Development requires activation but not phosphorylation of β1 integrins

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Named after the ability of many of them to “integrate” the extracellular matrix with the intracellular cytoskeleton, integrins are well-characterized mediators of cellular adhesion and signaling [Giancotti and Ruoslahti 1999, Hynes 2002]. Each integrin consists of an α and a β subunit and binds to a distinct, but often overlapping, spectrum of ligands. Whereas β1 integrins mediate cell adhesion to extracellular matrix components, β2 integrins play key roles in immune cell recognition and activation by interacting with counterreceptors of the Ig superfamily, and αIIbβ3 orchestrates platelet aggregation by binding to the polyvalent blood protein fibrinogen. Genetic studies using mouse models have demonstrated that β1 integrins have unique roles in embryonic development, hematopoiesis, wound healing, and cancer [Brakebusch et al. 1997; Grose et al. 2002; White et al. 2004]. Successful execution of a number of physiological processes requires precisely choreographed changes in the activity of several integrins. The regulation of integrin function is complex, and recent studies suggest that it hinges on long-range structural changes, which are propagated across the plasma membrane in both directions [Hynes 2002]. In spite of its importance, genetic analysis of this aspect of integrin function has lagged behind. A new paper by Chen et al. [2006], published in the April 15 issue of Genes and Development, intends to remedy this imbalance.

Many integrins are maintained in a default low-affinity state and, hence, need to be activated to exert their function. The past decade has seen the emergence and consolidation of two major paradigms for integrin activation and signaling. The first describes the process through which cytoplasmic signals trigger integrin activation (“inside-to-outside” integrin signaling) [Ginsberg et al. 1992]. Most, perhaps all, activating signals promote the binding of talin to the cytoplasmic portion of the integrin β subunit [Liddington and Ginsberg 2002]. Talin binding promotes a separation of the integrin cytoplasmic tails and triggers a series of long-range conformational changes, including a “switch-blade” movement of the integrin head domains, resulting in increased availability of the extracellular ligand-binding site [Shimaoka et al. 2002]. The immediate outcome of this form of regulation is an increase in the affinity of integrins for their matrix ligands. The second process, “outside-to-inside” integrin signaling, flows in the opposite direction. Upon binding to extracellular matrix components, integrins multimerize and recruit complexes of cytoskeletal and signaling molecules. Using this latter mechanism, integrins cooperate with receptors for cytokines and growth factors to control a multitude of cellular functions, including cell adhesion, migration, survival, differentiation, and proliferation [Assoian and Schwartz 2001; Miranti and Brugge 2002; Giancotti and Tarone 2003]. Both inside-to-outside and outside-to-inside integrin signaling rely on a combination of regulatory controls that involves alterations in the structure of the receptors’ cytoplasmic domains, long-range conformational changes in integrin structure, a steep increase in ligand-binding affinity, and, finally, clustering of integrins and downstream signal transduction.

Mutagenesis and structural studies have painted a particularly vivid picture of integrin activation. Integrins are maintained inactive by interactions between the transmembrane domains and the membrane-proximal segments of the cytoplasmic tails of their constituent subunits [Fig. 1; Liddington and Ginsberg 2002]. Substitution with Ala of an Asp residue in the membrane-proximal segment of the β subunit or an Arg residue in the corresponding region of the α subunit causes constitutive integrin activation, suggesting that a salt bridge between the side chains of the two amino acids stabilizes the inactive conformation [Hughes et al. 1996]. In agreement with this notion, forced dimerization of the α and β tails prevents integrin activation [Lu et al. 2001].

What is the mechanism through which cytoplasmic signals disrupt the α–β tail “clasp”?! The C-terminal portion of all β subunits contains two tandem NPxY motifs, and compelling evidence indicates that the N-terminal motif functions to “unclasp” the integrin tails [Fig. 1]. NMR structural analysis indicates that this motif forms a β turn, creating a binding interface for a phospho-tyro-
sine-binding (PTB) domain [Forman-Kay and Pawson 1999; Ulmer et al. 2001]. In fact, Calderwood et al. [1999] showed that binding of the PTB-containing FERM domain of talin to the N-terminal NPxY motif of β3 and other β subunits induces integrin activation. The importance of this interaction is demonstrated by the observation that small interfering RNA (siRNA)-mediated silencing of talin prevents integrin activation [Tadokoro et al. 2003]. In addition, mutation of the N-terminal NPxY motif inhibits integrin interaction with the actin cytoskeleton at focal contacts [Reszka et al. 1992]. NMR studies demonstrate that the talin head domain disrupts the association of α–β tail peptides, suggesting that talin binding to the N-terminal NPxY motif is sufficient to “unclasp” the integrin α and β subunit cytotails [Vino-gradova et al. 2002]. These results indicate that talin is key to both integrin activation and integrin association with the cytoskeleton.

The function of the C-terminal NPxY motif is less understood. However, binding of the PTB-domain protein ICAP-1α to this motif interferes with cell spreading and assembly of focal contacts, presumably by suppressing binding of talin to the upstream motif [Bouvard et al. 2003]. Moreover, the C-terminal motif of β3 has been implicated in the recruitment of the signaling adaptor Shc in platelets [Fig. 1; Cowan et al. 2000; Phillips et al. 2001]. In contrast to the β1 integrin, which is not detectably phosphorylated in normal cells [Johansson et al. 1994], the β3 subunit undergoes tyrosine phosphorylation during platelet aggregation [Blystone et al. 1997; Jenkins et al. 1998]. The importance of tyrosine phosphorylation of β3 has been examined in vivo using a knock-in mouse model, where each of the tyrosines in the NPY motifs of β3 integrin was replaced by a Phe [β3 diYA mice] [Law et al. 1999]. The mutations did not seem to affect inside-out signaling in cells, since initial ADP-induced aggregation rates and fibrinogen binding of stimulated platelets were not perturbed. Outside-to-inside signaling, however, was impaired, as platelets exhibited defects in maintaining their aggregation following ADP stimulation. Consequently, the mice displayed an increased propensity to rebleed after clotting.

The tyrosine residues in the NPxY motifs of integrin β subunits are putative sites for tyrosine kinase-mediated phosphorylation. In fact, early studies showed that β1 integrins become phosphorylated on tyrosine in cells transformed with oncogenic tyrosine kinases [Hirst et al. 1986]. Notably, this phosphorylation correlated with decreased integrin binding to both fibronectin and talin in vitro, suggesting a molecular mechanism for disruption of adhesion in neoplastically transformed cells [Tapley et al. 1989]. Accordingly, the substitutions at both NPxY motifs in β1 integrins reduced assembly of focal contacts and matrix deposition of fibronectin in cells transformed by the v-Src oncogene [Sakai et al. 2001].

The FERM domain of talin binds to integrins via a novel variant of the canonical PTB domain–NPxY ligand interaction [Garcia-Alvarez et al. 2003]. Phosphorylation of the NPxY motif is predicted to disrupt this interaction, as well as that of ICAP-1α, with integrins. By contrast, binding of the signaling adaptor Shc to β3 appears to require phosphorylation of the C-terminal NPxY motif [Fig. 1; Cowan et al. 2000]. Other physiologically relevant PTB domain interactions may be similarly regulated, as integrin β tails can bind in vitro to several PTB domain proteins, including Numb, Dok-1, EPS8, tensin, and Dab [Calderwood et al. 2003]. Based on these observations, it has been proposed that tyrosine phosphorylation of the NPxY motifs can function as a “molecular switch,” contributing to diversity of signaling following integrin-dependent adhesion [Liddington and Ginsberg 2002; Calderwood et al. 2003].

Chen et al. [2006] have chosen to address this question by studying the role of the NPxY motifs in β1 integrins. The β1 subunit is expressed in most cells from the earliest stages of development until adulthood, and it combines with 12 distinct α subunits to form receptors that bind to fibronectin, laminins, collagens, and a variety of other matrix components. Not surprisingly, ablation of β1 causes early, preimplantation embryonic lethality: The inner cell mass does not develop properly, the blastocoele collapses, and profound defects in endoderm morphogenesis and migration ensue [Fassler and Meyer 1995; Stephens et al. 1995].

To examine whether the Tyr residues in NPxY motifs are necessary for proper functioning of β1 integrins, Chen et al. [2006] first introduced Ala substitutions at both residues in ES cells. Targeting both NPxY motifs avoided the possibility of compensatory binding of cytoplasmic-interacting partners to the nonmutated tyrosine [Fig. 2]. Homozygous embryos (β1 diYA embryos) failed before implantation, essentially like β1-null embryos, demonstrating that the Tyr residues in NPxY motifs are absolutely required for β1 integrin function during early development.

They next addressed the significance of phosphorylation of these residues by generating another set of knock-
in mutants, this time featuring conservative substitutions of both Tyr residues with Phe residues ([β1 diYF mice] Fig. 2). This approach preserved the hydrophobicity conferred by tyrosines but eliminated the contribution of phosphorylation. Surprisingly, homozygous β1 diYF mice were born alive and grew into adulthood healthy, indicating that phosphorylation of the β1 cytoplasmic tail is not essential for development and normal adult life.

The importance of β1 integrin cytoplasmic tyrosines after embryogenesis was assessed using elegant chimera studies, where β1 diYA and diYF ES cells were injected into wild-type blastocysts. Whereas diYF cells were able to contribute to all tissues that were analyzed, diYA cells did not participate in the development of most organs, behaving essentially like β1-null cells in similar chimeras. These observations provide strong evidence that the tyrosine residues in NpxY motifs are necessary for β1 function during development. Phosphorylation of these tyrosines, however, seems to be dispensable.

What is the mechanism by which the diYA mutation disrupts integrin β1 function? Chen et al. (2006) provide evidence that cells bearing this mutation exhibit profound defects in binding to fibronectin and laminin. In addition, their β1 integrins were not recognized by the activation-specific monoclonal antibody 9EG7. Therefore, the diYA mutation disables the inside-to-outside arm of integrin signaling. Because loss of talin phenocopies the effect of ablation of β1 integrins in worms and flies (Brown et al. 2002; Cram et al. 2003), the profound effect of the diYA mutation is probably due to loss of binding of talin. Not surprisingly, β1 diYA platelets did not adhere well to collagen under flow. Defective outside-to-inside signaling may contribute to a small extent to the phenotype, as β1 diYF platelets showed marginally defective adhesion to collagen in the same assay. Albeit of much smaller intensity, this latter defect is reminiscent of the post-clotting defect observed in β3 diYF platelets, suggesting that tyrosine phosphorylation contributes to adhesion strengthening in platelets.

The conclusions drawn from the analysis of β1 diYF mutant mice underscore the necessity for a rigorous genetic analysis of integrin function. Whereas tyrosine phosphorylation may contribute to regulation of αIIbβ3 and, to a smaller extent, α2β1 in platelets, this modification does not appear to affect the function of most β1 integrins during development. It remains to be resolved whether this dichotomy arises from differences in integrin signaling between platelets and other cells or differences between β1 and β3 integrins. Notably, the substitution at both NpxY motifs does not impair integrin β1-mediated recruitment of Shc and activation of Ras–ERK signaling in mouse fibroblasts (Wary et al. 1996), in agreement with the observation that these motifs in β1 are not detectably phosphorylated in cultured cells (Johansson et al. 1994). Rather, β1 integrins recruit Shc through their α subunit (Wary et al. 1998) or through Focal Adhesion Kinase (Schlaepfer et al. 1998). A second question relates to the relative contribution of the C-terminal NpxY motif. Whereas the major function of the N-terminal motif is to recruit talin, the C-terminal motif can recruit ICAP-1α and possibly other PTB domain proteins. More specific loss-of-function mutations will be required to better define its function. Finally, we should not avoid considering the possibility that phosphorylation of the NpxY motifs becomes important under conditions of cellular distress, such as transformation or inflammation. The data linking tyrosine phosphorylation of β1 to disruption of adhesion and modified signaling in cells transformed by oncogenic tyrosine kinases are compelling. We speculate that oncogenic tyrosine kinases may hijack the integrin NpxY motifs to disrupt adhesion and promote constitutive signaling. Breeding the β1 diYF mice into relevant mouse models of cancer will reveal if this is indeed the case.

A genetic test of integrin activation

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