

# The Role of the Specificity-Determining Loop of the Integrin $\beta$ Subunit I-like Domain in Autonomous Expression, Association with the $\alpha$ Subunit, and Ligand Binding<sup>†</sup>

Junichi Takagi,<sup>\*,‡</sup> Daniel P. DeBottis,<sup>‡</sup> Harold P. Erickson,<sup>§</sup> and Timothy A. Springer<sup>‡</sup>

*The Center for Blood Research and Department of Pathology, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115, and Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710*

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**ABSTRACT:** Integrin  $\beta$  subunits contain a highly conserved I-like domain that is known to be important for ligand binding. Unlike integrin I domains, the I-like domain requires integrin  $\alpha$  and  $\beta$  subunit association for optimal folding. Pactolus is a novel gene product that is highly homologous to integrin  $\beta$  subunits but lacks associating  $\alpha$  subunits [Chen, Y., Garrison, S., Weis, J. J., and Weis, J. H. (1998) *J. Biol. Chem.* 273, 8711–8718] and a  $\sim 30$  amino acid segment corresponding to the specificity-determining loop (SDL) in the I-like domain. We find that the SDL is responsible for the defects in integrin  $\beta$  subunit expression and folding in the absence of  $\alpha$  subunits. When transfected in the absence of  $\alpha$  subunits into cells, extracellular domains of mutant  $\beta$  subunits lacking SDL, but not wild-type  $\beta$  subunits, were well secreted and contained immunoreactive I-like domains. The purified recombinant soluble  $\beta 1$  subunit with the SDL deletion showed an elongated shape in electron microscopy, consistent with its structure in  $\alpha\beta$  complexes. The SDL segment is not required for formation of  $\alpha 5\beta 1$ ,  $\alpha 4\beta 1$ ,  $\alpha V\beta 3$ , and  $\alpha 6\beta 4$  heterodimers, but is essential for formation of  $\alpha 6\beta 1$ ,  $\alpha V\beta 1$ , and  $\alpha L\beta 2$  heterodimers, suggesting that usage of subunit interface residues is variable among integrins. The  $\beta 1$  SDL is required for ligand binding and for the formation of the epitope for the  $\alpha 5$  monoclonal antibody 16 that maps to loop segments connecting blades 2 and 3 of  $\beta$ -propeller domain of  $\alpha 5$ , but is not essential for nearby  $\beta$ -propeller epitopes.

Integrins are ubiquitous cell adhesion receptors and bind ligands on the surface of other cells and in the extracellular matrix, connect the extracellular environment to the actin and keratin cytoskeletons, regulate cell migration and growth, and communicate signals bi-directionally across the plasma membrane (1). Integrins contain 2 noncovalently associated glycoprotein subunits with extracellular domains of  $>940$  ( $\alpha$ ) and  $>640$  ( $\beta$ ) residues. The N-terminal halves of both subunits comprise a ligand binding “head” region, which is connected to the cell membrane by “stalks” provided by the C-terminal halves of both subunits (2, 3). The headpiece contains a  $\beta$ -propeller domain in the  $\alpha$  subunit and an I-like domain in the  $\beta$  subunit. These two domains are correctly folded only when they are associated (4, 5). The basis for this is revealed in the crystal structure of the extracellular domain of integrin  $\alpha V\beta 3$  (3). The most extensive interaction present between the  $\alpha$  and  $\beta$  subunits is in the headpiece, between the  $\beta$ -propeller domain and the I-like domain.

The I-like domain is structurally homologous to the integrin I or A domain, which is present in a subset of  $\alpha$  subunits (6). Both domains have a metal-ion-dependent

adhesion site (MIDAS),<sup>1</sup> containing a DXSXS motif. In the I-like domain, there is an additional metal binding site called ADMIDAS, that is suggested to be involved in the regulation of ligand binding (3). The I domain is inserted between blades 2 and 3 of the  $\beta$ -propeller domain of the  $\alpha$  chain, and plays an important role in ligand binding in integrins in which it is present. The I domain can be expressed autonomously of other integrin domains. In contrast to the I domain, production of recombinant, isolated I-like domain has been difficult. One report claimed that the bacterially expressed  $\beta 1$  I-like domain can be refolded into a native structure; however, that report lacked assessment of structural integrity such as monoclonal antibody (mAb) binding (7), and we have been unable to reproduce this work and find that the reported I-like domain constructs cannot be refolded with retention of immunological activity or solubility.

Pactolus is a novel protein highly homologous ( $\sim 60\%$  identical) to the integrin  $\beta 2$  subunit (8). However, Pactolus is unlikely to be a novel integrin  $\beta$  chain because it is secreted, it lacks a critical Asp residue in the MIDAS motif that is conserved in all integrin  $\beta$  subunits and is thought to be involved in ligand binding, and associating  $\alpha$  subunits cannot be detected (8, 9). Interestingly, Pactolus lacks a short,  $\sim 30$ -residue segment between  $\beta$  strands 2 and 3 in the  $\beta$

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<sup>\*</sup> To whom correspondence should be addressed. Phone: (617) 278-3205, Fax: (617) 278-3232, E-mail: takagi@cbr.med.harvard.edu.

<sup>‡</sup> Harvard Medical School.

<sup>§</sup> Duke University Medical Center.

<sup>1</sup> Abbreviations: mAb, monoclonal antibody; MIDAS, metal-ion-dependent adhesion site; SDL, specificity-determining loop; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

subunit I-like domain, which also is missing in the  $\alpha$  subunit I domain (8, 10, 11). Moreover, this segment contains a small disulfide-bonded loop between cysteine residues 187 and 193 in  $\beta 1$  that is critical for determining ligand binding specificity, and hence predicted to be located in the ligand binding site close to the  $\alpha$ - $\beta$  interface (12). The fact that Pactolus is expressed as a monomer suggested to us that removal from I-like domains of the "specificity-determining loop", to mimic the deletion in Pactolus, might enable autonomous folding as seen with integrin I domains. To examine the function of this segment, we have examined the consequences of Pactolus-like deletions in integrin  $\beta$  subunits on expression, folding, association with  $\alpha$  subunits, and ligand binding.

## EXPERIMENTAL PROCEDURES

**Monoclonal Antibodies.** The murine anti-human  $\beta 1$  mAbs TS2/16 (13), AG89 (14),  $\beta 2$  mAb TS1/18 (15),  $\alpha L$  mAbs TS2/4, CBR LFA-1/1 (16), and rabbit anti-"velcro" (ACID/BASE coiled-coil) polyclonal antiserum (17) were previously described. Anti-coiled-coil mAb 2H11 (18),  $\beta 1$  mAb SG19 (19), and  $\beta 2$  mAbs KIM127 (20) and KIM185 (21) were gifts from Drs. E. L. Reinherz, K. Miyake, and M. Robinson, respectively. Rat anti-human  $\beta 2$  mAb YFC118.3 (22) was a gift from Dr. G. Hale. Murine anti- $\alpha 5$  mAbs P1D6, JBS5,  $\beta 1$  mAb 12G10,  $\beta 4$  mAb 3E1, and rat anti- $\alpha 6$  mAb GoH3 were purchased from Chemicon International Inc. (Temecula, CA). The hybridoma cell line for murine anti- $\beta 3$  mAb AP3 was obtained from American Type Culture Collection. All other mAbs were obtained from the Fifth International Leukocyte Workshop (23).

**DNA Construction for  $\beta$  Subunits.** The design for all constructs was essentially the same as for the soluble  $\beta 1$  reported previously ( $\beta 1$ -tev-BHCys) (17). A C-terminal segment coding for BASE-p1 peptide (24) with one residue mutated to Cys was PCR-amplified from  $\beta 1$ -tev-BHCys and fused to the C-termini of the extracellular domains of wild-type human  $\beta 2$  [1-678, (25)],  $\beta 3$  [1-692, (26)], and  $\beta 4$  [1-683, (27)]. The fusions were inserted in pEF1-puro (17). For  $\Delta$ SDL versions, SDL segments (172-198 in  $\beta 1$ , 154-181 in  $\beta 2$ , 160-188 in  $\beta 3$ , and 151-174 in  $\beta 4$ ) were eliminated by overlap extension PCR using primers that contained at least a 19 bp overlap at the region to be deleted. For large-scale expression,  $\beta 1\Delta$ SDL (residues 1-171 and 199-708) was also fused to a hexahistidine tag using the AgeI site of the pEF1/V5-HisA vector (Invitrogen) with an intervening short linker sequence (Ala-Thr-Gly).

**DNA Construction for  $\alpha$  Subunits.** Again the design for all  $\alpha$  subunit constructs was essentially the same as for the soluble  $\alpha 5$  reported previously ( $\alpha 5$ -AHCys) (17). A C-terminal segment coding for ACID-p1 peptide (24) with one mutation to Cys was PCR-amplified from  $\alpha 5$ -AHCys and fused to the C-termini of the extracellular domains of wild-type human  $\alpha 4$  [1-942, (28)],  $\alpha 6$  [1-988, (27)],  $\alpha v$  [1-960, (29)], and  $\alpha L$  [1-1063, (25)]. The fusions were inserted in the pEF1/V5-HisA vector (Invitrogen). All segments amplified by PCR were confirmed by DNA sequencing.

**Transfection and Immunoprecipitation.** DNAs (2  $\mu$ g/subunit/well) were transiently transfected into 293T cells using calcium phosphate precipitates (30). Metabolic labeling and immunoprecipitation were as follows. Transfected cells

in 6-well plates ( $\sim 80\%$  confluency after 24 h of transfection) were incubated with 0.3 mCi of [ $^{35}$ S]methionine and cysteine (NEN Life Science Products, Inc., Boston, MA) in 1.5 mL of labeling medium (methionine- and cysteine-free RPMI 1640 containing 10% dialyzed FCS) for 1 h and chased by adding the same volume of labeling medium containing 500  $\mu$ g/mL cysteine and 100  $\mu$ g/mL methionine for an additional 16 h. The culture supernatants were harvested and centrifuged to remove cell debris, and subjected to immunoprecipitation using integrin mAbs and Protein G agarose. Materials eluted from the beads in SDS sample buffer were resolved on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by fluorography.

**Production of Soluble Recombinant Monomeric  $\beta 1$  Mutant.** DNA (10  $\mu$ g,  $\beta 1\Delta$ SDL with a C-terminal hexahistidine tag) was transfected into CHO Lec 3.2.8.1 cells (31) and selected against 1 mg/mL Geneticin G418 (GIBCO). Stable clones were screened for secretion of mutant  $\beta 1$  by sandwich ELISA using AG89 as a capturing antibody and biotinylated TS2/16 as a detection antibody. A clone with the highest expression was cultured in roller bottles, and  $\sim 4$  L of culture supernatant was collected. Soluble monomeric mutant  $\beta 1$  ( $\beta 1\Delta$ SDL-His) was purified from the culture supernatant by Ni-NTA agarose (QIAGEN) followed by gel filtration on a Superdex 200 column (1.6  $\times$  60 cm) (Pharmacia) equilibrated with 50 mM Tris, 150 mM NaCl, pH 7.5 (TBS). Analytical gel filtration was performed on a Superdex 200 HR column (1  $\times$  30 cm) equilibrated with TBS at a flow rate of 0.5 mL/min. The purified sample was sedimented through a 15-40% (v/v) glycerol gradient in 0.2 M ammonium acetate, 1 mM Tris, pH 7.5, and the position of  $\beta 1\Delta$ SDL-His was determined by running each fraction on SDS-PAGE. The sedimentation coefficient was determined using calibrated standard proteins with known  $s$  values (32) sedimented in a separate gradient. Rotary shadowing and electron microscopy were performed as described previously (33).

**Binding of  $\alpha 5\beta 1$  to Fibronectin.** A recombinant fibronectin fragment encompassing the seventh through tenth FNIII repeat of human fibronectin (Fn $_{7-10}$ ) was prepared as described previously (34) and coupled to CNBr-activated Sepharose (Pharmacia) at 1.2 mg/mL of gel.  $^{35}$ S-labeled culture supernatants (100  $\mu$ L) from  $\alpha 5\beta 1$  or  $\alpha 5\beta 1\Delta$ SDL transfectants were added with 1 mM MnCl $_2$  and incubated with 25  $\mu$ L of Fn $_{7-10}$ -Sepharose beads for 2 h at 4  $^{\circ}$ C. The beads were washed 3 times with TBS, and the eluate with 10 mM EDTA was subjected to SDS-7.5% PAGE under nonreducing condition. Sepharose that was blocked with ethanolamine instead of Fn $_{7-10}$  was used as a control matrix.

## RESULTS

**Alignment of the Pactolus I-like Domain,  $\beta$  Integrin I-like Domains, and  $\alpha$  Integrin I Domains.** We aligned the I-like domains of Pactolus and integrin  $\beta$  subunits, and the I domains of integrin  $\alpha$  subunits by structure and sequence (Figure 1A). There are two inserts in the I-like domains that distinguish them from the I domains, and, importantly, these are in loops. Insert 1 is between  $\beta$  strands 2 and 3, and insert 2 is between  $\beta$  strand 4 and  $\alpha$  helix 5 (Figure 1). Each insertion is about 30 residues long. Insertion 1 corresponds to the specificity-determining loop (SDL) (11).

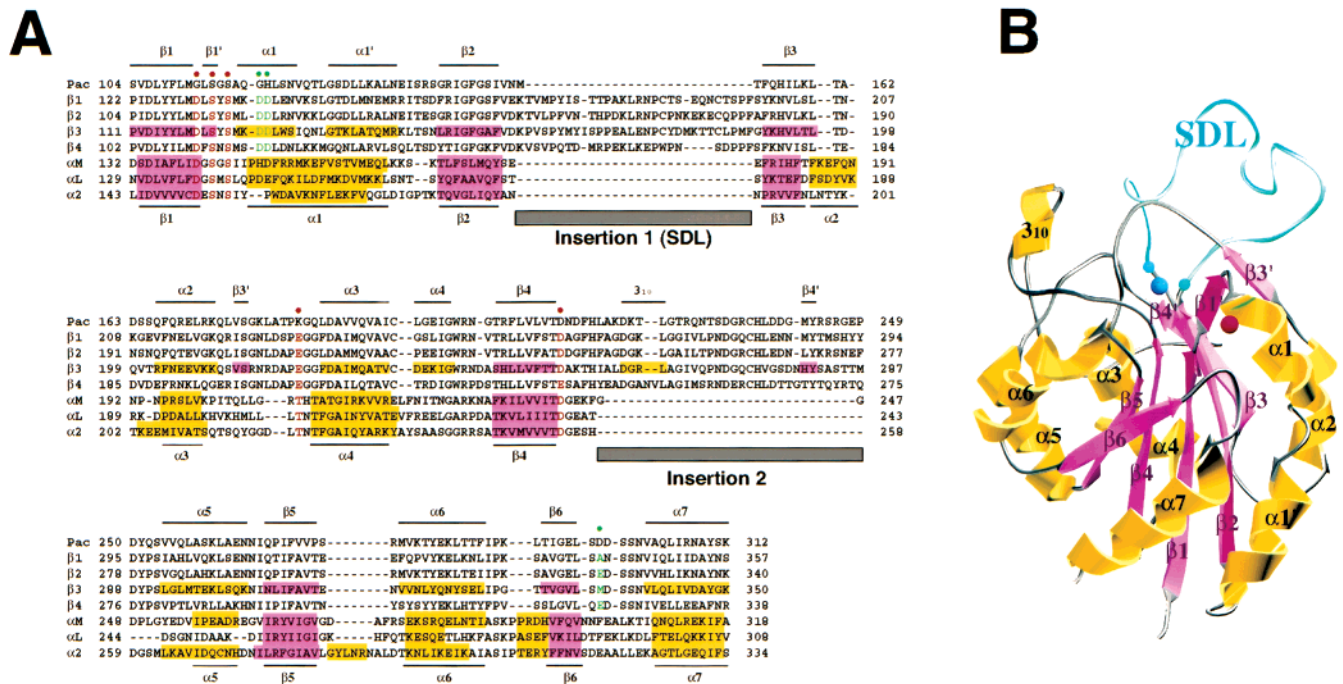


FIGURE 1: Structural features of integrin  $\beta$  subunit I-like domain. (A) Alignment of the I-like domains and Pactolus with I domains of integrin  $\alpha$  subunits. Structurally defined integrin  $\alpha$  subunit I-domains (1JLM for  $\alpha M$ , 1ZON for  $\alpha L$ , and 1AOX for  $\alpha 2$ ) and  $\beta 3$  I-like domain (1JV2) were superimposed with 3DMALIGN of MODELLER (52) to obtain a structure-based sequence alignment.  $\beta 1$ ,  $\beta 2$ ,  $\beta 4$ , and Pactolus I-like domain sequences are added to the alignment using their high homology to  $\beta 3$ .  $\beta$  strand and  $\alpha$  helix segments are highlighted in pink and yellow, respectively, as defined by crystal structures. Each segment is denoted according to the numbering used in  $\beta 3$  (above) or  $\alpha M$  (below). Residues shown in I domains or suggested in the I-like domain to coordinate the metal in the MIDAS are colored red; those that coordinate the  $Ca^{2+}$  in the ADMIDAS of the I-like domain are colored green, and are also denoted by dots above the alignment. Two regions that are present in I-like domains but not in  $\beta$  subunit I domains are denoted by gray bars at the bottom. (B) Structure of  $\beta 3$  integrin I-like domain. Secondary structure elements are color-coded as in (A) and labeled. SDL is shown in cyan with the location of truncation boundary residues (Lys159 and Gly189) as small balls. Also shown are  $Ca^{2+}$  bound to ADMIDAS (red sphere) and hypothetical  $Mg^{2+}$  bound to MIDAS (blue sphere) (3). The figure was prepared with SwissPdbViewer (53).

The Pactolus sequence completely lacks insertion 1, despite its very high overall sequence homology to integrin  $\beta$  subunits. In the structures of the I domains of  $\alpha M$ ,  $\alpha L$ , and  $\alpha 2$  integrin subunits, the loop between  $\beta$  strands 2 and 3 is only three residues long and makes a tight turn (6, 35, 36). Pactolus contains four residues in this loop, indicating a local structure around this position similar to the  $\alpha$  subunit I domain. In contrast, the SDL loop in this position in integrin  $\beta$  subunit I-like domains is 23–30 residues.

**Effect of SDL Deletion on the Expression of Soluble  $\beta$  Subunit Monomer.** The ability of Pactolus to fold on its own led us to speculate that the presence of the SDL segment in the  $\beta$  subunit might prevent it from being properly expressed in the absence of an  $\alpha$  subunit. Therefore, the SDL segment exactly corresponding to that missing in Pactolus was deleted from the  $\beta 1$ ,  $\beta 2$ ,  $\beta 3$ , and  $\beta 4$  integrin subunits (Figure 1A,B). Furthermore, the transmembrane and cytoplasmic domains were deleted, and a soluble BASE-p1 helical peptide was fused to the C-terminus. This allowed immunoprecipitation with “velcro” antiserum independently of appropriate folding of the  $\beta$  subunit. Material secreted by 293T cell transfectants was compared using anti-velcro polyclonal antibody (Figure 2). The secretion of wild-type  $\beta 1$  and  $\beta 2$  monomers was barely detectable, but upon SDL deletion, secretion was dramatically increased by 12-fold for  $\beta 1$  (Figure 2A, lanes 7 and 14) and by 25-fold for  $\beta 2$  (Figure 2B, lanes 4 and 8). In the case of  $\beta 3$  and  $\beta 4$ , the secretion of wild-type monomer was completely undetectable (Figure 2C, lane 6, and Figure 2D, lane 4), while the  $\Delta$ SDL mutants were produced

efficiently (Figure 2C, lane 12, and Figure 2D, lane 8). Immunoprecipitation from the cell lysate revealed that there was no accumulation of  $\beta$  subunit precursors within the cells (data not shown), suggesting that wild-type  $\beta$  subunits are susceptible to degradation, rather than blocked in transport along the secretion pathway. The expression levels of the  $\Delta$ SDL mutants were high and were comparable to those obtained when appropriate wild-type  $\alpha$  and  $\beta$  subunits were cotransfected (data not shown). Mutant  $\beta$  subunits were recognized by mAbs to different domains, including the I-like domain. Thus,  $\beta 1\Delta$ SDL was recognized by mAbs TS2/16, 13, and 12G10 to the I-like domain (Figure 2A, lanes 9–11), as well as mAbs to the stalk region (K20 and AG89, lanes 12 and 13) and to the N-terminal region (SG19, lane 8). One anti-I-like domain mAb (TS1/18, Figure 2B, lane 5) recognized  $\beta 2\Delta$ SDL. The amount of material precipitated by this I-like domain mAb is comparable to that precipitated by mAbs mapped more C-terminally (CLB LFA-1/1 to residues 332–339, and 6.7 to residues 344–432, respectively, Figure 2B, lanes 6 and 7). Although mAbs definitely mapped to the  $\beta 3$  and  $\beta 4$  subunit I-like domains were not available for these studies, the  $\beta 3$  mAb 23C6 and the  $\beta 4$  mAb UM-A9 both block ligand binding and thus are likely to bind to the I-like domain. These mAbs immunoprecipitated the  $\Delta$ SDL mutants of  $\beta 3$  (Figure 2C, lane 7) and  $\beta 4$  (Figure 2D, lane 5). Folding of portions of the I-like domain of wild-type  $\beta 2$  is dependent on association with  $\alpha$  subunit (4). For example, a mAb directed to combinatorial epitopes involving residues in the adjacent  $\alpha$ -1' and  $\alpha$ -7 helices (TS1/18) requires  $\alpha$

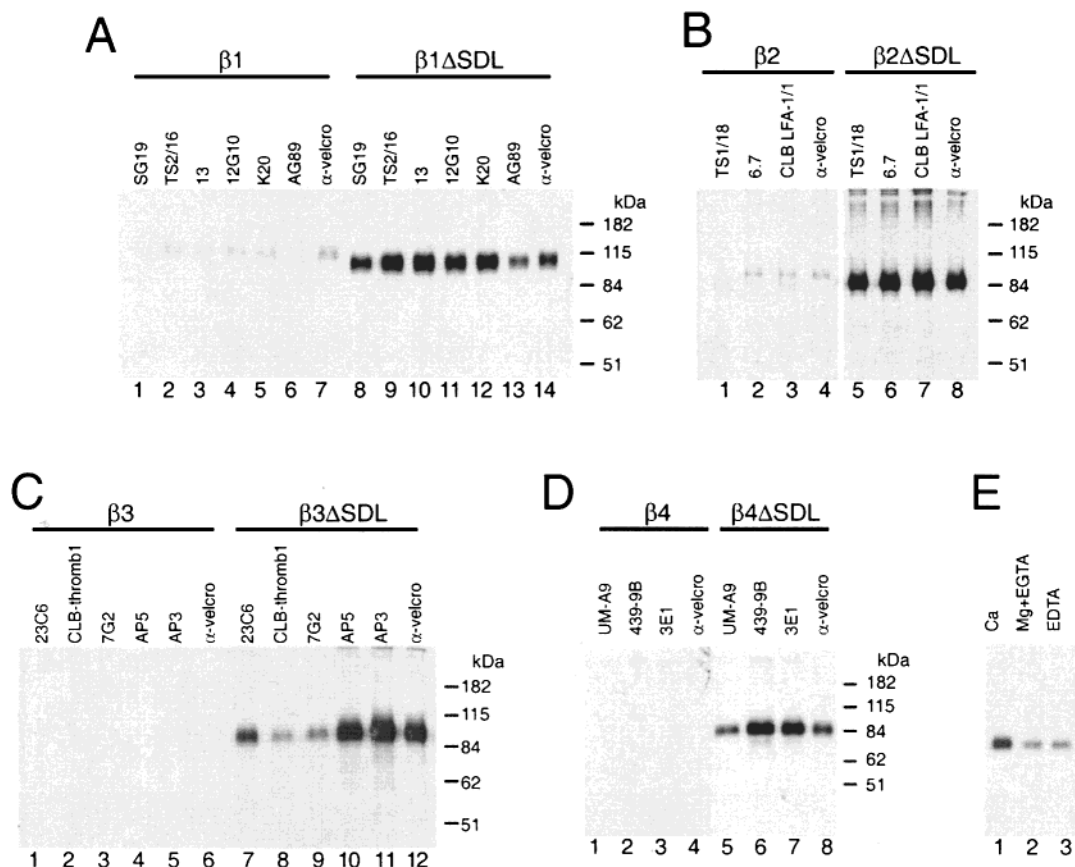


FIGURE 2: Effect of SDL deletion on the expression of monomeric  $\beta$  subunits. 293T cells were transiently transfected with cDNAs coding for wild-type or  $\Delta S D L$  mutants of  $\beta 1$  (A),  $\beta 2$  (B, E),  $\beta 3$  (C), and  $\beta 4$  (D). After metabolic labeling with [<sup>35</sup>S]cysteine and -methionine, culture supernatants were immunoprecipitated with the indicated mAbs to  $\beta$  subunits or anti-“velcro” antiserum to the C-terminal tag (17) and subjected to SDS–10% PAGE followed by fluorography. Positions of molecular weight markers are shown on the right. (E)  $\beta 2\Delta S D L$  was immunoprecipitated with anti- $\beta 2$  I-like domain mAb TS1/18 in the presence of 2 mM Ca<sup>2+</sup> (lane 1), 2 mM Mg<sup>2+</sup> + 1 mM EGTA (lane 2), or 3 mM EDTA (lane 3).

subunit association for reactivity, whereas a mAb to the  $\alpha$ -7 helix alone (CLB LFA-1/1) does not (4). Although wild-type  $\beta 2$  was not well expressed, mAb CLB LFA-1/1 and 6.7 to the C-terminal region were as reactive as anti-velcro (Figure 2B, lanes 2 and 3) to this minimally expressed material, while mAb TS1/18 to the I-like domain  $\alpha$ -1' and  $\alpha$ -7 helices was unreactive (Figure 2B, lane 1). By contrast, the  $\beta 2\Delta S D L$  mutant reacted equally well with TS1/18, 6.7, CLB LFA-1/1, and anti-velcro (Figure 2B, lanes 5–8). Therefore, SDL deletion enables folding of the TS1/18 epitope in the absence of  $\alpha$  subunit association.

Binding of TS1/18 mAb to  $\alpha L\beta 2$  is enhanced by Ca<sup>2+</sup>, leading to the suggestion of a Ca<sup>2+</sup> binding site in the I-like domain (37) as recently confirmed by the presence of a Ca<sup>2+</sup> bound to the ADMIDAS (3). Remarkably, maximum reactivity of  $\beta 2\Delta S D L$  with TS1/18 was only observed when Ca<sup>2+</sup> was present, while in 2 mM Mg<sup>2+</sup>/1 mM EGTA or 3 mM EDTA binding was reduced by 67 or 64%, respectively (Figure 2E, lanes 2 and 3). This strongly suggests that the Ca<sup>2+</sup> binding at the ADMIDAS (3) is intact in  $\beta 2\Delta S D L$ , again showing the I-like domain can assume a natively like structure when the SDL is deleted.

*Production of Soluble Monomeric  $\beta 1$  Integrin with the SDL Deletion.* To extend these studies, large quantities of recombinant soluble  $\beta 1\Delta S D L$  were produced in CHO Lec 3.2.8.1 cells. The extracellular domain of  $\beta 1$  with the  $\Delta S D L$  mutation was fused to a C-terminal hexahistidine tag, and

protein was purified from culture supernatant using Ni-chelate chromatography. SDS–PAGE of purified protein showed single bands of 86 and 87 kDa under nonreducing and reducing conditions, respectively (Figure 3A). Analytical gel filtration on Superdex 200 showed a single symmetrical peak at an elution position of  $K_{av} = 0.22$  (Figure 3B). This corresponded to a Stoke's radius ( $R_s$ ) of 4.75 nm (Figure 3B, inset). Analytical ultracentrifugation in a glycerol gradient gave a sedimentation coefficient of 5.08 S. Combining these numbers by the Siegel–Monte approach gives a molecular weight of 94 000, which is close to the calculated molecular weight of  $\beta 1\Delta S D L$  of 93 540 (78 734 for the protein portion + 14 806 for 11 Man<sub>5</sub>GlcNAc<sub>2</sub> sugar chains). A high frictional coefficient ( $f/f_0 = R_s/R_{min} = 4.75/3.04 = 1.56$ ) shows that  $\beta 1\Delta S D L$  has an elongated shape.

The overall molecular shape of  $\beta 1\Delta S D L$  was further examined by electron microscopy (Figure 3C). Rotary-shadowed images of  $\beta 1\Delta S D L$  show rods with a bend or expansion at one end. They measure about 17 nm in length, or about 21 nm when followed along the contour around the bend. This shape is consistent with the high frictional coefficient and, more importantly, corresponds very well to half of the structure of the intact  $\alpha 5\beta 1$  heterodimer (17, 38). Thus, all immunochemical, physicochemical, and morphological analyses on  $\beta 1\Delta S D L$  strongly suggest that it maintains structural integrity despite the lack of association with the  $\alpha$  subunit at the head region.

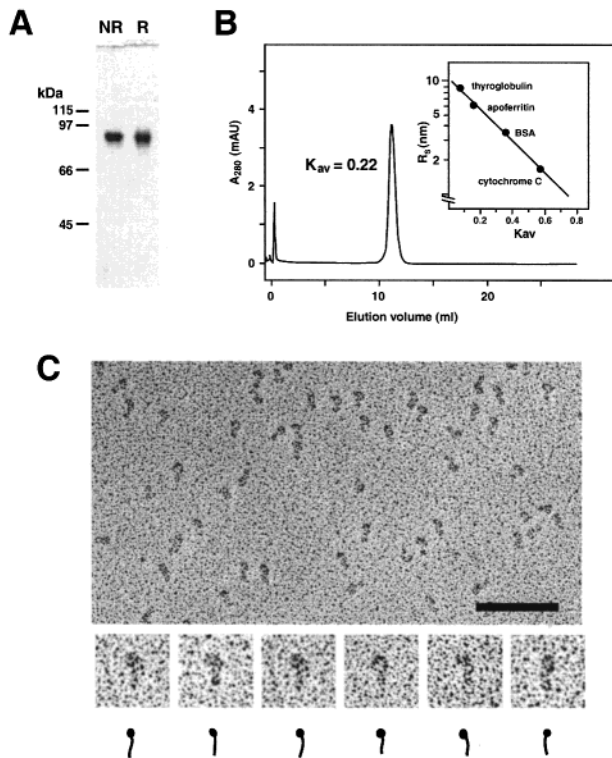


FIGURE 3: Characterization of purified soluble  $\beta 1\Delta\text{SDL}$ . (A) Purified  $\beta 1\Delta\text{SDL}$ -His ( $0.8 \mu\text{g}$  per lane) was subjected to SDS–10% PAGE under reducing (R) or nonreducing (NR) conditions and Coomassie Blue staining. (B) Analytical gel filtration. Purified  $\beta 1\Delta\text{SDL}$ -His ( $30 \mu\text{g}$ ) was analyzed on a Superdex 200 HR 10/30 column ( $V_t = 24 \text{ mL}$ ). The Stoke's radius was calculated from the elution positions of standard proteins with known  $R_s$  values (inset).  $R_s$  values were 8.5 nm for thyroglobulin, 6.1 nm for apoferritin, 3.55 nm for bovine serum albumin, and 1.70 nm for cytochrome *c*. (C) Electron micrographs. A field of rotary-shadowed  $\beta 1\Delta\text{SDL}$ -His (bar = 100 nm) and selected examples of the particles with interpretive drawings of the micrographs are shown.

**Effect of SDL Deletion on Heterodimer Formation and Ligand Binding.** To explore the role of the SDL in heterodimer formation, mutant  $\beta 1$  was coexpressed with the  $\alpha 5$  subunit. The extracellular domain of wild-type  $\alpha 5$  was fused with the ACID-p1 peptide, a short acidic  $\alpha$ -helix that forms a coiled-coil in association with the BASE-p1 peptide of the  $\beta 1$  subunit (24). The pairing of subunits was further stabilized by a disulfide formed by cysteines introduced at the beginning of each  $\alpha$  helix. In cotransfected 293T cells, soluble  $\alpha 5\beta 1$  heterodimer was formed and secreted into medium, and was readily immunoprecipitated with anti-ACID/BASE mAb 2H11 (Figure 4A, lane 1). This heterodimer appears as a single band in nonreducing SDS–PAGE because of the intersubunit disulfide in the coiled-coil (compare reducing SDS–PAGE in Figure 4B below). Secretion of  $\alpha 5$  or  $\beta 1$  monomers was not detectable, as shown by the lack of any extra bands even when anti-velcro antiserum was used (data not shown). Wild-type  $\alpha 5\beta 1$  was recognized by all anti- $\alpha 5$  and  $\beta 1$  mAbs tested (Figure 4B below and data not shown), and specifically bound to  $\text{Fn}_{7-10}$  in the presence of  $\text{Mn}^{2+}$  (Figure 4A, lane 3), indicating an active, native structure. Remarkably, the SDL deletion from  $\beta 1$  did not affect heterodimer formation, because a comparable amount of soluble  $\alpha 5\beta 1\Delta\text{SDL}$  was secreted and immunoprecipitated by mAb 2H11 (Figure 4A, lane 4). This dimerization was specific and dependent on the extracellular

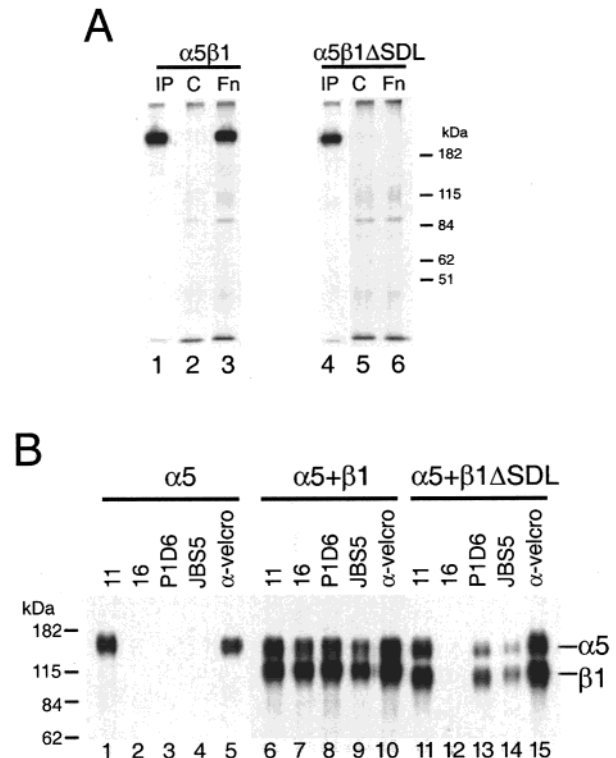
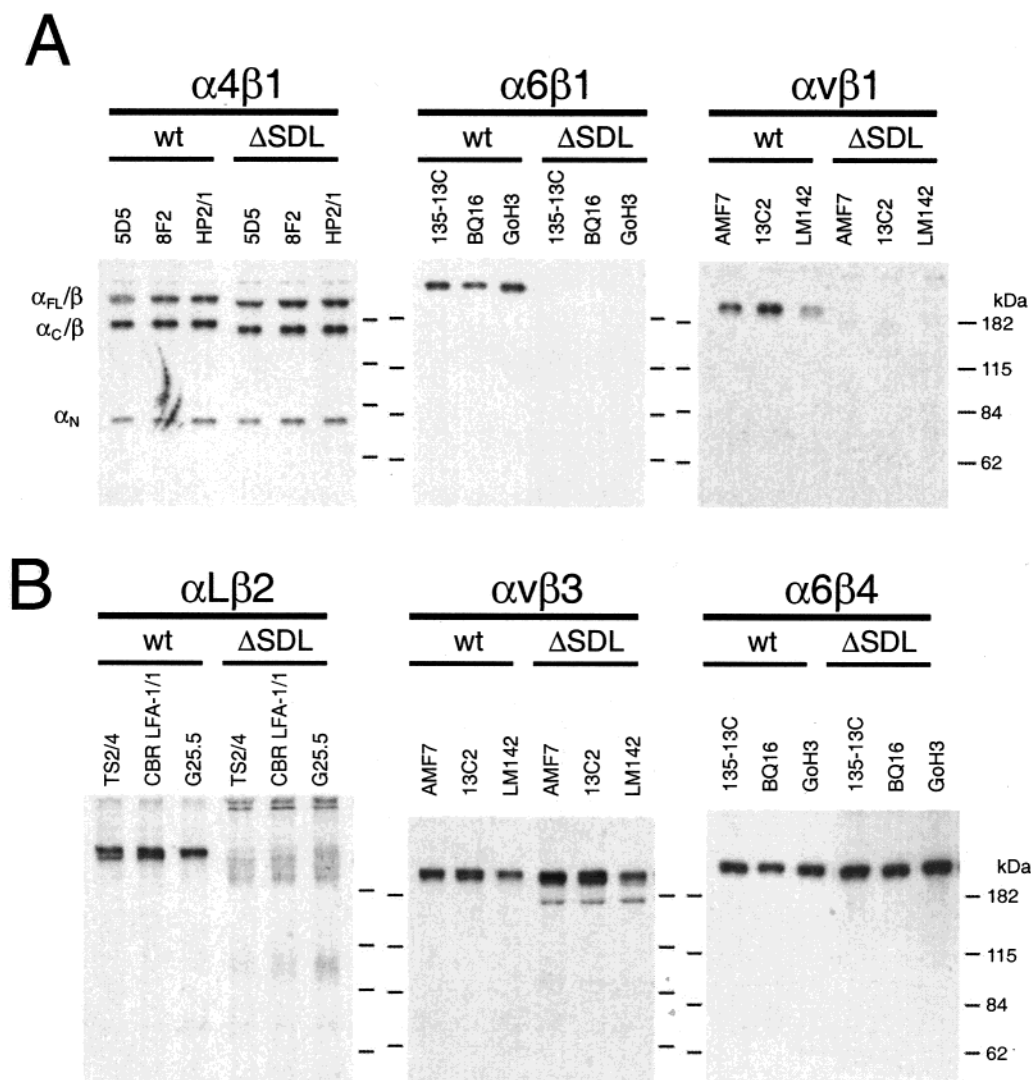


FIGURE 4: Pairing of  $\beta 1\Delta\text{SDL}$  with  $\alpha 5$  subunit. (A) Heterodimer formation and ligand binding activity. Wild-type and  $\Delta\text{SDL}$   $\beta 1$  extracellular domains were cotransfected with the  $\alpha 5$  extracellular domain as described under Experimental Procedures. Secreted materials from  $^{35}\text{S}$ -labeled cells were subjected to immunoprecipitation with anti-ACID/BASE coiled-coil mAb 2H11 (IP) or mixed with  $\text{Fn}_{7-10}$ -coupled Sepharose (Fn) or control Sepharose (C) in the presence of 1 mM  $\text{MnCl}_2$  followed by elution with EDTA. Amounts equivalent to  $100 \mu\text{L}$  of the secreted material from each experiment were subjected to nonreducing SDS–7.5% PAGE and fluorography. (B) Contribution of the  $\beta 1$  SDL segment to the folding of the  $\alpha 5$   $\beta$ -propeller domain. 293T cells were transfected with extracellular domains of  $\alpha 5$  alone,  $\alpha 5$  plus wild-type  $\beta 1$ , or  $\alpha 5$  plus  $\beta 1\Delta\text{SDL}$ .  $^{35}\text{S}$ -labeled culture supernatants were subjected to immunoprecipitation with mAbs to the  $\alpha 5$   $\beta$ -propeller domain. Immunoprecipitates were subjected to reducing SDS–7.5% PAGE and fluorography. Positions for the  $\alpha 5$  and  $\beta 1$  chains are shown on the right.

domains of both subunits, because incompatible combinations of  $\alpha$  and  $\beta$  (i.e.,  $\alpha\text{IIb} + \beta 1$ ,  $\alpha 5 + \beta 4$ , etc.; data not shown) did not form heterodimers even though they contained the same disulfide-forming ACID/BASE coiled-coil. These results show that the presence of SDL is not essential for  $\alpha 5$ – $\beta 1$  association; however, it is indispensable for the function of the heterodimer, because  $\alpha 5\beta 1\Delta\text{SDL}$  did not bind to fibronectin (Figure 4A, lane 6). This result is in agreement with the critical involvement of the SDL segment in ligand binding (12), and also strongly argues against the ability of the  $\alpha 5$  subunit to bind ligand independently of  $\beta 1$  (39).

Although SDL was not required for association of  $\alpha 5\beta 1$ , it may be important for other  $\alpha$ – $\beta$  pairs. Therefore, using similar constructs, the ability of  $\beta 1\Delta\text{SDL}$  to associate with other  $\alpha$  subunits was determined. Formation of folded heterodimers was assessed by immunoprecipitation with a panel of anti- $\alpha$  mAbs (Figure 5A). Some of the mAbs were mapped to the  $\beta$ -propeller domain (anti- $\alpha 4$  mAbs, 8F2 and HP2/1), and some are suggested to bind to the  $\beta$ -propeller because they are function-blocking (anti- $\alpha 6$  GoH3 and anti- $\alpha\text{V}$  AMF7 and 13C2). When cells were transfected with  $\alpha 4$ ,



**FIGURE 5:** Effect of SDL deletion on heterodimer formation. The extracellular domains of wild-type and  $\Delta$ SDL versions of  $\beta 1$  (A) or other  $\beta$  subunits (B) were cotransfected with the indicated  $\alpha$  subunit extracellular domains as described under Experimental Procedures. Culture supernatants from  $^{35}\text{S}$ -labeled transfectants were subjected to immunoprecipitation with  $\alpha$  subunit mAbs, nonreducing SDS-7.5% PAGE, and fluorography. Because of the intersubunit disulfide linkage introduced at the C-terminal coiled-coil peptide, intact heterodimers appeared as a single band of  $>200$  kDa. In the case of  $\alpha 4\beta 1$ , partial proteolytic processing of the  $\alpha 4$  subunit at Arg558 (54) resulted in a mixture of uncleaved, full-length  $\alpha 4\beta 1$  ( $\alpha_{FL}/\beta$ ,  $\sim 250$  kDa), the C-terminal  $\sim 70$  kDa fragment of  $\alpha 4$  disulfide linked to  $\beta 1$  ( $\alpha_C/\beta$ ,  $\sim 180$  kDa), and the N-terminal fragment of  $\alpha 4$  ( $\alpha_N$ , 80 kDa).

$\alpha 6$ , or  $\alpha V$  subunit alone, no immunoreactive materials were secreted into medium, even with anti-velcro antiserum (data not shown). Therefore, these  $\alpha$  subunits cannot be secreted from cells in the absence of appropriate  $\beta$  subunit even as a misfolded protein. Cotransfection of wild-type  $\beta 1$  with  $\alpha 4$ ,  $\alpha 6$ , or  $\alpha V$  subunits resulted in the secretion of disulfide-linked  $\alpha\beta$  heterodimers that could be immunoprecipitated by a panel of anti- $\alpha$  subunit mAbs (Figure 5A). As in the case of  $\alpha 5\beta 1$ , no  $\alpha$  or  $\beta$  monomer production was detected with anti-velcro (data not shown), indicating 100% efficiency in heterodimer formation. In contrast, when the  $\Delta$ SDL version of  $\beta 1$  was used, heterodimer formation was observed with  $\alpha 4$ , but not with  $\alpha 6$  or  $\alpha V$  (Figure 5A). Thus, the SDL segment in  $\beta 1$  is critical for  $\alpha V$ - $\beta 1$  as well as  $\alpha 6$ - $\beta 1$  interaction, but other region(s) in  $\beta 1$  can support association with  $\alpha 4$  and  $\alpha 5$ .

The differential involvement of SDL in  $\alpha$ - $\beta$  association was further demonstrated with other combinations of  $\alpha$ - $\beta$  pairs (Figure 5B). Soluble heterodimers of  $\alpha L\beta 2$ ,  $\alpha V\beta 3$ , and

$\alpha 6\beta 4$  were produced and secreted upon cotransfection with the corresponding  $\alpha$  and  $\beta$  subunits. Deletion of SDL impaired formation of the  $\alpha L\beta 2$  heterodimer, as judged by the lack of immunoprecipitation by three mAbs directed against the  $\beta$ -propeller domain of  $\alpha L$  (Figure 5B, left panel) and by anti-velcro antiserum (data not shown). In contrast,  $\alpha V$  and  $\alpha 6$  subunits could associate with  $\Delta$ SDL mutants of the  $\beta 3$  and  $\beta 4$  subunits, respectively, and were recognized well by anti- $\alpha 6$  and anti- $\alpha V$  mAbs (Figure 5B, center and right panels). This is in remarkable contrast with the inability of these  $\alpha$  subunits to associate with the  $\Delta$ SDL mutant of the  $\beta 1$  chain.

*Effect of SDL on the Folding of the  $\alpha$  Subunit  $\beta$ -Propeller Domain.* Among five different  $\alpha$  subunits tested in this study, four ( $\alpha 4$ ,  $\alpha 6$ ,  $\alpha V$ ,  $\alpha L$ ) could not be expressed and secreted when singly transfected into cells, as judged by the lack of immunoprecipitation using polyclonal antibody against the C-terminal tag (anti-velcro, data not shown). Although  $\alpha 5$  was expressed in the absence of a  $\beta$  subunit, this soluble  $\alpha 5$

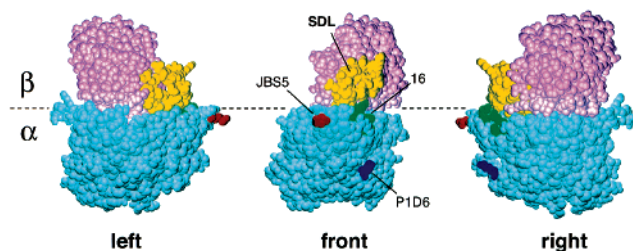


FIGURE 6: Location of the epitopes for function-blocking mAbs. The structure of the ligand binding head region of  $\alpha V\beta 3$  integrin (1JV2) (3) composed of the  $\beta$  subunit I-like domain (pink) and the  $\alpha$  subunit's  $\beta$ -propeller domain (cyan) is shown in a space-filling model. The SDL of  $\beta 3$  (Pro160–Phe188) is shown in yellow. The mAb 16, JBS5, and P1D6 epitopes are colored green, red, and blue, respectively. These residues are Lys119, Glu121, Ile147, and Asp148 corresponding to mAb 16 epitope residues Glu126, Leu128, Ser156, and Trp157 in  $\alpha 5$ ; Lys82 corresponding to JBS5 epitope residue Ser85 in  $\alpha 5$ ; and Lys203 corresponding to P1D6 epitope residue Leu212 in  $\alpha 5$ . The figure was prepared with SwissPdb-Viewer (53).

was recognized by mAb 11 directed against the C-terminal stalk region of  $\alpha 5$  (40) and by anti-velcro, but not by mAbs 16, P1D6, and JBS5 directed to the  $\beta$ -propeller domain (Figure 4B, lanes 1–5). This is consistent with data on  $\beta 2$  integrins that the  $\beta$ -propeller domain of the  $\alpha$  subunit cannot fold correctly on its own (5). Folding of the  $\beta$ -propeller domain was achieved when complexed with wild-type  $\beta 1$ , as shown by the reactivity of 16, P1D6, and JBS5 mAbs to the  $\alpha 5$   $\beta$ -propeller domain (Figure 4B, lanes 7–9). All three mAbs, which map to distinct regions within the  $\alpha 5$   $\beta$ -propeller domain (Figure 6) (41), are function-blocking antibodies, suggesting that they bind near the ligand binding pocket. When  $\alpha 5$  was complexed with  $\beta 1\Delta$ SDL, the heterodimer expressed epitopes for P1D6 and JBS5 (Figure 4B, lanes 13 and 14). The P1D6 and JBS5 epitopes were expressed to a lower extent in  $\alpha 5 + \beta 1\Delta$ SDL than in  $\alpha 5 + \beta 1$ , but were absent in  $\alpha 5$  alone. These results demonstrate that association with  $\beta 1\Delta$ SDL is sufficient for at least partial folding of the  $\alpha 5$   $\beta$ -propeller domain. However, mAb 16 did not react with  $\alpha 5\beta 1\Delta$ SDL at all (Figure 4B, lane 12). This strongly suggests that the SDL of  $\beta 1$  either constitutes a part of the epitope for mAb 16 or sits very close to it and maintains its local structure.

## DISCUSSION

The  $\beta$  integrin-like protein Pactolus gave us a hint to design  $\beta$  integrin subunits capable of folding in the absence of an  $\alpha$  subunit. Mutant  $\beta$  subunits with a deletion of the 24–29 amino acid segment corresponding to the specificity-determining loop that is missing in Pactolus were produced using a mammalian expression system. This enabled physicochemical characterization of an integrin  $\beta$  subunit monomer for the first time. Several groups have reported expression of  $\beta$  subunit extracellular fragments containing the I-like domain in the absence of associating  $\alpha$  subunits (42–45). However, the amounts secreted have been modest, none of the mAbs used for detection were specific for the I-like domain, and protein was not shown to be a single, monomeric species by physicochemical techniques or electron microscopy as described here. Deletion of the SDL segment of the I-like domain greatly improved (at least 1200%) expression of the  $\beta$  chain monomer, and all of the physico-

chemical and immunological properties of the mutant  $\Delta$ SDL  $\beta$  subunits suggest their native overall structure. The greatly improved expression upon deletion of the SDL segment was observed with all four different  $\beta$  chains tested, suggesting that this is a general feature of integrin  $\beta$  subunits.

Folding to a native conformation of the  $\Delta$ SDL mutants reported here was assessed by reactivity with mAb to different  $\beta$  subunit domains. Particularly important was the successful recognition of  $\Delta$ SDL mutants by mAbs against the I-like domain, which is known to be difficult to fold in the absence of the  $\alpha$  subunit. Recognition by multiple mAbs to the I-like domain of  $\beta 1$  and  $\beta 2$  strongly suggests the native structure of  $\beta 1$ - and  $\beta 2\Delta$ SDL mutants. The  $\text{Ca}^{2+}$ -sensitivity of TS1/18 binding to  $\beta 2\Delta$ SDL suggests that the ADMIDAS is intact, and provides further support for the native folding of the I-like domain in  $\beta 2\Delta$ SDL. Moreover, physicochemical characterization of purified  $\beta 1\Delta$ SDL protein revealed that it exists as a homogeneous monomer with an extended shape similar to half of a native heterodimer; by contrast, misfolded protein almost invariably gives rise to aggregation. Although none of the  $\beta 3$  and  $\beta 4$  mAbs used in this study have been precisely mapped to the I-like domain, 23C3 (46) to  $\beta 3$  and UM-A9 (47) to  $\beta 4$  are function-blocking, suggesting that they recognize the I-like domain. The only mAb we tested that failed to react with a  $\Delta$ SDL mutant, 7E3 to  $\beta 3$ , maps to a segment in the SDL itself (48).

Despite the partial folding of the  $\beta$ -propeller domain in the  $\alpha 5\beta 1\Delta$ SDL heterodimer, as judged by the recognition by mAbs P1D6 and JBS5, it did not bind fibronectin. Thus, the  $\alpha 5$  chain is insufficient for binding of fibronectin, even when its folding is largely supported by  $\beta 1$  association. In contrast, another group has reported an  $\alpha 5$  fragment encompassing only a portion of the predicted  $\beta$ -propeller domain that bound to fibronectin (39). No mAb reactivities were tested. Since the ligand binding assays are similar in methodology and hence in sensitivity, we are unable to explain this discrepancy. However, we note that the  $\beta$ -propeller fragment studied by Baneres et al. lacks the region from blades 1–3 which has been found by others to be important in ligand binding by integrin  $\alpha$  subunits (40, 49, 50).

During the preparation of this paper, a crystal structure of the integrin  $\alpha V\beta 3$  extracellular domain was reported (3). In the 3.1 Å resolution structure, the  $\beta$  subunit's I-like domain sits atop the  $\alpha$  subunit's  $\beta$ -propeller domain, offset from the center of the propeller, mainly above blades 3–5. The structure is remarkably consistent with what we found using SDL mutants. Lys159 and Gly189 are very close to one another with a C $\alpha$  atom distance of 4.85 Å (Figure 1B), enabling their connection after deletion of residues 160–188 with very minor main chain readjustment. In contrast, the first deletion we tested, residues 161–189, leaves residues 160 and 190 9.36 Å apart, which would require major main chain readjustment, and was not expressed (data not shown). The SDL residues comprise a bulge on the surface of the I-like domain (Figure 6), consistent with our finding that it is not essential for folding of the  $\beta$  subunit.

Our data on the function of SDL in  $\alpha\beta$  subunit association and ligand binding allow interpretation of the structure of the interface between the  $\beta$ -propeller domain and the I-like domain in the  $\alpha V\beta 3$  crystal structure. This interface is large, with a burial of more than 1600 Å<sup>2</sup> of solvent-accessible

surface on each subunit. The SDL of  $\beta 3$  buries 290 Å<sup>2</sup> in its interface with the  $\beta$ -propeller domain of  $\alpha V$ . However, this interface is not particularly hydrophobic, and the surface complementarity is low. By contrast,  $\beta 3$  segment in insertion 2 (residues 256–268) snugly nests in the dimple of the  $\alpha V$   $\beta$ -propeller, making a mainly hydrophobic interface that buries 900 Å<sup>2</sup>. A conserved hydrophobic stretch of residues in this region (GIVQP in  $\beta 3$ ) is replaced with a hydrophilic sequence in Pactus (TRQNT) (Figure 1A), and when the corresponding segment of  $\beta 1\Delta$ SDL was mutated to the hydrophilic Pactus sequence, association with the  $\alpha 5$  subunit was abrogated (data not shown). The relatively minor contribution of SDL to the  $\alpha\beta$  interface is consistent with our observation that SDL deletion did not affect heterodimer formation by several integrins, including  $\alpha V\beta 3$  (Figure 5). However, SDL is essential for the formation of several integrin heterodimers, including  $\alpha V\beta 1$ . This may reflect substantial differences in interaction surfaces dependent on the particular  $\alpha$ - $\beta$  combination. Moreover, the differential importance of the SDL for  $\beta 1$  association with different  $\alpha$  subunits may reflect substantial differences in the way the SDL packs against the  $\beta$ -propeller domain, depending on the associating  $\alpha$  subunit. In  $\alpha V$ , residues Gln120–Arg122 make a flat bed to accommodate  $\beta 3$  SDL residues Ile167–Pro170. The corresponding sequences are highly diverse among other  $\alpha$  subunits that pair with  $\beta 1$ , supporting the notion of  $\alpha$  subunit-specific conformations of the SDL.

Finally, our data on the effect of  $\beta 1$  SDL deletion on the formation of anti- $\alpha 5$  mAb epitopes correlate extremely well with integrin structure. The species-specific residues recognized by the function blocking  $\alpha 5$  mAbs 16, P1D6, and JBS5 have been precisely mapped in the  $\beta$ -propeller domain (41). When the corresponding residues in  $\alpha V$  are highlighted (Figure 6), all are located on the same side of the  $\beta$ -propeller, at the mutationally defined ligand binding site, consistent with the ability of the mAb to block ligand binding. However, among the three epitopes, only that of mAb 16 contacts the SDL (Figure 6). The direct contact of residues in  $\alpha V$  and  $\beta 3$  equivalent to Glu126–Ser129 in  $\alpha 5$  and Thr178–Ala181 in SDL of  $\beta 1$  explains the complete loss of the mAb 16 epitope in the  $\Delta$ SDL mutant. Indeed, because an antibody footprint is much larger than the area occupied by the species-specific residues of the mAb 16 epitope, it is possible that residues from SDL of  $\beta 1$  constitute a part of the epitope.

There are several mAbs that directly bind to the SDL portion in the  $\beta$  subunit. Of particular interest are anti- $\beta 2$  mAb m24 and anti- $\alpha IIb\beta 3$  mAb LJ-CP3 (48, 51). These mAbs can bind to their respective antigen only after the activation of the receptor, implying that they recognize specific conformations associated with the high-affinity receptor. Therefore, it is likely that the SDL can assume more than one conformation relative to other domains and may undergo conformational rearrangement during the conversion to the high-affinity receptor, thereby controlling both the specificity and the strength of ligand recognition.

In conclusion, we have found a segment of integrin  $\beta$  subunit that hampers autonomous folding of the I-like domain and efficient autonomous expression and have succeeded in producing soluble monomeric integrin  $\beta$  subunits. The present findings underscore the importance of SDL not only in specificity for ligands, but also in  $\alpha$  subunit association. SDL provides an important link between ligand binding and

the  $\alpha$ - $\beta$  interface, and the implication that SDL conformation is dependent on the associating  $\alpha$  subunit adds further complexity to the joint contribution of the integrin  $\alpha$  and  $\beta$  subunits in ligand recognition.

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## REFERENCES

- Hynes, R. O. (1992) *Cell* 69, 11–25.
- Humphries, M. J. (2000) *Biochem. Soc. Trans.* 28, 311–339.
- Xiong, J.-P., Stehle, T., Diefenbach, B., Zhang, R., Dunker, R., Scott, D. L., Joachimiak, A., Goodman, S. L., and Arnaout, M. A. (2001) *Science* 294, 339–345.
- Huang, C., Lu, C., and Springer, T. A. (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94, 3156–3161.
- Huang, C., and Springer, T. A. (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94, 3162–3167.
- Lee, J.-O., Rieu, P., Arnaout, M. A., and Liddington, R. (1995) *Cell* 80, 631–638.
- Baneres, J. L., Roquet, F., Martin, A., and Parello, J. (2000) *J. Biol. Chem.* 275, 5888–5903.
- Chen, Y., Garrison, S., Weis, J. J., and Weis, J. H. (1998) *J. Biol. Chem.* 273, 8711–8718.
- Margraf, R. L., Chen, Y., Garrison, S., Weis, J. J., and Weis, J. H. (1999) *Mamm. Genome* 10, 1075–1081.
- Tuckwell, D. S., and Humphries, M. J. (1997) *FEBS Lett.* 400, 297–303.
- Huang, C., Zang, Q., Takagi, J., and Springer, T. A. (2000) *J. Biol. Chem.* 275, 21514–21524.
- Takagi, J., Kamata, T., Meredith, J., Puzon-McLaughlin, W., and Takada, Y. (1997) *J. Biol. Chem.* 272, 19794–19800.
- Hemler, M. E., Sanchez-Madrid, F., Flotte, T. J., Krensky, A. M., Burakoff, S. J., Bhan, A. K., Springer, T. A., and Strominger, J. L. (1984) *J. Immunol.* 132, 3011–3018.
- Takagi, J., Isobe, T., Takada, Y., and Saito, Y. (1997) *J. Biochem. (Tokyo)* 121, 914–921.
- Sanchez-Madrid, F., Krensky, A. M., Ware, C. F., Robbins, E., Strominger, J. L., Burakoff, S. J., and Springer, T. A. (1982) *Proc. Natl. Acad. Sci. U.S.A.* 79, 7489–7493.
- Petrucelli, L., Maduzia, L., and Springer, T. A. (1995) *J. Immunol.* 155, 854–866.
- Takagi, J., Erickson, H. P., and Springer, T. A. (2001) *Nat. Struct. Biol.* 8, 412–416.
- Chang, H.-C., Bao, Z., Yao, Y., Tse, A. G. D., Goyarts, E. C., Madsen, M., Kawasaki, E., Brauer, P. P., Sacchetti, J. C., Nathanson, S. G., and Reinherz, E. L. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91, 11408–11412.
- Miyake, K., Hasunuma, Y., Yagita, H., and Kimoto, M. (1992) *J. Cell Biol.* 119, 653–662.
- Robinson, M. K., Andrew, D., Rosen, H., Brown, D., Ortlepp, S., Stephens, P., and Butcher, E. C. (1992) *J. Immunol.* 148, 1080–1085.
- Andrew, D., Shock, A., Ball, E., Ortlepp, S., Bell, J., and Robinson, M. (1993) *Eur. J. Immunol.* 23, 2217–2222.
- Pope, I., Hale, G., and Waldmann, H. (1989) in *Leukocyte Typing IV* (Knapp, W., Dorken, B., Gilks, W. R., Rieber, E. P., Schmidt, R. E., Stein, H., and von dem Borne, A. E. G. K., Eds.) pp 559–560, Oxford University Press, Oxford.
- Petrucelli, L., Luk, J., and Springer, T. A. (1995) in *Leukocyte typing V: White cell differentiation antigens* (Schlossman, S. F., Boumsell, L., Gilks, W., Harlan, J., Kishimoto, T., Morimoto, T., Ritz, J., Shaw, S., Silverstein, R., Springer, T., Tedder, T., and Todd, R., Eds.) pp 1581–1585, Oxford University Press, New York.
- O'Shea, E. K., Lumb, K. J., and Kim, P. S. (1993) *Curr. Biol.* 3, 658–667.



25. Huang, C., and Springer, T. A. (1995) *J. Biol. Chem.* 270, 19008–19016.
26. Zimrin, A. B., Gidwitz, S., Lord, S., Schwartz, E., Bennett, J. S., White, G. C., and Poncz, M. (1990) *J. Biol. Chem.* 265, 8590–8595.
27. Tamura, R. N., Rozzo, C., Starr, L., Chambers, J., Reichardt, L. F., Cooper, H. M., and Quaranta, V. (1990) *J. Cell Biol.* 111, 1593–1604.
28. Takada, Y., Elices, M. J., Crouse, C., and Hemler, M. E. (1989) *EMBO J.* 8, 1361–1368.
29. Suzuki, S., Pytela, R., Arai, H., Argraves, W. S., Krusius, T., Pierschbacher, M. D., and Ruoslahti, E. (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83, 8614–8618.
30. Oxvig, C., Lu, C., and Springer, T. A. (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96, 2215–2220.
31. Stanley, P. (1989) *Mol. Cell. Biol.* 9, 377–383.
32. Siegel, L. M., and Monty, K. J. (1966) *Biochim. Biophys. Acta* 112, 346–362.
33. Fowler, W. E., and Erickson, H. P. (1979) *J. Mol. Biol.* 134, 241–249.
34. Redick, S. D., Settles, D. L., Briscoe, G., and Erickson, H. P. (2000) *J. Cell Biol.* 149, 521–527.
35. Qu, A., and Leahy, D. J. (1995) *Proc. Natl. Acad. Sci. U.S.A.* 92, 10277–10281.
36. Emsley, J., King, S. L., Bergelson, J. M., and Liddington, R. C. (1997) *J. Biol. Chem.* 272, 28512–28517.
37. Xiong, Y. M., and Zhang, L. (2001) *J. Biol. Chem.* 276, 19340–19349.
38. Nermut, M. V., Green, N. M., Eason, P., Yamada, S. S., and Yamada, K. M. (1988) *EMBO J.* 7, 4093–4099.
39. Baneres, J. L., Roquet, F., Green, M., LeCalvez, H., and Parello, J. (1998) *J. Biol. Chem.* 273, 24744–24753.
40. Mould, P., Askari, J. A., and Humphries, M. J. (2000) *J. Biol. Chem.* 275, 20324–20336.
41. Burrows, L., Clark, K., Mould, A. P., and Humphries, M. J. (1999) *Biochem. J.* 344 Pt. 2, 527–533.
42. Bennett, J. S., Kolodziej, M. A., Vिलाire, G., and Poncz, M. (1993) *J. Biol. Chem.* 268, 3580–3585.
43. Weisel, J. W., Nagaswami, C., Vिलाire, G., and Bennett, J. S. (1992) *J. Biol. Chem.* 267, 16637–16643.
44. Honda, S., Tomiyama, Y., Pelletier, A. J., Annis, D., Honda, Y., Orchekowski, R., Ruggeri, Z., and Kunicki, T. J. (1995) *J. Biol. Chem.* 270, 11947–11954.
45. Tan, S. M., Hyland, R. H., Al-Shamkhani, A., Douglass, W. A., Shaw, J. M., and Law, S. K. (2000) *J. Immunol.* 165, 2574–2581.
46. Chang, M. C., Wang, B. R., and Huang, T. F. (1995) *Thromb. Haemostasis* 74, 764–769.
47. Lee, E. C., Lotz, M. M., Steele, G. D. J., and Mercurio, A. M. (1992) *J. Cell Biol.* 117, 671–678.
48. Puzon-McLaughlin, W., Kamata, T., and Takada, Y. (2000) *J. Biol. Chem.* 275, 7795–7802.
49. Irie, A., Kamata, T., and Takada, Y. (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94, 7198–7203.
50. Kamata, T., Tieu, K. K., Springer, T. A., and Takada, Y. (2001) *J. Biol. Chem.* 276, 44275–44283.
51. Lu, C., Shimaoka, M., Zang, Q., Takagi, J., and Springer, T. A. (2001) *Proc. Natl. Acad. Sci. U.S.A.* 98, 2393–2398.
52. Sali, A., and Blundell, T. L. (1993) *J. Mol. Biol.* 234, 779–815.
53. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis* 18, 2714–27123.
54. Pujades, C., Teixido, J., Bazzoni, G., and Hemler, M. E. (1996) *Biochem. J.* 313, 899–908.

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