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Small Molecule Integrin Antagonists that Bind to the β_2 Subunit I-like Domain and Activate Signals in One Direction and Block Them in the Other

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Summary

Leukocyte integrins contain an inserted (I) domain in their α subunits and an I-like domain in their β_2 subunit, which directly bind ligand and regulate ligand binding, respectively. We describe a novel mechanistic class of integrin inhibitors that bind to the metal ion-dependent adhesion site of the β_2 I-like domain and prevent its interaction with and activation of the α_L I domain. The inhibitors do not bind to the α_L I domain but stabilize α/β subunit association and can show selectivity for $\alpha_L\beta_2$ compared to $\alpha_M\beta_2$. The inhibitors reveal a crucial intersection for relaying conformational signals within integrin extracellular domains. While blocking signals in one direction to the I domain, the antagonists induce the active conformation of the I-like domain and stalk domains, and thus transmit conformational signals in the other direction toward the transmembrane domains.

Introduction

LFA-1 ($\alpha_L\beta_2$) is a member of the leukocyte integrin subfamily that shares the β_2 subunit with Mac-1 ($\alpha_M\beta_2$), p150,95 ($\alpha_X\beta_2$), and $\alpha_D\beta_2$ (Gahmberg et al., 1997; Harris et al., 2000). $\alpha_L\beta_2$ plays a critical role in leukocyte adhesion to and migration through endothelium by its ability to bind intercellular adhesion molecules (ICAMs), especially ICAM-1, on endothelial cells. $\alpha_L\beta_2$ is also crucial for lymphocyte adhesion and costimulation, and for formation of the immunological synapse between T and antigen-presenting cells (Grakoui et al., 1999). Furthermore, $\alpha_L\beta_2$ is an important pharmaceutical target for blocking rejection in organ transplantation and treating autoimmune diseases. Monoclonal antibodies to $\alpha_L\beta_2$ prolong graft survival in many animal models (Nicolls et al., 2002; Poston et al., 2000; Sarnacki et al., 2000) and dramatically alleviate the symptoms of psoriasis in clinical trials (Gottlieb and Bos, 2002; Gottlieb et al., 2002).

Leukocyte integrins contain one von Willebrand factor-type A domain in each subunit, the inserted (I) domain in the α subunit and the I-like domain in the β subunit (Shimaoka et al., 2002). Each domain adopts an α/β Rossmann fold with a metal ion-dependent adhesion site (MIDAS) on the "top" of the domain, whereas its C- and N-terminal connections to the neighboring domain are on the distal, "bottom" face (Huang et al., 2000; Lee et al., 1995; Shimaoka et al., 2002; Xiong et al., 2001). $\alpha_L\beta_2$ binds its immunoglobulin superfamily ligand ICAM-1 through the α_L I domain, with the binding site centered on the Mg^{2+} of the MIDAS which directly coordinates to Glu34 of ICAM-1 (Shimaoka et al., 2003). The affinity of the α_L I domain for ICAMs is regulated by downward axial displacement of its C-terminal helix, which is conformationally linked to alterations of MIDAS loops and Mg^{2+} coordination (Huth et al., 2000; Shimaoka et al., 2001, 2003). Compared to the default, low-affinity conformation, downward displacements by one and two turns of helix increase affinity ~500- and 10,000-fold, respectively (Shimaoka et al., 2003). The β_2 I-like domain does not directly bind ligand despite containing a MIDAS motif. Instead, it functions indirectly by regulating the activity of the I domain (Leitinger and Hogg, 2000; Lu et al., 2001b, 2001c; Shimaoka et al., 2001; Yalamanchili et al., 2000).

At least two distinct classes of potent, low molecular weight $\alpha_L\beta_2$ antagonists are under development as anti-inflammatory agents (Figure 1). One group of antagonists binds underneath the C-terminal α helix of the α_L I domain (e.g., LFA703 or BIRT377), blocks the downward axial displacement of the C-terminal helix, and inhibits ligand binding of $\alpha_L\beta_2$ allosterically by stabilizing the I domain in the low-affinity conformation (Kallen et al., 1999; Last-Barney et al., 2001; Liu et al., 2001; Lu et al., 2001c; Weitz-Schmidt et al., 2001). By contrast, a second class of compounds, which has been patented as $\alpha_L\beta_2$ antagonists by Genentech (Burdick, 1999; Burdick et al., 2001) and dual-acting $\alpha_L\beta_2$ and $\alpha_M\beta_2$ antagonists (Fotouhi et al., 1999) or $\alpha_L\beta_2$ antagonists (Fotouhi et al., 2001) by Roche, remains less defined with regard to the binding site and mode of action. Genentech compounds #3 and #4 (Figure 1A) were reported to be rationally designed based on the structure of ICAM-1 by grafting amino acid residue functionalities that were distant in amino acid sequence but contiguous in three-dimensional structure onto a small molecule antagonist (Gadek et al., 2002). Thus, it was implied, but not demonstrated, that the antagonists bound like ICAM-1 to the α_L I domain. However, studies with a structurally related, dual $\alpha_L\beta_2$ and $\alpha_M\beta_2$ inhibitor from Roche (Figure 1A) showed it did not perturb NMR chemical shifts of isolated α_L or α_M I domains, but did perturb antibody binding to the β_2 I-like domain in $\alpha_L\beta_2$ and $\alpha_M\beta_2$ holoreceptors (Welzenbach et al., 2002). Here we demonstrate that these inhibitors represent a novel class of antagonists of I domain-containing integrins, which bind to the β_2 I-like domain MIDAS near a key regulatory interface with the α_L and α_M subunits, and block communication of conformational change to the I domain, while at the same time activating conformational rearrangements elsewhere in integrins.

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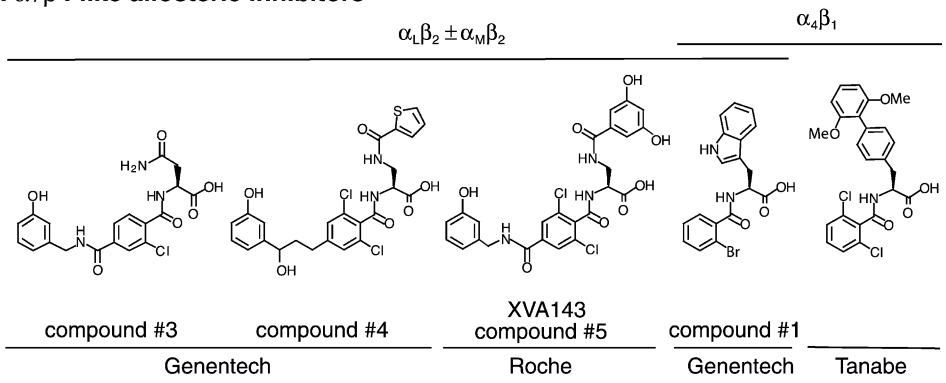
A. α/β I-like allosteric inhibitors



B. α I allosteric inhibitors

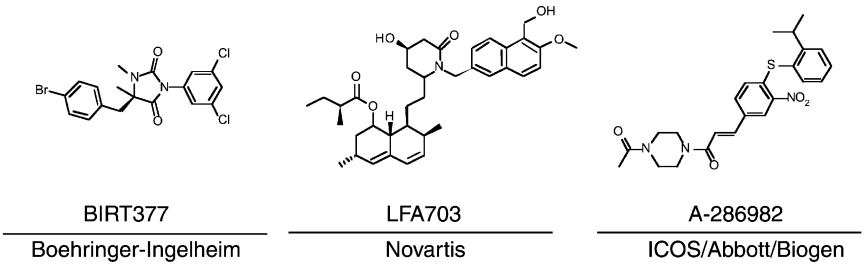


Figure 1. Chemical Structures of Small Molecule Integrin Inhibitors

(A) α/β I-like allosteric inhibitors to the β_2 integrin and comparison to α/β I-like competitive inhibitors to $\alpha_4\beta_1$. Compounds #1, #3, and #4 are as described (Gadek et al., 2002); compound #5 (XVA143) is identical to example 345 (Fotouhi et al., 1999) (Welzenbach et al., 2002); the Tanabe and other $\alpha_4\beta_1$ antagonists are described (Jackson, 2002).

(B) α I allosteric inhibitors are shown for comparison: BIRT377 (Last-Barney et al., 2001), LFA703 (Weitz-Schmidt et al., 2001), and A-286982 (Liu et al., 2001).

Results

Inhibition of Both $\alpha_L\beta_2$ and $\alpha_M\beta_2$ by Compounds #3, #4, and #5

XVA143 (compound #5, Figure 1A) and many related compounds patented by Roche inhibit both $\alpha_L\beta_2$ and $\alpha_M\beta_2$ (Fotouhi et al., 1999; Welzenbach et al., 2002). The Genentech compounds #3 and #4 (Figure 1A) and the Roche compounds are closely structurally related and are derived by optimization of the same drug discovery lead, compound #1 (J. Tilley, Roche, Nutley, NJ, personal communication) (Gadek et al., 2002). The Genentech compounds have been reported to inhibit $\alpha_L\beta_2$; however, the results of $\alpha_M\beta_2$ inhibition assays have not yet been disclosed in the research or patent literature (Burdick, 1999; Burdick et al., 2001; Gadek et al., 2002). We tested the hypothesis that Genentech compounds #3 and #4 would inhibit $\alpha_M\beta_2$ as well as $\alpha_L\beta_2$ (Figures 2A and 2B). Compounds #3 and #4 inhibited $\alpha_M\beta_2$ binding to iC3b with IC_{50} values of 3 ± 1 and 0.1 ± 0.01 μM, respectively. Compound #5 had an IC_{50} value of 0.06 ± 0.01 μM in the same assay, extending previous inhibition data on the $\alpha_M\beta_2$ interaction with fibrinogen and ICAM-1 to the $\alpha_M\beta_2$ interaction with iC3b. Thus, all of the tested compounds inhibited both $\alpha_L\beta_2$ and $\alpha_M\beta_2$. Compound #3 was less potent than #4 and #5 with both integrins, and some selectivity of compound #4 was evident for LFA-1, because compounds #4 and #5 inhibited $\alpha_L\beta_2$ equally well but compound #4 was less potent than #5 against $\alpha_M\beta_2$ (Figures 2A and 2B). By contrast to compounds #3, #4, and #5, the α_L I domain allosteric inhibitors BIRT377 and LFA703 (Figure 1B) blocked exclusively $\alpha_L\beta_2$ (Figures 2A and 2B and data not shown).

In the mixed lymphocyte reaction (MLR), the $\alpha_L\beta_2$-ICAM-1 interaction transmits costimulatory signals for proliferation. Remarkably, compound #5 compares in potency with the immunosuppressive drug cyclosporin A in its ability to inhibit the MLR (Figure 2C). Compounds #3 and #4 have previously also been shown to inhibit the MLR (Gadek et al., 2002). Thus, the class of antagonists that includes compounds #3, #4, and #5 are potent inhibitors of lymphocyte functional responses that require LFA-1 and also act as dual LFA-1 ($\alpha_L\beta_2$) and Mac-1 ($\alpha_M\beta_2$) antagonists.

Allosteric Inhibition by Compounds #3, #4, and #5

The mode of action of inhibitors was studied by using $\alpha_L\beta_2$ containing a mutant I domain, which is reversibly locked in the open, high-affinity conformation with K287C and K294C mutations to introduce cysteines that form an engineered disulfide bond (locked open I domain) (Lu et al., 2001c). Adhesion through $\alpha_L\beta_2$ con-

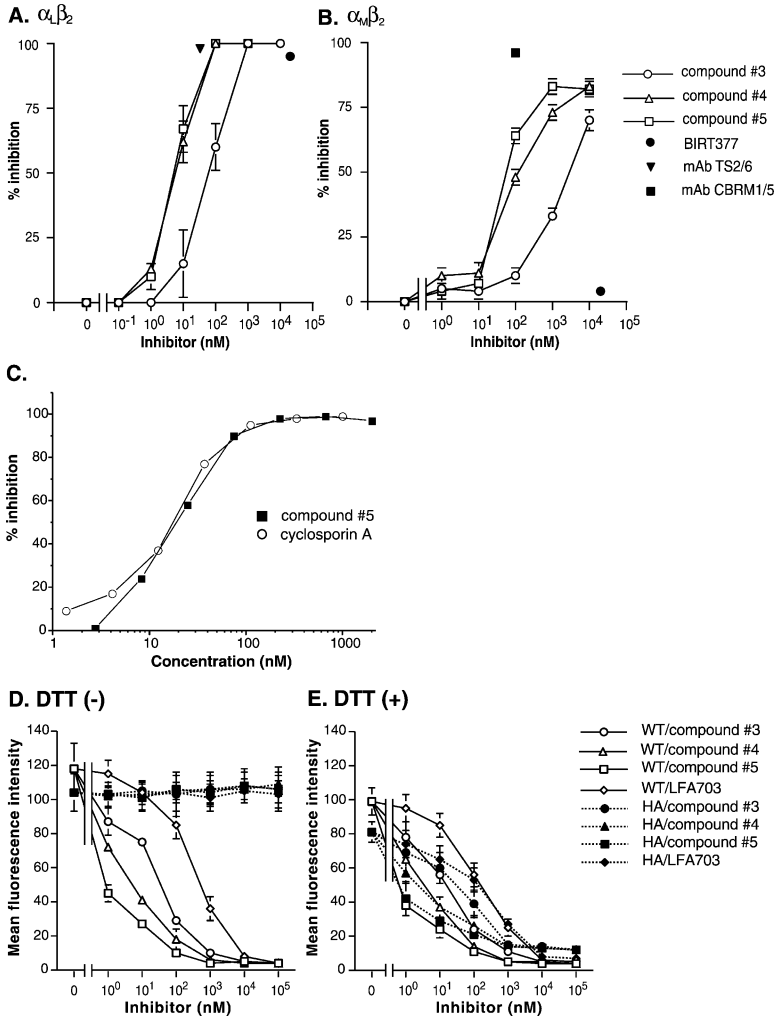


Figure 2. Inhibition by Compounds #3–#5 of $\alpha_L\beta_2$ and $\alpha_M\beta_2$

(A and B) Adhesion of $\alpha_L\beta_2$-expressing K562 cells to immobilized ICAM-1 (A) and $\alpha_M\beta_2$-expressing cells to immobilized iC3b on plastic (B) was as described (Lu and Springer, 1997; Shimaoka et al., 2000). $\alpha_L\beta_2$ was activated by 1 mM Mn^{2+}, and $\alpha_M\beta_2$ was activated by 1 mM Mn^{2+} in the presence of 2 μg/ml CBR LFA-1/2. Values are % inhibition of mock-treated cells and expressed as mean ± difference from the mean of two independent experiments. Specific binding of cells in the absence of inhibitors was 44.4 ± 1.8% and 52.8 ± 1.5% of input for $\alpha_L\beta_2$ and $\alpha_M\beta_2$ transfectants, respectively.

(C) Compound #5 and cyclosporin A inhibit the mouse MLR. Inhibition of [^{3}H]thymidine incorporation was measured as described in the Experimental Procedures. A representative experiment out of two independent experiments is shown.

(D and E) Defining the mechanism of inhibition of small molecule antagonists using $\alpha_L\beta_2$ heterodimers containing mutant α_L I domains. Binding of soluble ICAM-1-IgA/FITC anti-IgA to K562 transfectants expressing $\alpha_L\beta_2$ containing wild-type (WT) or the mutant high-affinity (HA) I domain in the presence of 1 mM $MnCl_2$. The binding assay was performed at room temperature in the absence (D) or presence (E) of 10 mM DTT. Results are mean ± SD of three independent experiments.

taining the locked open I domain can be inhibited by mAbs that map to the ICAM-1 binding site of the α_L I domain, but not by mAbs that map to other sites in the α_L I domain, or mAbs that map to the β_2 I-like domain (Lu et al., 2001c). By contrast, wild-type $\alpha_L\beta_2$ and locked open $\alpha_L\beta_2$ treated with dithiothreitol (DTT) to reduce the introduced disulfide bond and allow the I domain to shift to the closed conformation can be completely inhibited by both classes of mAbs to the α_L I domain and the mAbs to the β_2 I-like domain (Lu et al., 2001c). Similarly, the α I domain allosteric antagonists lovastatin and LFA703 cannot inhibit locked open $\alpha_L\beta_2$ but do inhibit wild-type $\alpha_L\beta_2$ and DTT-treated K287C/K294C mutant $\alpha_L\beta_2$ (Lu et al., 2001c; Salas et al., 2002). Thus, competitive antagonists to the ICAM-1 binding site in the α_L I domain and noncompetitive antagonists that bind elsewhere in $\alpha_L\beta_2$ can be discriminated using locked open $\alpha_L\beta_2$.

Compounds #3, #4, and #5 blocked binding to ICAM-1 of $\alpha_L\beta_2$ containing the wild-type I domain but not $\alpha_L\beta_2$ containing the locked open I domain (Figure 2D). Similar results were obtained with the I domain allosteric inhibitors LFA703 (Figure 2D) and BIRT377 (data not shown). By contrast, both wild-type and mutant $\alpha_L\beta_2$ were completely blocked by the direct inhibitor mAb TS2/6 to the I domain (data not shown). After mild DTT reduction, $\alpha_L\beta_2$ containing the K287C/K294C mutant I domain became susceptible to both classes of small molecule inhibitors (Figure 2E).

Lack of Inhibition by Compounds #3–#5 of the Isolated α_L I Domain

Compounds #3, #4, and #5 were further tested for their ability to inhibit binding of the isolated, soluble α_L I domain to ICAM-1. In addition to the K287C/K294C high-affinity mutant α_L I domain (Shimaoka et al., 2001), we used the L161C/F299C intermediate-affinity mutant α_L I domain (Shimaoka et al., 2003). Crystal structures of both of these mutant I domains have been determined, including the intermediate-affinity α_L I domain bound to ICAM-1 (Shimaoka et al., 2003). Furthermore, two different crystal forms of the mutant high-affinity α_L I domain demonstrate that even in the absence of ICAM-1, its ligand binding site surrounding the MIDAS is locked into the same conformation as seen when ICAM-1 is bound (Shimaoka et al., 2003). We tested binding of these mutant I domains to ICAM-1 using surface plasmon resonance (Figures 3A–3J). The plasmon resonance curves in the absence of inhibitors (Figures 3A and 3F) and the amount of inhibition by soluble ICAM-1 (Figures 3E and 3J) were consistent with previ-

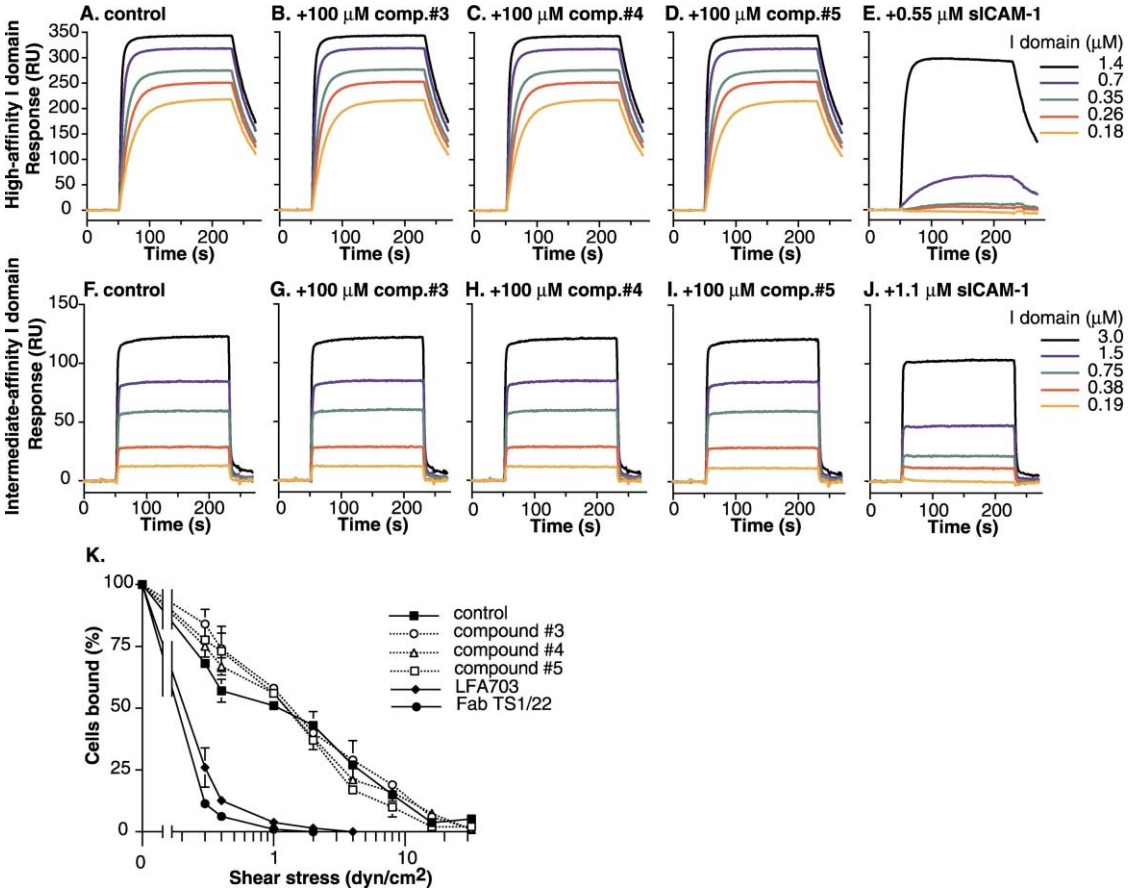


Figure 3. Lack of Inhibition by Compounds #3–#5 of α_L I Domain Binding to ICAM-1

(A–J) Surface plasmon resonance analysis of binding by the α_L I domains to ICAM-1. The high-affinity (K287C/K294C) or the intermediate-affinity (L161C/F299C) I domain was perfused onto immobilized ICAM-1 in the presence of 100 μM of compound #3 (B and G), #4 (C and H), #5 (D and I), or 0.55 (E) or 1.1 μM (J) of soluble ICAM-1. DMSO (1:1000), the vehicle for compound #5, and BSA (100 μg/ml), the control for ICAM-1, had no effect on I domain binding (data not shown).

(K) Detachment in shear flow of transfectants bound to ICAM-1 substrates. K562 transfectants expressing the isolated wild-type α_L domain were incubated with or without 100 μM compounds or 10 μg/ml Fab fragment of TS1/22 for 10 min at RT in Hank's balanced salt solution, 1 mM Mg^{2+}, 1 mM Ca^{2+}, infused in the flow chamber, and allowed to settle on the ICAM-1 substrate for 5 min, and the wall shear stress was then increased in steps every 10 s as shown (Salas et al., 2002). Cells interacting with the ICAM-1 substrate, including both firmly adherent and rolling cells, were counted and expressed as percentage of input at the end of each 10 s interval.

ous measurements of the K_D for ICAM-1 of 150 nM for the high-affinity I domain and 3 μM for the intermediate-affinity I domain (Shimaoka et al., 2001, 2003). Compounds #3, #4, and #5 had no effect on binding of high-affinity (Figures 3A–3D) or intermediate-affinity (Figures 3F–3I) I domains to immobilized ICAM-1, despite use of the compounds at 100 μM, a concentration 10^4 to 10^5 higher than the compounds' reported IC_{50} values for inhibition of intact $\alpha_L\beta_2$ (Fotouhi et al., 1999; Gadek et al., 2002). By contrast, soluble ICAM-1 used at 0.55 μM with the high-affinity I domain (Figure 3E) or 1.1 μM with the intermediate-affinity I domain (Figure 3J) markedly inhibited binding to immobilized ICAM-1.

To test the effect of the compounds on ligand binding by the wild-type α_L I domain, the isolated α_L I domain was fused to an artificial transmembrane domain and a 5 residue cytoplasmic domain and expressed on K562 transfectants (Lu et al., 2001b). Interaction with ICAM-1 of the isolated wild-type α_L I domain is most readily detected by its ability to support rolling of cells in shear flow, where force exerted on the C terminus of the I domain helps stabilize it in the high-affinity conformation (Salas et al., 2002). Rolling of transfectants on ICAM-1 substrates was blocked by TS1/22 Fab to the I domain and by the I domain allosteric antagonist compounds LFA703 and BIRT377 but not by compounds #3, #4, and #5 (Figure 3K and data not shown). Differential inhibition of rolling mediated by the isolated α_L I domain thus distinguishes between these two classes of LFA-1 antagonists. Compounds #3, #4, and #5 clearly fail to inhibit binding of ICAM-1 to the mutant locked open $\alpha_L\beta_2$ holoreceptor, to mutant soluble high-affinity and intermediate I domains, and to the isolated wild-type I domain on cell surfaces. These findings demonstrate that compounds #3, #4, and #5 do not mimic ICAM-1.

Stabilization of α/β Subunit Association

Inhibitors of integrins that lack I domains, such as those that are based on the Arg-Gly-Asp (RGD) sequence, bind to both the integrin α and β subunit. The Asp car-

boxylic acid side chain coordinates directly to the metal of the β subunit I-like domain MIDAS, while the Arg side chain binds to the α subunit β-propeller domain (Xiong et al., 2002). Several of this class of antagonists to integrins that lack I domains have been shown to stabilize αβ association sufficiently to make it resistant to SDS at room temperature (Thibault, 2000; Zolotarjova et al., 2001). Compounds #3, #4, and #5 (Figure 1A), but not I domain allosteric antagonists such as LFA703 (Figure 1B), resemble non-I domain integrin antagonists in the absolute requirement for a free carboxyl group and often in other structural features as well. We reasoned that if compounds #3–#5 bound to a similar site they might also stabilize the αβ integrin complex to SDS. In the absence of added compounds, $\alpha_L\beta_2$ was dissociated by SDS, and the α_L and β_2 subunits migrated individually in SDS-PAGE, in both reducing and nonreducing conditions (Figure 4A, lanes 1 and 6). By contrast, when treated with compounds #3–#5, but not with LFA703, an SDS-stable $\alpha_L\beta_2$ complex was formed, and no free α_L or β_2 subunit was present (Figure 4A, lanes 2–5). Identical results were obtained after reduction with DTT (Figure 4A, lanes 7–9). Stabilization by the inhibitors to SDS treatment required divalent cations because EDTA abolished complex formation (Figures 4A, lanes 11–13, and 4D).

Stabilization of mutant αβ complexes was utilized to test compound binding to $\alpha_L\beta_2$ mutants and locate the inhibitor binding site. Compounds #3–#5 formed SDS-stable αβ complexes with $\alpha_L\beta_2$ containing the mutant locked open I domain (Figure 4B, lanes 3 and 4), demonstrating that, despite their lack of ability to inhibit binding to ICAM-1, they bind to locked open $\alpha_L\beta_2$. Furthermore, compounds #3–#5 stabilized association in SDS of $\alpha_L\beta_2$ with the I domain deleted from the α_L subunit (I-less, Figure 4B, lanes 7 and 8) (Yalamanchili et al., 2000), demonstrating that the α_L I domain is not required for compound binding. Quantitative analysis further demonstrated that EC_{50} values for compound binding to wild-type $\alpha_L\beta_2$, $\alpha_L\beta_2$ containing the high-affinity I domain, and I-less $\alpha_L\beta_2$ are indistinguishable (Figures 4C and 4D). To test whether the MIDAS of the β_2 I-like domain was required for binding, we mutated one of the metal-coordinating side chains in β_2, Ser114, to Ala. Although the α_L and mutant β_2 chains were well expressed and noncovalently associated prior to SDS addition, as demonstrated by coprecipitation with mAb TS2/4 (Figure 4B, lane 5), none of the compounds stabilized SDS-resistant αβ complexes with the β_2-S114A mutant (Figure 4B, lane 6), demonstrating the crucial role of the β_2 MIDAS in the action of these compounds. In all cases, compound binding was dependent on metal ions, and both Ca^{2+}/Mg^{2+} and Mn^{2+} were permissive for complex formation (Figure 4D). Interestingly, compound #3 stabilized complex formation better in Mn^{2+} than Mg^{2+}/Ca^{2+} (Figure 4D), again showing the importance of metal ions for compound binding.

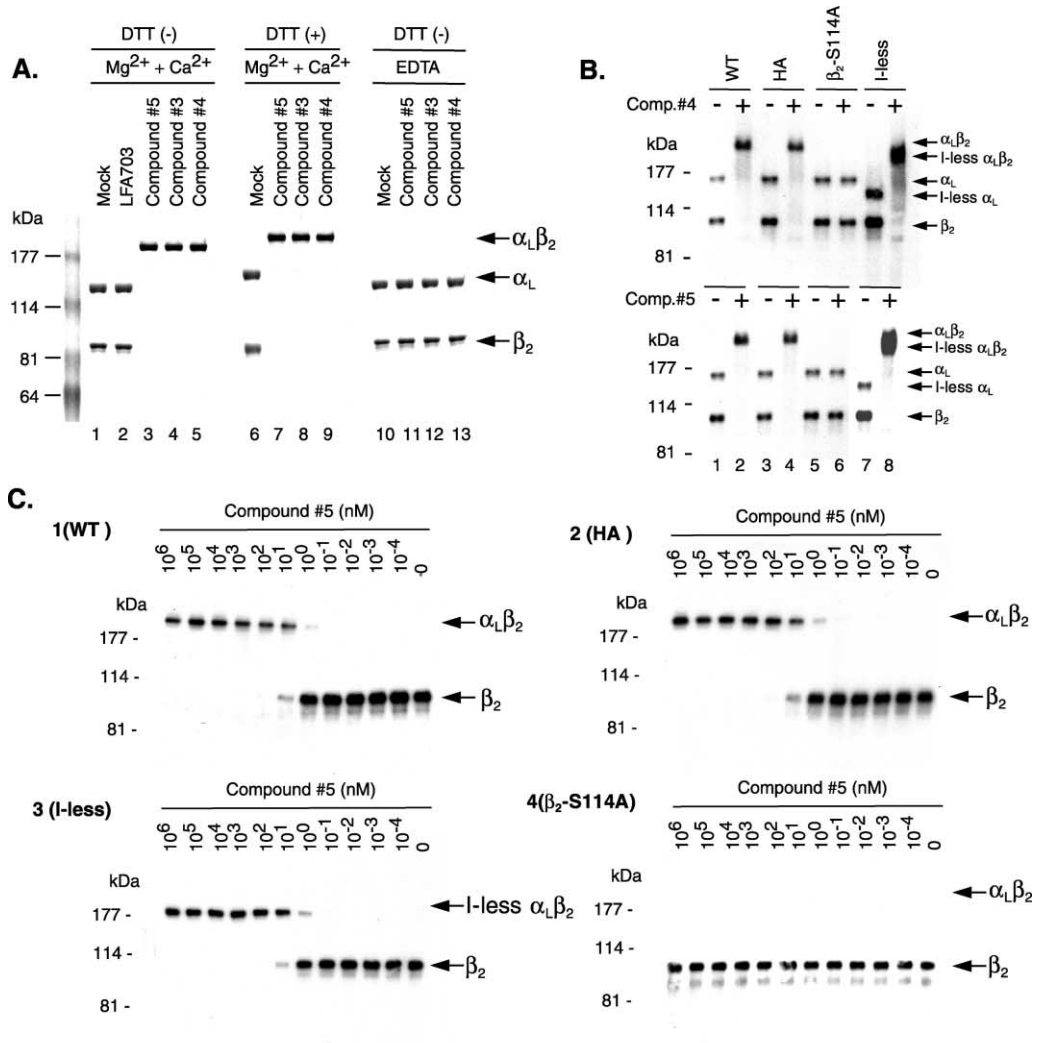
The ability to stabilize αβ association and the lack of requirement for the α I domain suggest that compounds #3–#5 may bridge the β_2 I-like domain MIDAS to the α subunit β-propeller domain. The β-propeller shares an extensive interface with the I-like domain and comes close to its MIDAS in the three-dimensional structure (Xiong et al., 2001; Zang et al., 2000). Thus, it should be possible to build α subunit specificity into the compounds, as is indeed illustrated by the compound series. Compound #5 stabilizes $\alpha_L\beta_2$ and $\alpha_M\beta_2$ complexes with equal potency, both in Mg^{2+}/Ca^{2+} and in Mn^{2+} (Figure 4D). Compound #4 was equipotent in stabilizing $\alpha_L\beta_2$ and $\alpha_M\beta_2$ in Mn^{2+}; however, it was 50-fold more potent in stabilizing $\alpha_L\beta_2$ than $\alpha_M\beta_2$ in Mg^{2+}/Ca^{2+} (Figure 4D). Finally, compound #3 was 100-fold more potent in stabilizing $\alpha_L\beta_2$ than $\alpha_M\beta_2$ in Mn^{2+}, and the difference in potency increased to 1000-fold in Mg^{2+}/Ca^{2+} (Figure 4D). On the basis of the evidence that compounds #3–#5 bind to the β_2 I-like MIDAS, stabilize αβ association, and can show selectivity for the α subunit, we propose to call them α/β I-like allosteric antagonists. We propose the designation α I allosteric antagonists for the other class of inhibitors to the I domain.

Impacts on Integrin Conformation

In order to obtain further mechanistic insights into the α/β I-like allosteric antagonists, their impacts on integrin conformation were studied using purified recombinant and cell surface $\alpha_L\beta_2$. We tested three activation-dependent mAbs mapped to distinct sites. The m24 epitope maps to the β_2 I-like domain, and its exposure shows that the I-like domain is activated (Dransfield and Hogg, 1989; Lu et al., 2001c). The KIM127 epitope involves residues in integrin EGF domain 2 in the β_2 subunit that are buried in the headpiece-tailpiece interface in the bent integrin conformation and exposed in the extended conformation (Beglova et al., 2002; Lu et al., 2001a). The NKI-L16 epitope maps to the thigh domain and genu of the α_L leg (Huang and Springer, 1995) (C. Xie and T.A.S., unpublished data), and its exposure is associated with activation (van Kooyk et al., 1991). The exposure of these epitopes is usually induced by inside-out signaling as well as ligand binding. Remarkably, the α/β I-like allosteric antagonists potently induced m24, KIM127, and NKI-L16 epitope expression in $\alpha_L\beta_2$ (Figures 5A and 5B and Table 1). By contrast, the α I domain allosteric antagonists BIRT377 (Table 1) and LFA703 (data not shown) did not enhance KIM127 and m24 epitopes expression. To the contrary, these compounds suppressed induction of activation epitopes by Mn^{2+} (Woska et al., 2001) (Table 1). Induction by α/β I-like allosteric antagonists of the KIM127 epitope was divalent cation dependent, as shown by inhibition by EDTA, whereas activating mAb CBR LFA-1/2 induced KIM127 expression in the presence of EDTA (Figure 5C). Consistent with previous results (Welzenbach et al., 2002), binding of the β_2 I-like domain mAbs, TS1/18 and IB4, was reduced by α β I-like antagonists (Figure 5A and Table 1). Induction of activation-dependent epitopes by the antagonists was also observed with $\alpha_L\beta_2$ containing the locked open I domain and with I-less $\alpha_L\beta_2$, but not with the $\alpha_L\beta_2$-S114A mutant (Figure 5D). These results confirmed that the α_L I domain is not required and that the β_2 I-like MIDAS is required for compound activity.

Discussion

Regulation of $\alpha_L\beta_2$ activation is a pivotal process for controlling leukocyte trafficking and immune responses in health and diseases (Anderson et al., 1989; Harlan et



D.

Integrins	Cation	EC_{50} (μM) compound #3	compound #4	compound #5
WT $\alpha_L\beta_2$	Mg + Ca	0.7 ± .2	0.007 ± .002	0.006 ± .002
HA $\alpha_L\beta_2$	Mg + Ca	0.7 ± .2	0.006 ± .002	0.005 ± .002
I-less $\alpha_L\beta_2$	Mg + Ca	0.6 ± .2	0.005 ± .001	0.004 ± .001
$\alpha_L\beta_2$-S114A	Mg + Ca	>1,000	>1,000	>1,000
WT $\alpha_M\beta_2$	Mg + Ca	900 ± 100	0.3 ± .1	0.009 ± .001
WT $\alpha_L\beta_2$	Mn	0.03 ± .01	0.004 ± .002	0.004 ± .001
HA $\alpha_L\beta_2$	Mn	0.04 ± .01	0.004 ± .001	0.005 ± .001
WT $\alpha_M\beta_2$	Mn	2.5 ± .3	0.004 ± .001	0.003 ± .001
WT $\alpha_L\beta_2$	EDTA	>1,000	>1,000	>1,000
WT $\alpha_M\beta_2$	EDTA	>1,000	>1,000	>1,000

Figure 4. Effect of Compounds on $\alpha_L\beta_2$ Mutants and $\alpha_M\beta_2$ Measured by Stabilization of $\alpha\beta$ Complexes in SDS-PAGE

(A) SDS-PAGE and Coomassie blue staining of soluble recombinant $\alpha_L\beta_2$ in the presence or absence of the inhibitors and 10 mM DTT.

(B) Immunoprecipitation of wild-type and mutant $\alpha_L\beta_2$ heterodimers. Lysates of surface-biotinylated K562 cell transfectants, which were pretreated with inhibitors (100 μM) or DMSO (1:1000), were immunoprecipitated with mAb TS2/4 to $\alpha_L\beta_2$ and detected on Western blots by streptavidin and luminescence.

(C) Dose response for formation of the SDS-stable $\alpha\beta$ complex by wild-type and mutant $\alpha_L\beta_2$. Lysates from transfectants, adjusted to contain 35 pM of $\alpha_L\beta_2$, were incubated with the indicated concentrations of the inhibitors. The complexed and free β_2 subunit was detected by Western blotting with mAb CBR LFA-1/2. Representative Western blots are shown. Because of its larger size, the $\alpha\beta$ complex is transferred less efficiently out of the gel than free β, resulting in weaker maximal intensity.

(D) EC_{50} values for complex stabilization by the inhibitors. Values were calculated from experiments performed identically to those shown in Figure 3C. $\alpha_L\beta_2$ complex and free β_2 were quantitated by NIH image software, the differences in maximal intensity of $\alpha_L\beta_2$ and free β_2 were normalized using conditions in which only $\alpha_L\beta_2$ or free β_2 were present, and the free β_2 was calculated as % of total normalized $\alpha_L\beta_2$ and free β_2 in each lane. Results are mean and difference from the mean of two independent experiments. >1000, no complex stabilization seen at 1 mM compound. The ability of compound #3 to stabilize $\alpha_M\beta_2$ in Mg^{2+} and Ca^{2+} was confirmed by complete stabilization of recombinant, purified $\alpha_M\beta_2$ with 10 mM compound #3.

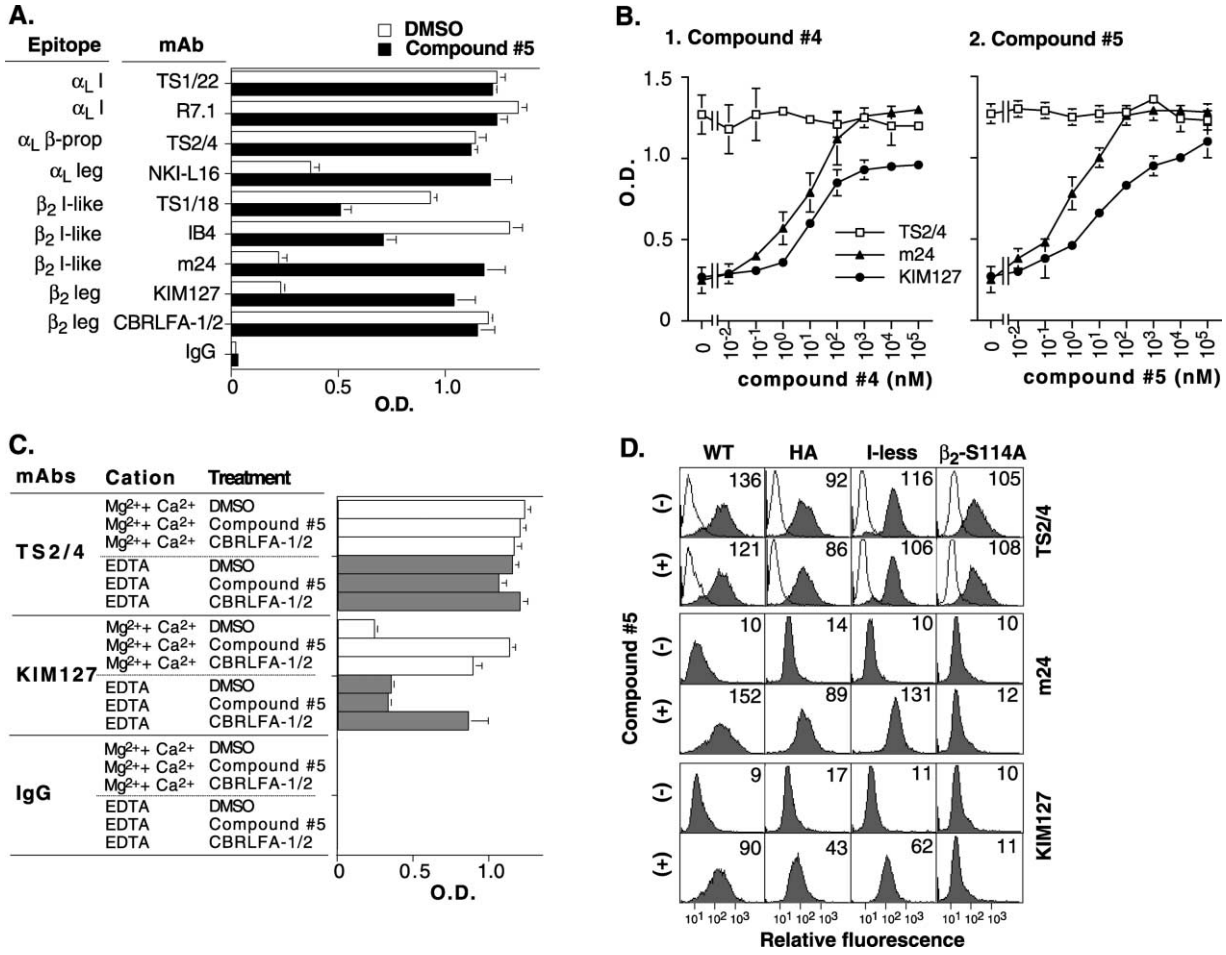


Figure 5. Effect of Compounds on Expression of Activation Epitopes

(A–C) Recombinant soluble $\alpha_L\beta_2$. (A) Induction of activation epitopes and partial inhibition of expression of β_2 I-like domain epitopes by compound #5. Soluble $\alpha_L\beta_2$ indirectly captured onto plastic with antibody to the C-terminal coiled-coil tag was incubated with mAbs (2 μg/ml) in HBS, 1 mM $MgCl_2$, 1 mM $CaCl_2$ in the presence of 10 μM compound #5 or 1:10,000 DMSO. Binding of mAbs was detected by peroxidase-labeled secondary antibody. Results are mean ± SD of three independent experiments (A–C). (B) Dose-dependent induction of KIM127 and m24 activation epitopes by compounds #4 and #5. (C) Divalent cation-dependence of KIM127 epitope induction.

(D) Induction of epitopes of $\alpha_L\beta_2$ mutants on K562 cell transfectants. Cells were stained with mAbs (2 μg/ml) in HBS, 1 mM $MgCl_2$, 1 mM $CaCl_2$, containing compound #5 (1 μM) or DMSO at 37°C for 30 min, followed by immunofluorescence flow cytometry. Representative histograms and mean values of fluorescence intensity in three independent experiments are shown. Binding of the positive control TS2/4 mAb (filled histogram) and nonbinding IgG (open histogram) are shown in the upper panels. Compound #5 failed to enhance KIM127 and m24 epitopes in mutant β_2 S114A; these epitopes were induced in the same mutant by activating mAb CBR LFA-1/2 (data not shown).

al., 1992; Springer, 1990). Upon integrin activation, the affinity of the ligand binding I domain is allosterically regulated by an axial downward displacement of its C-terminal helix, which is proposed to be induced by a neighboring regulatory domain, the I-like domain (Shimaoka et al., 2002, 2003). Transmission of conformational signals through regulatory domains (e.g., the I-like domain) to the I domain is a key step in integrin activation and a novel target for development of allosteric inhibitors.

Here, we report a novel mechanistic class of antagonists to integrins that contain I domains that we term α/β I-like allosteric antagonists. Interestingly, we have found that the small molecule compounds #3 and #4 reported by Genentech to antagonize ICAM-1 binding to integrin $\alpha_L\beta_2$ are not ligand mimetics that function competitively as originally suggested (Gadek et al., 2002). While these compounds are potent antagonists of wild-type $\alpha_L\beta_2$, they fail to inhibit binding to ICAM-1 of $\alpha_L\beta_2$ containing a mutant I domain locked in the high-affinity conformation with a disulfide bond. These compounds also fail to inhibit binding to ICAM-1 of soluble, mutant intermediate or high-affinity I domains as shown by surface plasmon resonance, or binding to ICAM-1 of the isolated, surface-displayed, wild-type I domain as shown by cell rolling assays in shear flow. The same results were obtained with compound #5, a representative of a structurally closely related group of compounds patented by Roche (Fotouhi et al., 1999, 2001). Allosteric antagonists that bind underneath the C-terminal α helix of the α_L I domain such as LFA703 and BIRT377 also blocked wild-type $\alpha_L\beta_2$ but not $\alpha_L\beta_2$ containing the mutant high-affinity I domain. Crystal structures of the mutant high-affinity α_L I domain demonstrate that it is stabi-

Table 1. Effects of Small Molecule Inhibitors on mAb Binding

		Mg^{2+} + Ca^{2+}			Mn^{2+}		
		Mean Fluorescence Intensity					
mAb	Epitope	DMSO	Comp. #5	BIRT377	DMSO	Comp. #5	BIRT377
TS1/22	α_L I domain	113 ± 4	116 ± 3	109 ± 3	120 ± 3	121 ± 3	109 ± 3
R7.1	α_L I domain	143 ± 5	136 ± 4	28 ± 3	147 ± 8	143 ± 3	34 ± 3
TS2/4	α_L β-propeller	127 ± 3	118 ± 4	124 ± 3	136 ± 2	128 ± 3	130 ± 4
NKI-L16[a]	α_L thigh/genu	108 ± 10	156 ± 5	111 ± 3	81 ± 5	154 ± 7	55 ± 5
TS1/18	β_2 I-like domain	114 ± 5	92 ± 4	112 ± 3	79 ± 2	17 ± 3	77 ± 3
IB4	β_2 I-like domain	138 ± 9	127 ± 8	139 ± 4	139 ± 5	80 ± 6	139 ± 3
m24[a]	β_2 I-like domain	7 ±1	140 ± 5	3 ± 1	142 ± 7	152 ± 3	51 ± 6
KIM127[a]	β_2 I-EGF2	6 ± 1	86 ± 7	3 ± 1	92 ± 2	98 ± 3	29 ± 4
CBRLFA-1/2	β_2 I-EGF3	110 ± 4	108 ± 7	110 ± 2	111 ± 5	107 ± 6	117 ± 3

Cells were stained with mAbs (2 μg/ml) in HBS with 1 mM $MgCl_2$ and 1 mM $CaCl_2$, or with 1 mM $MnCl_2$, in the presence of compound #5 (1 μM), BIRT377 (10 μM), or DMSO (1:1000) at 37 °C for 30 min, followed by immunofluorescence flow cytometry. Values are means ± SEM of three independent experiments. Values significantly different from the DMSO control are underlined.
[a] Activation-dependent antibody.

lized in the same open, high-affinity conformation that is seen when the α_L I domain is bound to ICAM-1 (Shimaoka et al., 2003). Thus, compounds #3–#5, BIRT377, and LFA703 do not compete for the ICAM-1 binding site. However, compounds #3–#5 clearly bind to a site distinct from BIRT377 and LFA703, as demonstrated by the ability of BIRT377 and LFA703 but not compounds #3–#5 to inhibit interactions of the wild-type, isolated, surface-expressed I domain with ICAM-1 substrates in shear flow.

Further studies emphasize the similarities in the mode of action of compounds #3 and 4 from Genentech and compound #5 from Roche, their distinction from α_L I domain allosteric antagonists, and define the binding site for compounds #3–#5. We find that compounds #3 and #4 inhibit $\alpha_M\beta_2$ in addition to $\alpha_L\beta_2$, as previously described for compound #5. Compound #5 inhibits the MLR, as previously described for compounds #3 and #4. Compounds #3–#5 induce activation epitopes on $\alpha_L\beta_2$, whereas LFA703 and BIRT377 suppress epitope induction by Mn^{2+}. Compounds #3–#5, but not LFA703 or BIRT377, stabilized association between the α_L and β_2 subunits of LFA-1 and the α_M and β_2 subunits of Mac-1 in SDS-PAGE. The binding site for compounds #3–#5 does not include the I domain because studies with mutant $\alpha_L\beta_2$ receptors from which the I domain was deleted showed that these compounds still stabilized association between the α_L and β_2 subunits and induced exposure of activation epitopes. In contrast, the compounds did not stabilize $\alpha_L\beta_2$ association or induce activation epitopes when residue Ser114 of the β_2 MIDAS was mutated to Ala, strongly suggesting that the β_2 MIDAS forms part of the binding site for these compounds. Since the α subunit β-propeller domain and the linker segments between the β-propeller domain and I domain are near the β_2 MIDAS in the integrin three-dimensional structure, and some compounds differ in affinity for $\alpha_L\beta_2$ and $\alpha_M\beta_2$, the α subunit may also participate in selectivity. Therefore, we designate compounds #3–#5 "α/β I-like allosteric antagonists," in contrast to LFA703 and BIRT377 which are α I allosteric antagonists.

The α/β I-like allosteric antagonists described here to β_2 integrins share chemical and functional properties with ligand-mimetic, α/β I-like competitive antagonists to integrins $\alpha_V\beta_3$, $\alpha_{IIb}\beta_3$, and $\alpha_4\beta_1$ that lack an I domain. RGD-mimetic competitive antagonists of $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ induce exposure of activation epitopes (Du et al., 1991; Peter et al., 2001). This is a consequence of the conformational change induced in the I-like domain that results in a movement at its interface with the hybrid domain, and conversion of the low-affinity, bent integrin conformation to the high-affinity, extended conformation (Takagi et al., 2002). Exposure of epitopes in the I-like domain, α subunit genu, and β subunit I-EGF-2 domain suggests that α/β I-like allosteric antagonists induce a similar conformational change in $\alpha_L\beta_2$ (Figure 6). Remarkably, a number of $\alpha_4\beta_1$ integrin antagonists are chemically similar to compounds #3–#5 in being polysubstituted (*S*)-2-benzoylamino propionic acids (Figure 1A); indeed, compounds #3–#5 were developed from compound #1, which was initially recognized as an $\alpha4\beta1$ antagonist and later found to inhibit $\alpha_L\beta_2$ (J. Tilley, personal communication). We propose that the α/β I-like antagonists to integrins that lack and contain I domains act analogously, except that they inhibit binding to extrinsic ligands and intrinsic ligands, respectively. The polypeptide linker between the C terminus of the I domain and β sheet 3 of the β-propeller domain including α_L residue Glu310 has been shown to be important in I domain activation (Huth et al., 2000). I domain activation is induced by a downward pull on the C-terminal α helix or linker (Salas et al., 2002; Shimaoka et al., 2002, 2003). Binding of the activated β_2 I-like domain to the "intrinsic ligand" α_L Glu310 has been proposed to pull the C-terminal α helix of the α_L and α_M I domains downward and activate high affinity for ligand (Alonso et al., 2002; Shimaoka et al., 2002; Takagi and Springer, 2002). The induction of activation-dependent epitopes by the α/β I-like allosteric antagonists described here suggests that they act as intrinsic ligand-mimetics that bind to the β_2 MIDAS, stabilize the I-like domain in the liganded state, and induce the switchblade-like opening that results in conversion of the bent to extended integrin conformation (Figure 6). Thus, the interface between the I domain linker and the I-like domain appears to be

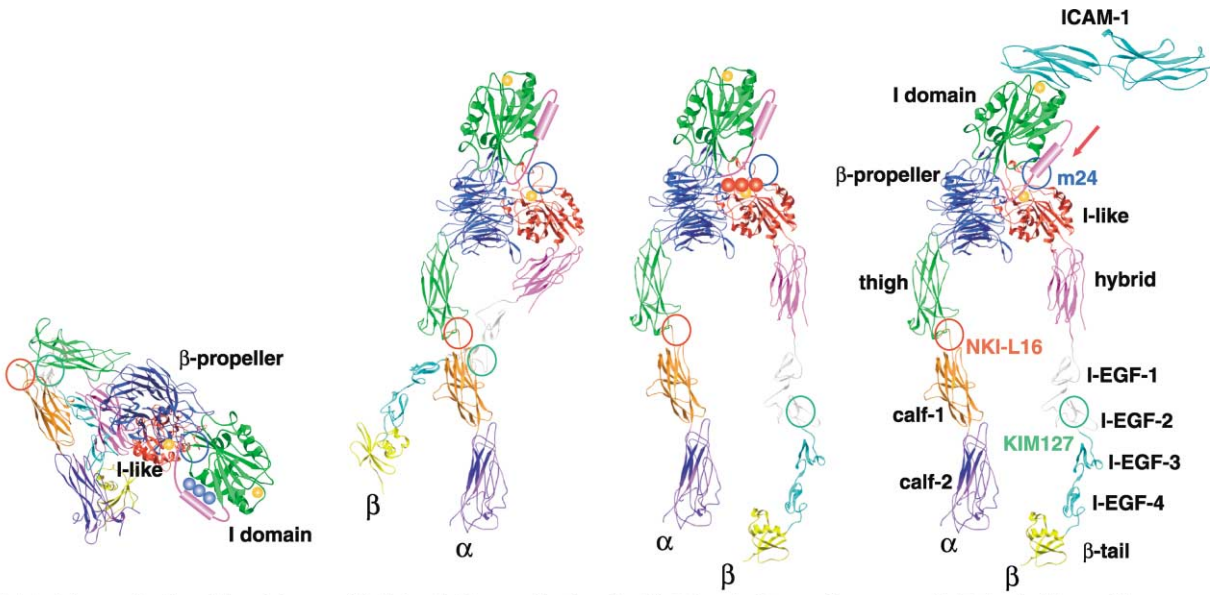


Figure 6. Model for Conformational States of the Extracellular Domain of $\alpha_L\beta_2$

The models for all of the extracellular domain except for the I domain (Springer, 2002) are based on conformational states of $\alpha_V\beta_3$ and $\alpha_L\beta_2$ defined by negative stain electron microscopy (Takagi et al., 2002), crystallography (Xiong et al., 2002), NMR (Beglova et al., 2002), and mapping of activation epitopes (Lu et al., 2001a, 2001c). The α_L I domain and its complex with ICAM-1 are cartoons based on crystal structures (Shimaoka et al., 2003); the C-terminal I domain α helix is represented by a cylinder. The I domain is joined at the point of its insertion in the β-propeller domain, but its orientation is arbitrary; the I domain and ICAM-1 are shown at slightly larger scale for emphasis. The orientation of all other domains is based on three distinct conformations of $\alpha_V\beta_3$ visualized by electron microscopy (Springer, 2002; Takagi et al., 2002). Regions to which activation epitopes map are circled. The metal ions at the MIDAS of the I and I-like domains are shown as gold spheres.

(A) Bent conformation with low affinity. This conformation is stabilized by bound α I allosteric antagonists, symbolized by three cyan spheres.

(B) Extended conformation with closed headpiece. This conformation is in equilibrium with the bent conformation and is stabilized by Mn^{2+} as shown with $\alpha_V\beta_3$ (Takagi et al., 2002).

(C) Extended conformation with open headpiece induced by bound α/β I-like allosteric antagonist (symbolized by three red spheres), which blocks conformational communication to the I domain and leaves it in the low-affinity state. The extended conformation with the open headpiece is induced by α/β I-like competitive antagonists to $\alpha_V\beta_3$ (Takagi et al., 2002).

(D) Extended conformation with open headpiece, high-affinity I domain, and bound ICAM-1. Both activation signals from within the cell and binding to ICAM-1 stabilize this active, high-affinity conformation.

important to convey bidirectional activation signals between the I and I-like domains.

Interestingly, the integrin β_1 subunit associates both with α subunits that lack and contain I domains. Antibodies to the I domains of the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins effectively block animal models of autoimmune diseases (de Fougerolles et al., 2000), yet small molecule antagonists have not yet been reported. Our findings suggest that it should be possible to develop α/β I-like allosteric antagonists of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins starting from α/β I-like competitive antagonists of $\alpha_4\beta_1$.

The α/β I-like allosteric antagonists inhibit I domain activation, whereas they stabilize the rest of the integrin in the active conformation. The antagonists appear to block ligand binding by a novel mechanism, in which they competitively inhibit binding of the I domain linker to the I-like domain and leave the I domain in the default inactive closed conformation (Figure 6). At the same time, the α/β I-like inhibitors stabilize the I-like domain in its active configuration, mimicking internal ligand binding to the β_2 MIDAS, and as a consequence of I-like domain activation stabilize the extended integrin conformation. The results of this study highlight a pathway of interdomain communication between I and I-like domains as a novel target for integrin antagonism.

Experimental Procedures

Cells

K562 transfectants expressing wild-type and mutant $\alpha_L\beta_2$, $\alpha_M\beta_2$, and isolated α_L I domain were described (Lu et al., 2001b). cDNAs encoding β_2 S114A mutations were constructed and subcloned in pcDNA 3.1 (+)/neo (Invitrogen) by standard molecular biology techniques. The plasmids containing β_2 S114A were cotransfected with vector containing wild-type α_L subunit into K562 cells and selected with 1 mg/ml G418 (Lu and Springer, 1997).

Small Molecule Inhibitors

Compound #5 (XVA143) (Welzenbach et al., 2002) was synthesized according to example 345 of the patent (Fotouhi et al., 1999) and was also obtained from Dr. Paul Gillespie (Roche, Nutley, NJ). Compounds #3 and #4 were obtained from Genentech (South San Francisco, CA) through the research reagents program (http://www.gene.com/gene/about/collaborations/contracts.jsp). LFA703 (Weitz-Schmidt et al., 2001) was from Novartis Pharma AG (Basel, Switzerland), and BIRT377 (Last-Barney et al., 2001) was from Dr. Terence Kelly (Boehringer Ingelheim Pharmaceuticals Inc, Ridgeway, CT).

Mouse Mixed Lymphocyte Reaction

The two-way murine MLR was as described (Grassberger et al., 1999). In brief, single-cell suspensions were isolated from the spleens of CBA (H-2^k) and BALB/c (H-2^d) mice, mixed, and incubated with serial dilutions of test compounds for 4 days at 37°C. [^{3}H]thymidine was added and incubated for 16 hr. Cells were harvested on filter paper, and radioactivity was counted after the addition of scintillation fluid. The background reading from proliferation of BALB/c cells alone was subtracted from all values. The proliferation of mixed cells in absence of compound was taken as the maximum activity (100%). The test compounds were dissolved in DMSO and then further diluted in assay buffer.

Binding of Soluble ICAM-1

K562 transfectants were harvested, dispensed to 5 ml polystyrene tubes (Becton Dickinson) (1×10^5 cells/tube), and washed once with HEPES-buffered saline (HBS), 10 mM EDTA, and three times with HBS. Cells were incubated in 50 μl of HBS in the presence or absence of 10 mM DTT at room temperature for 10 min. Then, 50 μl HBS containing 20 μg/ml ICAM-1-IgA fusion protein (Martin et al., 1993), 100 μg/ml FITC-labeled anti-human IgA polyclonal antibody (Pierce), 2 mM $MnCl_2$, and small molecule inhibitors, inhibitory antibodies, or appropriate controls were added. For control, ICAM-1-IgA fusion protein was replaced with purified human IgA at the same concentration. Cells were further incubated at room temperature for 20 min and washed once by 4 ml of HBS and resuspended in 100 μl HBS containing 2% paraformaldehyde. Binding of ICAM-1-IgA was analyzed by flow cytometry.

Surface Plasmon Resonance

The high-affinity (K287C/K294C) or the intermediate-affinity (L161C/F299C) I domain was perfused into a CM-5 chip bearing immobilized ICAM-1 or BSA in Tris-buffered saline containing 2 mM $MnCl_2$ at a flow rate of 10 μl/min at 25°C as described (Shimaoka et al., 2003). I domains were preincubated with 100 μM of the antagonists or 0.55 or 1.1 μM of soluble ICAM-1 in the same buffer for 30 min at room temperature and perfused onto the chip in a BIAcore 2000 instrument. The background response on the BSA surface is subtracted from each sensorgram.

Controlled Detachment in Shear Flow

Detachment in shear flow of transfectants bound to ICAM-1 substrates was as described (Salas et al., 2002).

Immunofluorescent Cytometry

Immunofluorescent cytometry was as described (Lu et al., 2001a; Lu and Springer, 1997). KIM127, m24, and NKI-L16 antibodies were kindly provided by M. Robinson, N. Hogg, and Y. van Kooyk, respectively.

Soluble Recombinant LFA-1

cDNAs encoding extracellular domains of α_L (residues 1 to 1063) and β_2 (residues 1 to 678) subunits fused to the N terminus of ACID and BASE α-helical coiled coils, respectively, were constructed and subcloned into pEF1-puro plasmids (Takagi et al., 2001). CHO lec 3.2.8.1 cells were cotransfected with the vectors containing the α and β subunits and selected in 4 μg/ml puromycin. Clones with the highest secretion level were cultured in CHO serum-free media (Sigma) supplemented with 2.5% FCS in roller bottles, and culture supernatants were harvested weekly, pooled, and stored at −80°C. Pooled culture supernatant (5 liters) was purified with TS2/4 mAb Sepharose CL-4B (Dustin et al., 1992). Soluble LFA-1 was further purified by gel filtration on a Superdex 200 column (Pharmacia) in Tris-buffered saline, 1 mM $MgCl_2$, 1 mM $CaCl_2$. Typical yield was 0.3 mg/l.

ELISA

Soluble LFA-1 (1 μg/ml) was immobilized indirectly on plastic with rabbit antibody to the ACID BASE α-helical coiled coil (Takagi et al., 2001). A panel of mouse monoclonal anti-LFA-1 antibodies (2 μg/ml in HBS, 1 mM $MgCl_2$, 1 mM $CaCl_2$) were tested for binding to immobilized LFA-1 in the presence or absence of the small molecule inhibitors. Binding of antibodies was detected by peroxidase-labeled goat anti-mouse antibody and substrate. In some experiments that included EDTA and activation by mAb CBRLFA-1/2, biotinylated KIM127 and TS2/4 as a control were used and detected by peroxidase-streptavidin and substrate.

SDS-PAGE

Soluble LFA-1 (5 μl, 0.2 mg/ml) was incubated in HBS containing 10 μM inhibitors, or DMSO (1:10000) in the presence of 1 mM $MgCl_2$, 1 mM $CaCl_2$ or 5 mM EDTA at room temperature for 10 min. Sample buffer containing 0.2% SDS with or without 10 mM DTT (5 μl) was added, mixed, and incubated 5 min at room temperature. Samples were subjected to Tris-Glycine SDS 7.5% PAGE (BioWhittaker Molecular Applications, Rockland, ME), and stained with Coomassie brilliant blue R.

Immunoblotting

K562 transfectants were washed with Hank's balanced salt solution (HBSS), 1 mM $MgCl_2$, 1 mM $CaCl_2$. Cells (2×10^7 /ml) were lysed with an equal volume of 2× lysis buffer (HBSS, 2% Triton X-100, 0.2% NP-40, 1 mM $MgCl_2$, 1 mM $CaCl_2$) supplemented with proteinase inhibitors (complete EDTA-free, Roche), incubated for 15 min on ice, and centrifuged at 14,000 rpm for 15 min. Supernatant was collected, the concentration of $\alpha_L\beta_2$ was measured by ELISA using recombinant soluble LFA-1 as standard, the concentration was adjusted to 35 nM with 1× lysis buffer, and stored at −80°C. Lysate samples diluted to 35 pM with HBS containing 1 mM $MgCl_2$/$CaCl_2$, 1 mM $MnCl_2$, or 1 mM EDTA were incubated with inhibitors at room temperature for 10 min. Samples were mixed with SDS-sample buffer and subjected to nonreducing SDS-PAGE as described above, and proteins were blotted to PVDF membrane (Bio-Rad). Blots were probed with 1 μg/ml CBR LFA-1/2 antibody to β_2, then with peroxidase-goat anti-mouse IgG (Petruzzelli et al., 1995), and visualized by luminescence (Lumi Glo, New England Biolabs, Beverly, MA).

Cell Surface Biotinylation and Immunoprecipitation

K562 transfectants washed with HBSS, 1 mM $MgCl_2$, 1 mM $CaCl_2$ (2×10^7 /ml) were incubated with 100 μM inhibitors or DMSO (1:1000) as control at 4°C for 30 min. Cell surface proteins were labeled with biotin by adding 0.5 mg/ml of Sulfo-NHC-LC-biotin (Pierce) for 1 hr at 4°C with rotation. Cells were washed three times with HBSS, 5 mM Tris HCl (pH 7.4), 1 mM $MgCl_2$, 1 mM $CaCl_2$, and resuspended in the same buffer containing the appropriate inhibitor at 2×10^7 cells/ml. Cell lysates were prepared as described above and subjected to immunoprecipitation with mAb TS2/4 to $\alpha_L\beta_2$ and protein A agarose (Pharmacia). The protein A beads were washed with Tris-buffered saline, 1 mM $MgCl_2$, 1 mM $CaCl_2$ containing 100 nM inhibitors or 0.0001% DMSO. Beads were incubated with sample buffer containing 0.2% SDS at room temperature for 5 min and centrifuged at 14,000 rpm for 30 s. Supernatant was subjected to SDS-PAGE and blotting as described above. Blots were probed with peroxidase-streptavidin and visualized by luminescence as described above.

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