RESEARCH COMMUNICATION

In vivo β 1 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 β 1 integrin cytoplasmic tyrosines with alanines, resulting in the loss of all PTB domain interaction, causes complete loss of β 1 integrin function in vivo. In contrast, replacement of β 1 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.

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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 (Calderwood 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 (Calderwood 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. β 3 integrins in platelets are critical for hemostasis, and substitution of the two β 3 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 β 3 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 β 1 integrins. Since β 1 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. *Itgb1*^{YA/+} and *Itgb1*^{YF/+} mice were healthy and fertile (data not shown) and were intercrossed to generate β 1YA (*Itgb1*^{YA/YA}) and β 1YF (*Itgb1*^{YF/YF}) animals. Analysis of the progeny of heterozygous matings revealed expected numbers of *Itgb1*^{YA/+} mice but failed to reveal live-born β 1YA animals (Supplementary Table 1), indicating that β 1YA animals die during embryonic life. Timed *Itgb1*^{YA/+} matings identified resorptions that

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Figure 1. Integrin $\beta 1$ YA mice experience early embryonic lethality identical to that of integrin $\beta 1$ -deficient mice. (A) *Itgb1* gene targeting to generate $\beta 1$ YA and $\beta 1$ YF animals. Targeting constructs were generated to replace *Itgb1* exon 15, in which both cytoplasmic tyrosines are encoded, with mutant exons encoding Y-A ($\beta 1$ YA) and Y-F ($\beta 1$ YF) mutations as shown. Removal of the FRT-flanked neomycin selection cassette from the targeted locus was performed in vivo using CMV-Flope transgenic animals. (B) $\beta 1$ YA embryos fail to develop past blastocyst implantation. The progeny of *Itgb1*^{YA/+} matings at E10.5 and E7.5 are shown. (C) The early embryonic lethality of $\beta 1$ YA animals phenocopies that of $\beta 1$ -deficient animals. The progeny of *Itgb1*^{YA/+} and *Itgb1*^{+/-} crosses were analyzed at E6.5 using hematoxylin and eosin staining of embryo sections. Presumptive $\beta 1$ YA and $\beta 1$ -deficient E6.5 embryos failed to develop an inner cell mass and were identified by the presence of persistent trophoblastic cells. Images shown are representative of four to five separate experiments.

could be genotyped as *Itgb1*^{YA/YA} animals but no live $\beta 1$ 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 $\beta 1$ YA embryos were resorbed with only a residual population of trophoblastic cells visible, a phenotype similar to that reported for $\beta 1$ integrin-deficient embryos (Fig. 1C; Fassler and Meyer 1995; Stephens et al. 1995). Mouse embryos lacking $\beta 1$ integrins form normal blastocysts but fail to develop an inner cell mass, a critical early step in mammalian embryogenesis (Fassler and Meyer 1995;

Stephens et al. 1995). Direct histologic comparison with E6.5 embryos from *Itgb1*^{+/-} intercrosses revealed identical findings in presumptive *Itgb1*^{-/-} embryos (Fig. 1C), demonstrating that $\beta 1$ cytoplasmic tyrosines are required for integrin function in early mouse embryonic development.

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

Essential roles for $\beta 1$ 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 $\beta 1$ -deficient embryonic stem (ES) cells into wild-type blastocysts. These studies utilized lineage tracing to demonstrate that $\beta 1$ 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 $\beta 1$ cytoplasmic tyrosines in vivo was restricted to early embryogenesis or if these residues were more broadly required for $\beta 1$ integrin function during development we injected $\beta 1$ YA (*Itgb1*^{YA/YA}) and $\beta 1$ YF (*Itgb1*^{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 (>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 $\beta 1$ YA ES cells and control $\beta 1$ YF ES cells (Fig. 2C; data not shown). $\beta 1$ YA and $\beta 1$ 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, $\beta 1$ YF cells contributed robustly to all tissues, but $\beta 1$ 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 $\beta 1$ YA embryos and $\beta 1$ YA chimeras phenocopy those lacking $\beta 1$ entirely, indicating that $\beta 1$ cytoplasmic tyrosines are universally required for integrin function during development.

Loss of $\beta 1$ integrin function in $\beta 1$ YA embryos and $\beta 1$ 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 β 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 $\beta 1$ integrins (Priddle et al. 1998). To address this question, mutant $\beta 1$ integrin expression and function were next studied in homozy-

Figure 2. $\beta 1YA$ cells exhibit cell-autonomous lineage restriction in chimeric animals. (A) Generation of homozygous $\beta 1YA$ and $\beta 1YF$ ES cells. $Itgb1^{YA/YA}$ (YA/YA) and $Itgb1^{YF/YF}$ (YF/YF) ES cells were generated by selection in high G418 concentration. Southern blot analysis using the probe shown in Figure 1 reveals loss of the wild-type 13-kb band and exclusive detection of the 5-kb targeted band in homozygous knock-in cells. Two clones were identified for both $\beta 1YA$ and $\beta 1YF$ genotypes. (B) Lentiviral GFP expression in $\beta 1YF$ and $\beta 1YA$ ES cells. Homozygous knock-in ES cells were exposed to GFP-expressing lentivirus and subclones were identified that expressed high levels of GFP. (C) Generation of chimeric animals using $\beta 1YF$ and $\beta 1YA$ ES cells. Homozygous $\beta 1YA$ and $\beta 1YF$ ES cells expressing GFP were injected into wild-type C57Bl/6 blastocysts. Chimeric animals with high-level ES cell contribution to the skin, determined by the extent of agouti coat color, were identified following injection of both cell types. (D) $\beta 1YA$ cells do not contribute to the hematopoietic lineages. GFP-expressing CD45⁺ bone marrow cells were measured in chimeric animals generated with GFP-tagged $\beta 1YF$ and $\beta 1YA$ ES cells using flow cytometry. Results shown are representative of three chimeric animals studied following injection of two distinct $\beta 1YA$ ES cell clones. (E) $\beta 1YA$ cells contribute to brain and gut but not liver or spleen. $\beta 1YF$ and $\beta 1YA$ ES cell contribution to different tissues was measured by immunoblot analysis using anti-GFP antibody. The results shown are representative of the data summarized in Supplementary Table 2.

gous $\beta 1YA$ and $\beta 1YF$ ES cells. $\beta 1YA$ ES cells expressed levels of total and cell surface $\beta 1$ integrin that were indistinguishable from those of $\beta 1YF$ and wild-type ES cells (Fig. 3A,B). $\beta 1YA$ ES cells appeared more loosely adherent than wild-type ES cells when grown on gelatin or embryonic fibroblasts (Fig. 3C; data not shown), however, and were unable to bind laminin, a $\beta 1$ -specific ligand, or fibronectin, a ligand for $\beta 1$ and $\beta 3$ integrins, when $\beta 3$ integrin function was blocked by RGD peptide (Fig. 3D). Finally, in contrast to wild-type or $\beta 1YF$ ES cells, $\beta 1$ integrins on the surface of $\beta 1YA$ ES cells were not recognized by the activation-specific anti- $\beta 1$ antibody 9EG7 (Fig. 3B). Thus $\beta 1YA$ integrins are expressed normally on the cell surface, but are in an inactive conformation and unable to bind matrix protein ligands.

The existing model of bidirectional integrin signaling has been developed primarily on the basis of studies performed in platelets where integrin $\alpha IIb\beta 3$, a fibrinogen receptor, is maintained in a strictly inactive conforma-

tion 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 $\beta 3$ subunit is tyrosine phosphorylated, an event that initiates a secondary wave of integrin signaling referred to as outside-in. In vivo replacement of the $\beta 3$ 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 $\alpha 2\beta 1$ integrin, which functions as a receptor for vessel wall collagen. The $\alpha 2\beta 1$ 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 $\alpha IIb\beta 3$ (Jung and Moroi 2000). To address the role of $\beta 1$ cytoplasmic tyrosines in the context of defined inside-out and outside-in integrin signaling, we

Figure 3. $\beta 1YA$ integrins are expressed on the cell surface but are in an inactive conformation and fail to mediate ES cell adhesion. (A) $\beta 1YA$ and $\beta 1YF$ integrins are expressed at normal levels. $\beta 1$ integrins in wild-type (WT), $\beta 1YF$ (YF), and $\beta 1YA$ (YA) ES cell lysates were probed with HMB1-1, a monoclonal antibody that recognizes the N terminus of the $\beta 1$ integrin. Actin staining is shown as a control for loading. (B) $\beta 1YA$ and $\beta 1YF$ integrins are expressed on the cell surface but $\beta 1YA$ integrins are poorly bound by the activation-state-specific antibody 9EG7. Flow cytometry of live cells was performed using the activation-state-insensitive monoclonal antibody HMB1-1 and the activation-state-specific antibody 9EG7. (C) $\beta 1YA$ ES cells fail to adhere to gelatin. Wild-type, $\beta 1YF$, and $\beta 1YA$ ES cells were grown on gelatin overnight. Results are representative of five experiments. (D) $\beta 1YA$ integrins fail to adhere to matrix ligands. Wild-type, $\beta 1YF$, and $\beta 1YA$ ES cells were plated on fibronectin, with or without RGD peptide to inhibit $\beta 3$ integrin function, the $\beta 1$ ligand laminin, or collagen, for which no receptors are expressed on ES cells. OD595 measures binding of adherent cell nuclei acid by crystal violet. $N = 5$; mean and standard deviation are shown.

Figure 4. Inside-out and outside-in signaling defects in platelets expressing $\beta 1$ YA and $\beta 1$ YF integrins. (A) *Itgb1* gene targeting to generate $\beta 1$ -conditional YA ($\beta 1$ cYA) 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 3' 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). (B) $\beta 1$ YA is expressed from the $\beta 1$ cYA allele in the presence of Cre recombinase. RT-PCR was performed to amplify $\beta 1$ integrin cDNA between exons 13 and 15 as shown from the spleen of wild-type (+/+), *Itgb1*^{+/YA} (+/YA), *Itgb1*^{+/cYA} (+/cYA), *Itgb1*^{cYA/cYA} (cYA/cYA), and *Itgb1*^{+/cYA}; CMV-Cre (+/YA; Cre) mice. The $\beta 1$ YA allele can be distinguished from the wild-type $\beta 1$ allele by the presence of a 282-bp band following MwoI digestion. Note the absence of $\beta 1$ YA transcript in $\beta 1$ cYA animals lacking Cre expression and the expression of $\beta 1$ YA in $\beta 1$ cYA mice carrying the Cre transgene. (C) Expression of $\beta 1$ YA on the surface of platelets from induced $\beta 1$ cYA; MX1-Cre transgenic mice. Surface $\beta 1$ was measured with the activation-state-insensitive antibody HMb1-1. (D) $\beta 1$ integrins in wild-type but not $\beta 1$ YA platelets undergo extracellular conformational changes following ADP stimulation. Surface $\beta 1$ was measured with the activation-state-sensitive antibody 9EG7 following platelet exposure to 50 μ M ADP. (E) $\beta 1$ YA integrins cannot be activated by inside-out signals in platelets. Platelets derived from wild-type (WT), $\beta 1$ YF (YF), $\beta 1$ YA (YA), and $\alpha 2\beta 1$ -deficient ($\alpha 2$ KO) mice were activated with 50 μ M ADP and integrin activation measured through binding of either FITC-soluble collagen ($\beta 1$ ligand) or Alexa Fluor488-fibrinogen ($\beta 3$ ligand). $N = 5$; mean and standard deviation are shown. (F) $\beta 1$ YA and $\beta 1$ YF integrins confer defective platelet adhesion to collagen under flow. Heparinized blood derived from wild-type (WT), $\beta 1$ YF (YF), $\beta 1$ YA (YA), and $\alpha 2\beta 1$ -deficient ($\alpha 2$ 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.

therefore studied platelet collagen responses mediated by $\alpha 2\beta 1$.

$\beta 1$ YA platelets were generated using conditional $\beta 1$ YA knock-in ($\beta 1$ cYA) animals that grew to adulthood without phenotypic abnormalities (Fig. 4; data not shown). Cre-mediated excision of the endogenous *Itgb1* terminal exon in the $\beta 1$ cYA allele resulted in splicing to the mutant terminal exon and expression of the $\beta 1$ YA mRNA (Fig. 4B). Cre expression was induced in the hematopoietic cells of $\beta 1$ cYA animals carrying the MX1-Cre transgene. Cre-induced $\beta 1$ cYA animals had circulating platelets with normal surface levels of $\beta 1$ integrin

(Fig. 4C). Following ADP stimulation, $\beta 1$ integrins on the surface of wild-type but not $\beta 1$ YA platelets became 9EG7 positive (Fig. 4D), however, consistent with an inability to activate the $\beta 1$ YA integrin. Inside-out activation of $\alpha 2\beta 1$ was directly measured using the $\alpha 2\beta 1$ ligand soluble collagen. In contrast to wild-type and $\beta 1$ YF platelets, $\beta 1$ YA platelets failed to bind soluble collagen after ADP stimulation, although binding of the α Ib β 3 ligand fibrinogen was preserved in $\beta 1$ YA platelets (Fig. 4E; Supplementary Fig. 1). Thus cytoplasmic tyrosines are required for inside-out activation of $\beta 1$ integrins in platelets.

Platelet adhesion to collagen under flow conditions requires ligand binding by $\alpha 2\beta 1$, 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 $\alpha IIb\beta 3$ to stabilize platelet-fibrinogen binding within the thrombus (Law et al. 1999). To further test the role of $\beta 1$ integrin tyrosines, we next tested the ability of $\beta 1YA$ and $\beta 1YF$ platelets to mediate collagen adhesion under flow. Consistent with a complete loss of integrin function, $\beta 1YA$ platelets exhibited severe defects in collagen adhesion under flow similar to those of platelets lacking $\alpha 2\beta 1$ entirely (Fig. 4F; Supplementary Fig. 1). Despite normal inside-out responses, however, $\beta 1YF$ 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 $\beta 1$ 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 $\beta 1$ integrin function observed in $\beta 1YA$ animals and cells and the many essential roles played by $\beta 1$ integrins *in vivo*, the normal phenotype of $\beta 1YF$ mice is a particularly striking and unexpected finding. Although less utilized β integrin subunits such as $\beta 2$ contain NPxF motifs, $\beta 1$ integrin tyrosine residues are evolutionarily conserved from nematodes to man and $\beta 1$ 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 $\beta 1YF$ animals demonstrates that tyrosine phosphorylation is not an essential mechanism by which integrin function is regulated *in vivo*. Whether phosphorylation of $\beta 1$ tyrosine residues plays a critical role in more specialized functions that require the transmission of outside-in signals from $\beta 1$ 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

CMVcre, Mx1cre, FLPe transgenic mice, and C57BL6 were purchased from the Jackson Laboratory. $\alpha 2$ knockout mice were generously provided by Drs. Sam Santoro and Mary Zutter (Vanderbilt University, Nashville, TN). Conditional $\beta 1$ mice were obtained from the Jackson Laboratory and generously provided by Dr. Cord Brakebusch (Max Planck Institute, Martinsreid, Germany). $\beta 1YA$, $\beta 1YF$, and conditional $\beta 1YA$ 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 $\beta 1$ 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 treated with 0.25 mg/mL GRGDS peptide or control peptide GRGES (American Peptide Co.) or nothing for 30 min. ES cells were plated onto 96-well plates coated with 0.5 μ g/well of fibronectin, laminin, or collagen at 1×10^6 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 $\beta 1YA$ -GFP and $\beta 1YF$ -GFP chimeric mice were stained with 1 μ g/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 $\beta 1YA$ mRNA in conditional Y-A mice

RT-PCR was performed using the PCR primers 5'-GCAAGGAGAAG GACATTGATGA-3' and 5'-CAAGTCTTCTGTCTCAGTCCCTG-3', encompassing exon 13 to exon 15 (mutation-targeted exon) were 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 $\beta 1YA$ integrin expression in platelets in conditional YA mice

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

Platelet integrin $\beta 1$ expression and integrin inside-out activation assays

Platelet-rich plasma was stimulated with 0 or 50 μ M ADP and staining reagents (50 μ g/mL FITC-collagen or 100 μ g/mL Alexa Fluor 488-fibrinogen or 3 μ g/mL Alexa Fluor 647-9EG7 antibody or 3 μ g/mL Alexa Fluor

647-HM β 1-1) added. The platelets were fixed with 4% paraformaldehyde for 5 min and analyzed by flow cytometry.

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.

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