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# C-terminal opening mimics 'inside-out' activation of integrin α5β1

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**Integrins are adhesion molecules that convey signals both to and from the cytoplasm across the plasma membrane. In resting cells, integrins in a low affinity state can be activated by 'inside-out signaling', in which signals affecting integrin heterodimer cytoplasmic domains cause a conformational change in the integrin ligand-binding headpiece connected to the membrane by two long, ~16 nm stalks. Here we demonstrate a mechanism for conveying a conformational change over the long distance from the plasma membrane to the headpiece. We prepared soluble, α5β1 integrin heterodimer extracellular fragments in which interactions between α- and β-subunit cytoplasmic domains were replaced with an artificial clasp. Release of this C-terminal clasp by specific protease cleavage resulted in an ~14 nm separation of the stalks coupled to increased binding to fibronectin. This activation did not require any associated molecules or clustering and was observed with physiological concentrations of divalent cations. These findings suggest that the overall mechanism for integrin inside-out activation involves the spatial separation of the cytoplasmic and/or transmembrane domains.**

Integrin cell adhesion receptors are composed of noncovalently associated α- and β-subunits. Each subunit comprises a large extracellular domain, a single transmembrane domain and a short cytoplasmic tail. Similar to other cell surface receptors, integrins transduce signals to the cytoplasm upon binding to extracellular ligands (outside-in signaling)[1]. However, integrins can also modulate the affinity for ligand of their extracellular domain by intracellular signals (inside-out signaling)[2]. Modulation of this affinity is most likely not controlled by chemical modification of the receptor molecule but mainly accomplished by conformational change[3,4]. Experiments using monoclonal antibodies (mAb) that bind to the integrin stalk region suggested long-range, bidirectional conformational communication with the ligand-binding headpiece[4,5]. Cytoplasmic tail mutations of various integrin α–β pairs have revealed the importance of the cytoplasmic region in supporting association of the α- and β-subunits and in affinity regulation[3,6]. During activation, the membrane-proximal region of the α- and β-cytoplasmic tails may act like a fulcrum or 'hinge' during the scissor-like motion of the α–β complex[7–9]. However, other mechanisms for conveying inside-out signals are also possible. A number of molecules associate both intra- and extracellularly with integrins and may modulate the adhesive properties of cells. Therefore, the results of integrin cell surface ligand-binding experiments should be interpreted with caution, especially when cell adhesion is the final assay. In addition to integrin conformational change, associations with other molecules and clustering of receptors may modulate adhesion activity . More importantly, it is difficult to determine if affinity regulation is caused by the observed conformational change, or if conformational change is a consequence of ligand binding, as in the appearance of ligand-induced binding sites on integrins recognized by mAbs[4,10].

## Introduction of a 'releasable' C-terminal clasp

To avoid the above complications, we used soluble, recombinant integrins to study the spatial relationship between the membrane-proximal segments of the α- and β-subunits in relation to the ligand-binding activity of the integrin headpiece. Interactions

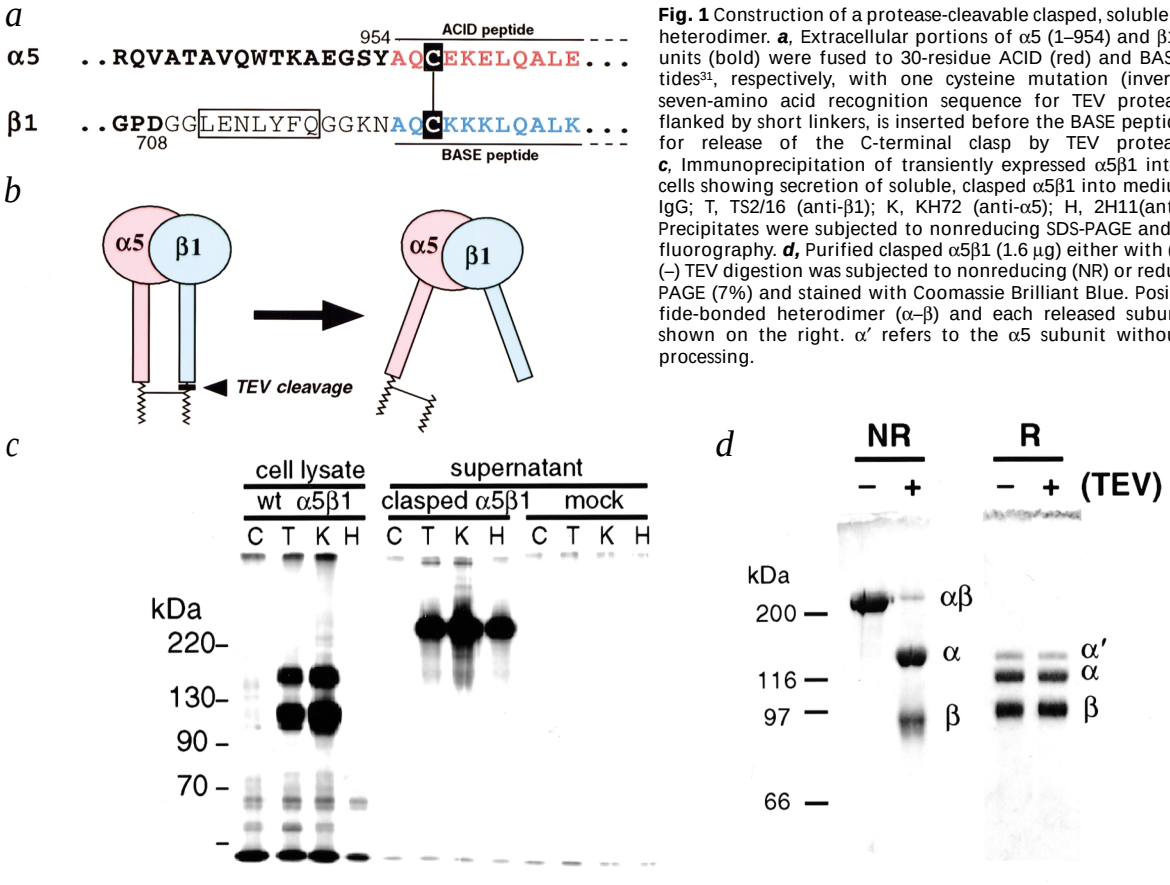


**Fig. 1** Construction of a protease-cleavable clasped, soluble integrin α5β1 heterodimer. ***a***, Extracellular portions of α5 (1–954) and β1 (1–708) subunits (bold) were fused to 30-residue ACID (red) and BASE (blue) peptides[31], respectively, with one cysteine mutation (inverse). In β1, a seven-amino acid recognition sequence for TEV protease[32] (boxed), flanked by short linkers, is inserted before the BASE peptide. ***b***, Scheme for release of the C-terminal clasp by TEV protease cleavage. ***c***, Immunoprecipitation of transiently expressed α5β1 integrin in 293T cells showing secretion of soluble, clasped α5β1 into medium. C, control IgG; T, TS2/16 (anti-β1); K, KH72 (anti-α5); H, 2H11(anti-coiled coil). Precipitates were subjected to nonreducing SDS-PAGE and visualized by fluorography. ***d,*** Purified clasped α5β1 (1.6 μg) either with (+) or without (–) TEV digestion was subjected to nonreducing (NR) or reducing (R) SDS - PAGE (7%) and stained with Coomassie Brilliant Blue. Positions of disulfide-bonded heterodimer (α–β) and each released subunit (α, β) are shown on the right. α′ refers to the α5 subunit without proteolytic processing.

between the α- and β-subunit cytoplasmic/transmembrane domains restrain integrins in an inactive state[6–8]. After activation of integrins[4,11–14], epitopes in the β-subunit stalk region become exposed. We hypothesized that association/dissociation of the α- and β-subunits at the membrane-proximal region could regulate ligand binding of the headpiece. Therefore, we introduced a releasable constraint at the C-terminal end of the α- and β-subunit stalks. We used α5β1 as a representative integrin. To obtain a covalently linked heterodimer, the α5 and β1 subunits were truncated before the transmembrane domains and fused to complementary 'ACID' and 'BASE' α-helical coiled coil peptides, with the residue in the fourth position of the first heptad mutated to cysteine[15] (Fig. 1*a*). This covalently linked coiled coil 'clasp' maintained close apposition between the membrane proximal regions of α5 and β1. A recognition sequence for tobacco etch virus (TEV) protease was included at the junction between the β1 subunit and the coiled coil to enable release of the constraint provided by the clasp. (Fig 1*b*). When transfected into 293T cells, this soluble, clasped version of α5β1 was expressed as a secreted ~250 kDa covalent heterodimer with an efficiency comparable to that of the membrane-bound, wild type noncovalent heterodimer (Fig. 1*c*). A panel of antibodies mapped to different regions of the α5 and β1 subunits recognized the soluble, clasped α5β1 (data not shown), confirming that it is structurally intact.

To obtain a large quantity of recombinant, soluble α5β1, a stable CHO Lec 3.2.8.1 transfectant was established. Soluble α5β1 was purified using an anti-β1 column and by gel filtration. α5β1 eluted as a homogeneous, single molecular species of ~350 kDa without aggregates. Ultracentrifugation on a glycerol gradient gave a sedimentation coefficient of 9.1 S. Incubation of this protein with 250 units $ml^{-1}$ TEV protease at 30 °C for 6 h resulted in almost complete cleavage of the clasp, as indicated by conversion of the 220 kDa species to 140 kDa and 95 kDa species, corresponding to the α5 and β1 subunits, respectively, on nonreducing SDS-PAGE (Fig. 1*d*). Consistent with release of a short peptide containing an intersubunit disulfide linkage, reducing SDS-PAGE revealed no apparent change in molecular weight of α- and β-subunits after TEV cleavage (Fig. 1*d*). The sedimentation coefficient of the unclasped integrin decreased to 8.3 S, indicating a more open conformation.

## Activation of ligand binding

Ligand binding by soluble, clasped α5β1 before and after clasp release was compared in real time using surface plasmon resonance. A cysteine introduced into the C-terminus of a recombinant fibronectin fragment containing the α5β1 binding sites in domains 9 and 10 (Fn9–10) was used to immobilize the fragment and provide a uniform orientation by immobilizing the fragment. When intact clasped α5β1 was infused in the presence of 1 mM $Ca^{2+}$ and $Mg^{2+}$, only a small amount of binding to Fn9–10 was observed (Fig. 2*a*). However, release of the C-terminal constraint by TEV protease treatment markedly increased binding to Fn9–10 (Fig. 2*a*). Binding to Fn9–10 was specific because (i) neither the clasped or unclasped α5β1 preparations bound Fn9–10 in which the Arg-Gly-Asp-Ser (RGDS) sequence was deleted (Fig. 2*a*); (ii) addition of 100 μM RGD peptide abolished binding (Fig. 2*a*); and (iii) preincubation of α5β1 with known blocking mAbs against the α5 and β1 subunits inhibited binding (data not shown). The activation of binding was proportional to the extent of conversion from the clasped to the unclasped form (Fig. 2*b*). Addition of 1 mM $Mn^{2+}$ increased binding by both clasped and unclasped α5β1. More importantly,

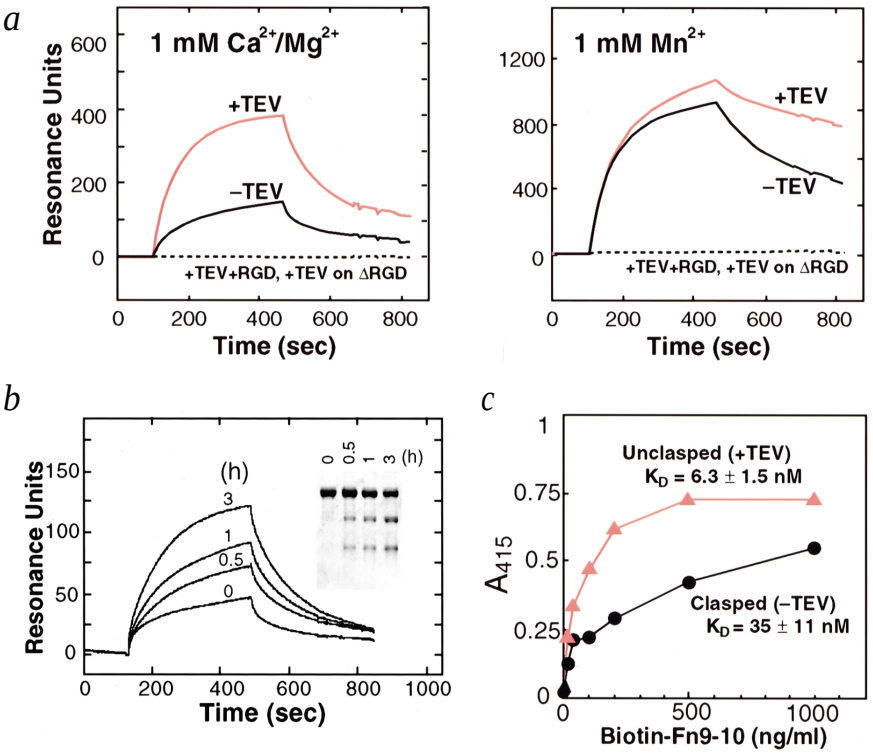


**Fig. 2** Activation of clasped α5β1 by C-terminal cleavage. ***a***, Surface plasmon resonance measurement of binding of α5β1 preparations to ligand. Purified clasped α5β1 (50 nM) before (–TEV, black tracings) or after (+TEV, red tracings) TEV cleavage was infused over a sensorchip with immobilized fibronectin fragment Fn9–10 in the presence of 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (left panel) or 1 mM $MnCl_2$ (right panel). Tracings (dashed line) are shown for binding to Fn9–10 in the presence of 100 μM GRGDSP peptide (+TEV, +RGD) and to Fn9–10ΔRGDS (ref. 28). ***b***, Time course of digestion and activation of ligand binding. Clasped α5β1 (0 h) was digested with 75 units $ml^{-1}$ TEV protease for 0.5–3 h and subjected at a concentration of 25 nM to surface plasmon resonance analysis as in (*a*) in the presence of 1mM $Ca^{2+}/Mg^{2+}$ or subjected to nonreducing 7% SDS-PAGE (inset). ***c***, Solid phase equilibrium binding using immobilized α5β1, either before (–TEV, circles) or after (+TEV, triangles) C-terminal cleavage, and solution phase biotinylated Fn9–10. Data from one representative of four independent experiments is shown. Steady-state dissociation constants ($K_D$) were calculated for each independent experiment and shown as mean ± s.e.m. (n = 4).

the binding curves of the association phase of clasped and unclasped α5β1 were almost superimposable in the presence of $Mn^{2+}$ (Fig. 2*a*, right). This confirms the activating effect of this divalent cation[12] and suggests that binding of $Mn^{2+}$ to α5β1 overcomes structural constraints that maintain the low affinity state. Thus, the clasped α5β1 has the full potential to bind ligand, and the disturbance in the overall structure of clasped α5β1 does not result in low ligand-binding activity in $Ca^{2+}$ and $Mg^{2+}$ compared to the released version.

To estimate steady state affinity for ligand, an ELISA-like solid-phase binding assay was employed. This assay was employed because the binding kinetics of α5β1 to Fn9–10 obtained by sensorgrams, either with or without C-terminal release, did not fit a simple Langmuir binding model and are likely to fit a more complicated model (J.T. and T.A.S., unpublished observations). Clasped or unclasped α5β1 was captured onto microtiter wells using a polyclonal antibody to the coiled coil; binding to biotin-labeled Fn9–10 was measured in the presence of 1 mM $Ca^{2+}$ and $Mg^{2+}$ after 1 h. Equilibrium binding revealed a five-fold increase in the apparent affinity of α5β1 after TEV treatment (Fig. 2*c*). This confirmed that activation after release of the C-terminal constraint can be observed regardless of which binding partner is in the immobilized phase.

## Opening of the stalk region

Electron micrographs examined the effects of release of the C-terminal clasp on the overall shape of the α5β1 heterodimer. The rotary-shadowed image of clasped α5β1 is ring-like, with a globular head of ~12 × 8 nm having two thin stalks of ~16 nm connected at their ends (Fig. 3, upper panel). By contrast, unclasped — that is, TEV-treated — α5β1 has a globular head with two tails that are widely separated at their ends (Fig. 3, lower panel). The mean distance of their separation was 14.1 ± 4.7 nm. In unclasped α5β1, the angle between the two stalks varied from ~0° (roughly parallel) to ~90°. Very few heterodimers exhibited a stalk angle of >90°, suggesting a conformational restriction exerted by the subunit interface in the headpiece.

Additional evidence for opening in the stalk region after cleavage of the clasp came from neoepitope exposure. The AG89 antibody to the β1 stalk region reacts with the resting α5β1 heterodimer but is hindered by the associating α5 subunit[14]. The α5 subunit modulates the amount of AG89 binding, but not its affinity[14]. When clasped α5β1 was partially cleaved and immunoprecipitated with an antibody to the β1 headpiece domain, TS2/16, the cleaved and uncleaved molecular species were recognized equally well (Fig. 4). In contrast, AG89 immunoprecipitated the cleaved form much more efficiently (~90%) than the uncleaved form (~30%), demonstrating the full exposure of the AG89 epitope in the β1 stalk after the removal of the clasp (Fig. 4).

## Integrin activation model

Receptor conformational change has recently been intensively studied in the field of transmembrane signaling. Ligand-induced conformational change is postulated to be a key step in signaling through G protein-coupled receptor[16], cytokine receptor dimers[17], guanylate-cyclase-coupled receptor dimers[18] and Fas–Fas ligand trimers[19]. In the bacterial aspartate receptor, a piston-like motion of one transmembrane helix relative to the other transmits signals from the extracellular domain to the cytoplasmic kinase cascade upon ligand binding[20]. This 'relay mechanism' is possible because the ligand binding domain is close to the membrane and is connected by a continuous α-helix to the cytoplasmic domain. In all of the above receptors, the ligand binding domain sits very close to the plasma membrane with stalks too short to be seen. By contrast, the integrin conformational signal must travel through long, 16 nm stalks. Because of the inherent flexibility of proteins and the long lengths of these stalks, small rotational or translational movements of integrin α- and β-chain transmembrane/cytoplasmic regions may be difficult to propagate all the way to the headpiece at the top of the integrin molecule.

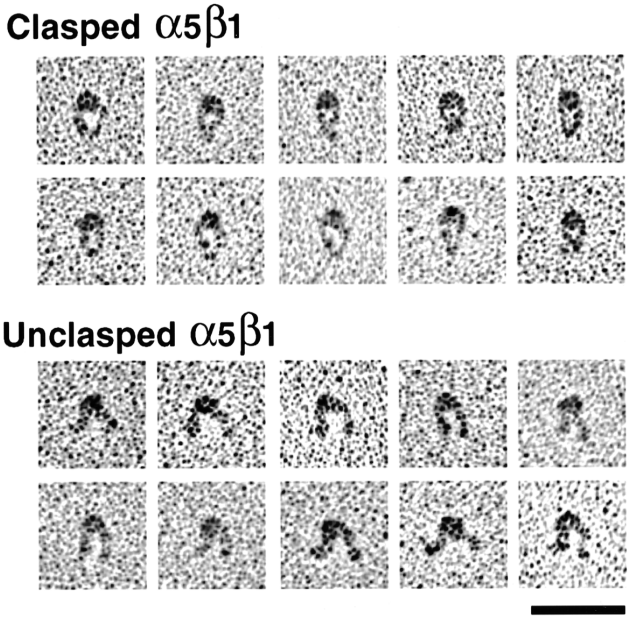


**Fig. 3** Electron micrographs of rotary-shadowed α5β1 showing opening of α5 and β1 C-terminal stalks after TEV cleavage. A gallery of representative images of soluble, clasped α5β1 is shown before (upper panel) and after (lower panel) release of the C-terminal constraint. Bar = 50 nm.

Our findings show that unclasping of the integrin C-terminal juxtamembrane region activates ligand binding, and that a separation of the juxtamembrane segments of ~14 nm accompanies unclasping. This separation is large, compared both to the diameter of the headpiece (~12 nm), the length of the stalks (16 nm) and the thickness of the plasma membrane (5 nm). Our findings suggest that a transition of the α- and β-subunit transmembrane and cytoplasmic domains from an associated to an unassociated form, thus becoming widely separated in the membrane, could solve the problem of transmitting signals over the long distance from the membrane to the headpiece. One hypothesis for integrin activation is the scissor-like motion around the hinge region at the α–β interface in the cytoplasmic domain[9]. The data presented here generally agree with the role of intersubunit orientation in this model. However, our findings suggest a different model in which the hinge is in the N-terminal ligand binding region, rather than near the junction of the transmembrane and cytoplasmic domains as the other model proposed. Our data suggests that the cytoplasmic domains keep the stalks closed rather than act as a hinge. Headpiece separation between the α- and β-subunits has been observed for detergent-solublized αIIbβ3 (ref. 21) treated with the RGD peptide. However, the same integrin shows separation in the stalks and not in the headpiece when bound to the physiological macromolecular ligands fibrinogen and von Willebrand factor[22]. Also, ligand-mimetic mAbs recognize residues in both the αIIb and β3 head regions simultaneously[23], strongly arguing against head separation during ligand binding.

Our results with the clasped α5β1 heterodimer suggest that major movements are required for activation, because the insertion of the TEV cleavage site and flanking linkers should allow considerable flexibility at the C-terminal clasp even in the absence of cleavage (Fig. 1*a*). This linker would allow much greater movements than what are known to transmit activation of the bacterial aspartate receptor[20]. When a version of the clasped α5β1 that lacks the 13-residue cleavage site was expressed and compared with the cleavable clasped version in the absence of cleavage, we found no difference in ligand binding activity (data not shown). This implies that the stalks are flexible enough to absorb the extra insert without affecting ligand binding and that large movements are required for activation.

Activation by unclasping is consistent with evidence from a number of studies. First, disruption of an α–β interaction site in the cytoplasmic tail converts resting integrins into constitutively active integrins[7]. Second, many activating antibodies map to the stalk region of the β-subunit and are suggested to act like a 'wedge' that loosens association between the α- and β-subunits at the membrane-proximal region[4,24]. Third, specific, limited cleavage of αIIb on platelets at the membrane-proximal region of αIIb (Val 837) by neutrophil elastase converts αIIbβ3 from a resting to a ligand-binding form in the absence of signal transduction events[25]. Fourth, substitution of heterodimer-promoting α-helical coiled coils in the cytoplasmic domains of the integrin αL and β2 subunits maintains αLβ2 in a nonligand binding state[6]. Finally, Weisel *et al.*[22] found more separation between the stalks of αIIbβ3 when bound to fibrinogen than when unbound, which agrees with the association of the opening of the stalk region with ligand binding and/or activation.

In summary, we have shown for the first time that 'inside-out' activation of integrins can be reproduced in a purified, soluble receptor and have demonstrated that unclasping of the membrane-proximal region is sufficient for integrin activation. The increase in ligand binding affinity observed in physiological buffer, without addition of artificial agents, is accompanied by a major conformational change indirectly indicated by sedimentation and directly visualized by electron micrography. Recent preliminary experiments indicate that the release of a similar C-terminal clasp also activates αIIbβ3 (J.T. and T.A.S., unpublished results), suggesting that this mechanism can be generalized to other classes of integrins. Important questions remain to be addressed. These include: Does stalk-opening represent the major affinity modulation mechanism on the cell surface? What kind of local structural rearrangement in the ligand-binding site is induced by the long-range conformational change? What kind

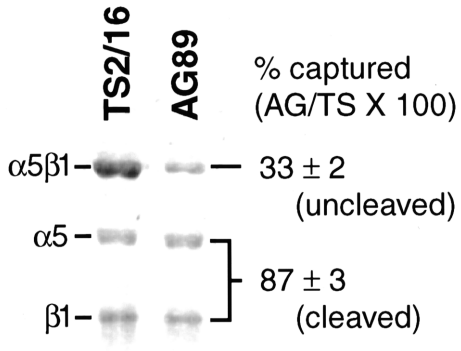


**Fig. 4** Differential exposure of a β1 stalk epitope in clasped and unclasped α5β1. Partially digested α5β1(~50% cleavage) was subjected to direct immunoprecipitation with TS2/16- or AG89-Sepharose. The amount of protein in each band was quantitated by NIH Image 1.62 software using a series of defined amounts of α5β1 protein bands run on the same gel as standards (not shown). Amounts of each molecular species (α5β1 band for uncleaved, α5 and β1 bands for cleaved) are compared between TS2/16- and AG89-immunoprecipitates and expressed as a ratio (mean ± SEM, n = 3).

of cellular machinery affects the separation of the cytoplasmic tails of the α- and β-subunits? Understanding the activation mechanism of integrins will provide insights into the rapid modulation of cell adhesion that is vital to a wide range of cellular processes, and could allow development of therapeutics that control cell adhesion, aggregation and migration in metastatic, thrombotic and inflammatory diseases.

## Methods

**Preparation of soluble integrin α5β1.** Soluble, clasped α5 (α5-AHCys) and β1 (β1-tev-BHCys) constructs were prepared from wild type human α5 and β1 cDNAs using overlap extension PCR. The C-terminal portions of the α5 and β1 extracellular sequences, a TEV cleavage site with flanking linker segments and ACID or BASE α-helical coiled-coils with Cys mutations were assembled (Fig. 1*a*) by extension PCR. The PCR fragments were digested with *Bam*HI and *Not*I (α5) or *Spe*I and *Not*I (β1) and cloned into the same enzyme sites of wild type constructs. The α5 and β1 inserts were transferred, respectively, into the *Kpn*I and *Not*I sites of pEF1/V5-HisA (Invitrogen) and *Bam*HI and *Not*I sites of pEF1-puro (J.T., unpublished) vectors. For transient expression, 293T cells were cotransfected with wild type α5 and β1 (wt α5β1) or soluble clasped mutants of α5 and β1(clasped α5β1) cDNAs or vector alone (mock) using calcium phosphate precipitates. Cells were metabolically labeled with [$^{35}$S] methionine and cysteine and culture supernatants (for clasped α5β1 and mock) or detergent cell lysates (for wt α5β1) were adjusted to the same volume and subjected to immunoprecipitation.

For stable expression, CHO lec 3.2.8.1 cells[26] were cotransfected with 10 μg each of α5–AHCys and β1–tev–BHCys plasmids by electroporation, plated onto 96-well plates and selected with medium containing 1 mg ml$^{-1}$ G418 (Gibco) and 10 μg ml$^{-1}$ puromycin (Sigma). Surviving clones were screened for expression of soluble α5β1 using a sandwich ELISA with KH72 as the capture antibody and biotinylated TS2/16 as the probe antibody. The clone with the highest secretion level was cultured in roller bottles and culture supernatants were harvested every week. The supernatants were passed through TS2/16-Sepharose and bound protein was eluted with 50 mM triethylamine, pH 11.5, containing 1 mM $CaCl_2$ followed by neutralization with 1 M Tris, pH 7.0. Eluted material was concentrated with Centriprep-30 (Amicon) to ~1 ml, and immediately subjected to gel filtration on a Superdex 200 HR column (1.6 × 60 cm, Pharmacia) equilibrated with 50 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.5. The peak fraction was concentrated by Centriprep-30 and stored at –80 °C. To obtain unclasped α5β1, purified clasped α5β1 was treated with recombinant TEV protease (Gibco) at 4 or 25 °C depending on the experiment, followed by inactivation of protease by addition of 2.5 mM iodoacetamide.

**Ligand binding assay.** A segment corresponding to fibronectin type III domains 9 and 10 (1,326–1,509) of human fibronectin, with one cysteine residue added after residue 1509, was prepared from the Fn7–10 construct[27], cloned into pET11c vector (Novagen) and expressed in *E. coli*. An inactive version of the fragment which lacks the critical RGDS sequence[28] was also prepared. Proteins were purified from bacterial lysates by anion-exchange chromatography on a HiTrap Q column (Pharmacia) and biotinylated *via* the sulfhydryl group of the cysteine with PEO-maleimide activated biotin (PIERCE) according to the manufacturer's recommendation. Biotinylated fragments were purified by MonoQ chromatography and shown to be free from unmodified protein. Biotinylated Fn9–10 was directly captured on streptavidin-conjugated Sensor Chip SA (BIAcore) with an average density of 500 RU. This surface was quite stable and maintained the same integrin-binding capacity over repeated binding/regeneration cycles. α5β1 integrin heterodimers were used as analytes at a flow rate of 20 μl min$^{-1}$ and bound material was completely stripped-off by regeneration of the surface with 50 mM NaOH containing 20 mM EDTA.

The solid phase binding assay was performed as follows. Purified soluble α5β1 (0.5 pmol) was incubated with or without 75 U ml$^{-1}$ TEV for 3 h and added to microtiter wells for capture with immobilized polyclonal antibody against ACID/BASE peptide[29]. Varying concentrations of biotinylated Fn9–10 were incubated in the presence of 1 mM $Ca^{2+}$ and $Mg^{2+}$ for 1 h at room temperature. After washing, bound Fn9–10 was chromogenically detected by peroxidase–streptavidin conjugate and substrate. To minimize dissociation, all wash and secondary reagent buffers contained 1 mM $Mn^{2+}$, and <15 min elapsed between the end of binding and beginning of color development. Steady-state dissociation constants ($K_D$) were calculated by nonlinear regression analysis of A415 *versus* Fn9–10 concentration by BIAevaluation software 3.0 (BIAcore).

**Electron microscopy.** Prior to electron micrography, integrin samples were sedimented through a 15–40% (v/v) glycerol gradient in 0.2 M ammonium acetate, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM Tris, pH 7.5. The clasped and unclasped forms of α5β1 sedimented at 8.3 S and 9.1 S, respectively. Peak fractions were sprayed onto mica and rotary shadowed[30]. Specimens were examined in a Phillips 301 electron microscope and photographed at 50,000×.

**Acknowledgments**

We thank E. Rouslahti, Y. Takada, E.L. Reinherz, K. Miyake and P.S. Kim for providing cDNAs, antibodies, and peptides; and C.V. Carman for helpful discussions. This work was supported by a grant from National Institute of Health.

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Received 1 December, 2000; accepted 5 March, 2001.

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