling defect.

These studies provide an in vivo test of a model of integrin function that has arisen from extensive biochemical and in vitro studies. In the present model integrin α- and β-chain interaction is believed to hold the receptor in an inactive conformation that requires allosteric release by intracellular molecular events (Takagi et al. 2002; Vinogradova et al. 2002). Protein binding to conserved cytoplasmic tyrosine (NPxY) motifs in the β chain has been proposed as a key step and point of regulation in this process (Calderwood et al. 2003; Tadokoro et al. 2003). Our studies reveal that cytoplasmic tyrosines play an essential role in the regulation of β1 integrin function in virtually all cells, regardless of whether they are known to tightly regulate integrin adhesion or not. What is the critical function of these tyrosines? Genetic studies in lower organisms suggest that one critical function may be to mediate interaction with the cytoskeletal protein talin, as the FERM domain of talin requires the aromatic ring of tyrosine or phenylalanine to bind β integrin cytoplasmic tails (Garcia-Alvarez et al. 2003), and loss of talin in both flies and nematodes confers phenotypes that closely mimic integrin β subunit loss (Brown et al. 2002; Cram et al. 2003). Given the large number of other proteins capable of binding these motifs, however, more specific loss-of-function mutations will be required to determine which play critical roles for β integrin function in vivo.

In light of the complete loss of β1 integrin function observed in β1YA animals and cells and the many essential roles played by β1 integrins in vivo, the normal phenotype of β1YF mice is a particularly striking and unexpected finding. Although less utilized β integrin subunits such as β2 contain NPxY motifs, β1 integrin tyrosine residues are evolutionarily conserved from nematodes to man and β1 tyrosine phosphorylation has been demonstrated to drive cell migration and transformation in vitro (Sakai et al. 2001). The ability of cells to regulate integrin adhesion and the large number of PTB domain-containing proteins that can potentially interact with β integrins have led to the hypothesis that tyrosine phosphorylation might provide a mechanism by which cells dynamically regulate integrin function. Binding of the cytoskeletal protein talin to β integrin NPxY motifs is thought to be necessary for inside-out activation of integrins (Tadokoro et al. 2003) and is predicted to be disrupted by tyrosine phosphorylation (Garcia-Alvarez et al. 2003). As a result, tyrosine phosphorylation has been proposed as a molecular switch by which the β integrin–talin interaction, and thereby integrin adhesion, may be regulated (Calderwood et al. 2003). The viability of β1YF animals demonstrates that tyrosine phosphorylation is not an essential mechanism by which integrin function is regulated in vivo. Whether phosphorylation of β1 tyrosine residues plays a critical role in more specialized functions that require the transmission of outside-in signals from β1 integrins will require further investigation.

This finding supports a model of integrin function in which NPxY–PTB domain interactions are critical for integrin function but are either not dynamically regulated or are regulated through other mechanisms, e.g., through structural changes driven by events at neighboring regions of the integrin cytoplasmic domain, or more indirectly through modulation of critical intermediary proteins such as talin itself. Further studies to determine how integrin interaction with intracellular proteins is dynamically regulated will provide fresh insight into the biological roles of these receptors as well as new therapeutic possibilities.

Materials and methods

Animals

CMVεcre, Mc1cre, FLPe transgenic mice, and CS7BL6 were purchased from the Jackson Laboratory. α2 knockout mice were generously provided by Drs. Sam Santoro and Mary Zutter (Vanderbilt University, Nashville, TN). Conditional β1 mice were obtained from the Jackson Laboratory and generously provided by Dr. Cord Brakebusch (Max Planck Institute, Martinsried, Germany). β1YA, β1YF, and conditional β1YA knock-in ES cells and mice were generated using standard protocols. Targeting constructs contain a 4.4-kb 5′ arm, a 4-kb 3′ arm, and a 1.5-kb target fragment covering exon 15 of the β1 gene, where sequences encoding Tyr 783 and Tyr 795 were mutated to alanines or phenylalanines. Homozygous targeted ES cells were generated by selection in 1.0 mg/mL G418.

ES cell adhesion assays

ES cells were trypsinized and cultured on gelatin plates for 1 h for removal of mouse embryonic fibroblast (MEF) cells. ES cells were then plated onto 96-well plates coated with 0.5 µg/well of fibronectin, laminin, or collagen at 1 × 10⁴ cells per well. After 1 h incubation at 37°C, the plates were washed three times in PBS. Adherent cells were fixed in 95% ethanol, stained in 0.1% crystal violet, washed in water, and lysed in 0.2% Triton X-100 for 10 min for each step, and OD 595 was recorded.

Chimeric mouse studies

GFP-expressing double knock-in YA, YF, or wild-type ES cells were analyzed by flow cytometry. Bone marrow cells of β1YA-GFP and β1YF-GFP chimeric mice were stained with 1 µg/mL PE-Cy5 anti-CD45 antibody and then analyzed by flow cytometry. GFP signals in chimeric tissues were detected by Western blot following standard protocols.

Detection of β1YA mRNA in conditional Y-A mice

RT–PCR was performed using the PCR primers 5′-GCAAGGAGAAG GACATTGATGA-3′ and 5′-CAATGCCCTCCTGCAGTCGTTG-3′, encompassing exon 13 to exon 15 (mutation-targeted exon) used to generate a 521-base-pair (bp) product. The MwoI enzyme was added directly to the PCR reactions and the products analyzed by gel electrophoresis.

Induction of β1YA integrin expression in platelets in conditional YA mice

Cre was induced by six intraperitoneal injections of 0.25 mg polynosinic acid–polycytidylic acid [Fluka #81354] at 2-d intervals. Treated mice were used in experiments at least 4 wk after induction.

Platelet integrin β1 expression and integrin inside-out activation assays

Platelet-rich plasma was stimulated with 0 or 50 µM ADP and staining reagents [50 µg/mL FITC-collagen or 100 µg/mL Alexa Fluor 488-fibrinogen or 3 µg/mL Alexa Fluor 647-9EG7 antibody or 3 µg/mL Alexa Fluor 948-acid.
Platelet flow assays
Whole anticoagulated blood was perfused over a collagen-coated glass slide in a tapered plate chamber, and platelet adhesion was quantified using ImagePro software as previously described (Sarratt et al. 2005).

Statistics
Means and standard deviations or standard error of the mean are shown with the number of samples for each group indicated as N. P values shown were calculated using the two-tailed Student’s t-test.

Acknowledgments
We thank Joel Bennett, Clayton Buck, Skip Brass, Gary Koretzky, Charles Abrams, and Ed Morrisey for their thoughtful comments on the manuscript, Cord Brakebusch for IrigA+/− mice, and Sam Santoro and Mary Zutter for Itgα2−/− mice.

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Genes Dev. 2006, 20: Access the most recent version at doi:10.1101/gad.1408306

Supplemental Material
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