Structural basis for distinctive recognition of fibrinogen γC peptide by the platelet integrin αIIbβ3

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Published Version</td>
<td>doi:10.1083/jcb.200801146</td>
</tr>
<tr>
<td>Citable link</td>
<td><a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:4878928">http://nrs.harvard.edu/urn-3:HUL.InstRepos:4878928</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA">http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA</a></td>
</tr>
</tbody>
</table>
Structural basis for distinctive recognition of fibrinogen γC peptide by the platelet integrin αIIbβ3

Timothy A. Springer,1,2 Jianghai Zhu,1,2 and Tsan Xiao1,2

1Immune Disease Institute and 2Department of Pathology, Harvard Medical School, Boston, MA 02115

Hemostasis and thrombosis (blood clotting) involve fibrinogen binding to integrin αIIbβ3 on platelets, resulting in platelet aggregation. αIIbβ3 binds fibrinogen via an Arg-Asp-Gly (RGD) motif in fibrinogen’s α subunit. αIIbβ3 also binds to fibrinogen; however, it does so via an unstructured RGD-lacking C-terminal region of the γ subunit (γC peptide). These distinct modes of fibrinogen binding enable αIIbβ3 and αIIbβ3 to function cooperatively in hemostasis. In this study, crystal structures reveal the integrin αIIbβ3-γC peptide interface, and, for comparison, integrin αIIbβ3 bound to a lamprey γC primordial RGD motif. Compared with RGD, the GAKQA-GDV motif in γC adopts a different backbone configuration and binds over a more extended region. The integrin metal ion–dependent adhesion site (MIDAS) Mg2+ ion binds the γC Asp side chain. The adjacent to MIDAS (ADIMIDAS) Ca2+ ion binds the γC C terminus, revealing a contribution for ADIMIDAS in ligand binding. Structural data from this natively disordered γC peptide enhances our understanding of the involvement of γC peptide and integrin αIIbβ3 in hemostasis and thrombosis.

Introduction

Integrins are formed from α and β subunits, each with a large extracellular domain and a single, more C-terminal transmembrane domain. The subunits come together in a large interface between the α subunit β propeller domain and the β subunit I domain to form the ligand-binding head. Other domains form upper and lower legs in each subunit to connect the head to the membrane. Integrins have an overall bent conformation in the low affinity state, with the bend between the upper and lower legs. Upon activation, integrins extend, and a major reorientation at the interface between the I and hybrid domains occurs that is linked to remodeling of the ligand-binding site in the β I domain (Luo et al., 2007). The β I domain has three metal ion–binding sites, with a Mg2+ ion in the central metal ion–dependent adhesion site (MIDAS) flanked by two Ca2+ ions, one of which is in a site termed adjacent to MIDAS (ADIMIDAS). Structures of αIIbβ3 and αIIbβ3 bound to cyclic RGD-like peptides show binding across the α subunit β propeller interface with the β subunit I domain. The Asp side chain coordinates the Mg2+ ion at the β subunit I MIDAS, whereas the Arg side chain binds to Asp residues in the α subunit β propeller domain (Xiong et al., 2002; Xiao et al., 2004).

Early in hemostasis and thrombosis, the integrin αIIbβ3 on platelets is activated and binds to its ligand fibrinogen. Fibrinogen is a dumbbell-shaped molecule (Fig. 1 a). Integrin αIIbβ3 binds specifically to the distal ends of the dimeric fibrinogen molecule in a natively unstructured region at the C terminus of the γ subunit (γC peptide; Yang et al., 2001). The large separation between the two αIIbβ3-binding sites on fibrinogen of ~440 Å is well suited for cross-linking of platelets, which results in platelet aggregation and formation of platelet plugs in hemostasis and thrombosis. In a later event in hemostasis, cleavage of peptides from the N termini of the fibrinogen α and β subunits stimulates assembly of fibrinogen into fibrin. Yet later, adjacent fibrinogen molecules within fibrin are cross-linked through their γC peptides by the factor XIIIa transglutaminase (Hawiger, 1995; Bennett, 2001).

One of the best established paradigms in integrin biology is the recognition of protein ligands through Arg-Gly-Asp (RGD) sequences, the majority of which are present within flexible loop regions. Eight vertebrate integrins, including αIIbβ3 and αIIbβ3, recognize RGD sequences in ligands, and crystal structures are beginning to reveal how RGD is recognized, at least in cyclic peptides (Xiong et al., 2002; Xiao et al., 2004).
be anchored on endothelium through $\alpha_{v}\beta_3$ (Cheresh et al., 1989; Smith et al., 1990).

Whether RGD and $\gamma C$ peptides bind to distinct or similar sites on $\alpha_{IIb}\beta_3$ is controversial. Several studies show competition for the same binding site (Lam et al., 1987; Santoro and Lawing, 1987; Bennett, 2001). Interestingly, although $\gamma C$ and RGD peptides were found to cross-compete, a $\gamma C$ dodecapeptide was photo-cross-linked only to the $\alpha_{IIb}$ subunit, whereas a RGD hexapeptide was cross-linked to both the $\alpha_{IIb}$ and $\beta_3$ subunits (Santoro and Lawing, 1987). Binding has been described of RGD and $\gamma C$ peptides to distinct, nonoverlapping, allosterically linked sites (Hu et al., 1999). RGD and RGD-like peptides have also been described to bind to distinct sites on $\alpha_{IIb}\beta_3$ (Cierniewski et al., 1999). Furthermore, $\gamma C$ peptide was reported to cross-link to an $\alpha_{IIb}$ site (D’Souza et al., 1990) that is distal from the binding site for RGD shown in crystal structures (Xiao et al., 2004).

Because of the biological and clinical importance of the $\gamma C$ peptide in hemostasis and thrombosis, there has been great interest in determining its biologically relevant integrin-bound...
**Results**

**Overall complex structure**

We soaked human γC deca- and dodecapeptides into α1β3β1 headpiece/Fab crystals (Xiao et al., 2004). Examination of fibrinogen γ subunit sequences in a diverse range of vertebrates (fibrinogen is found only in vertebrates) revealed that RGD is found in γC in frog and lamprey (Fig. 1 b). This observation, which, to our knowledge, is previously unremarked and therefore was a surprise to us, suggests an obvious evolutionary pathway from promiscuous integrin recognition of RGD in the primordial jawless vertebrate the lamprey to monospecific integrin recognition of non-RGD sequences in most higher vertebrates. Therefore, for comparison to human γC, we also soaked crystals with chimeric deca- or dodecapeptides containing the RGD sequence present in lamprey (Fig. 1 b). Structures were determined at 2.4–2.8-Å resolution (Table I), and previous structures with antagonists were reefined to lower Rfree (Table II). The structures contain the β propeller domain in the α1β3 subunit; the I, hybrid, plexin-semaphorin integrin, and integrin EGF-like (I-EGF) domain 1 in the β1 subunit; 15 carbohydrate residues; and bound ligand and Fab. The structures all have an open headpiece (i.e., with the hybrid domain swung out) and the β I domain in the high affinity state (Xiao et al., 2004).

The γC peptide binds at the interface between the α1β3 β propeller and β1 I domains (Fig. 2 a). The binding site overlaps with, but is more extensive on both the α and β subunits, than previously described for cyclic RGD peptides (Xiong et al., 2002; Xiao et al., 2004). The contacts on the β3 subunit include not only MIDAS but also a novel water-mediated coordination of the γC C terminus with ADMIDAS, as described in more detail in the next section. The γC peptide contacts with α1β3 lie in a groove between two long loops that connect blades (β sheets of the α1β3 β propeller) 2 and 3 and blades 3 and 4. The cap subdomain of the β propeller (Xiao et al., 2004) forms one side of this groove (Fig. 2 b). In the region of the γC peptide N terminal to residue 404, extension along the groove

**Table I. α1β3 γC peptide complex x-ray diffraction and refinement data**

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>HHLGGAKGAGDV</th>
<th>LGGAKQAGDV</th>
<th>HHLGGAQRQGDV</th>
<th>LGGAKQRQGDV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Space group</strong></td>
<td>P2₁,2₁</td>
<td>P2₁,2₁</td>
<td>P2₁,2₁</td>
<td>P2₁,2₁</td>
</tr>
<tr>
<td><strong>Unit cell (a, b, c) (Å)</strong></td>
<td>148.3, 148.3, 176.6</td>
<td>148.4, 148.4, 177.2</td>
<td>148.5, 148.5, 176.4</td>
<td>148.3, 148.3, 176.8</td>
</tr>
<tr>
<td>α, β, γ (degree)</td>
<td>90, 90, 120</td>
<td>90, 90, 120</td>
<td>90, 90, 120</td>
<td>90, 90, 120</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.07223</td>
<td>1.07223</td>
<td>1.07223</td>
<td>1.07223</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>50–2.5</td>
<td>50–2.8</td>
<td>50–2.6</td>
<td>50–2.4</td>
</tr>
<tr>
<td><strong>Number of reflections (total/unique)</strong></td>
<td>557,015/77,023</td>
<td>362,650/55,536</td>
<td>416,764/68,878</td>
<td>609,049/87,704</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.8/100h</td>
<td>99.4/99.6h</td>
<td>98.5/87.4</td>
<td>99.7/99.0h</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>17.6/3.5h</td>
<td>16.2/2.8h</td>
<td>19.1/2.2h</td>
<td>17.3/2.3h</td>
</tr>
<tr>
<td><strong>Ramachandran statistics</strong></td>
<td>96.9/3.0/0.1</td>
<td>96.5/3.4/0.1</td>
<td>97.1/2.6/0.2</td>
<td>97.1/2.7/0.2</td>
</tr>
<tr>
<td>(% favored/allowed/outlier)*</td>
<td>2VD code</td>
<td>2VDQ</td>
<td>2VDR</td>
<td></td>
</tr>
<tr>
<td>Rwork (%)</td>
<td>14.8</td>
<td>15.6</td>
<td>14.1</td>
<td>14.8</td>
</tr>
<tr>
<td>Rfree (%)</td>
<td>19.0</td>
<td>20.4</td>
<td>19.0</td>
<td>19.3</td>
</tr>
</tbody>
</table>

*These numbers correspond to the last resolution shell.

**Overall complex structure**

We soaked human γC deca- and dodecapeptides into α1β3β1 headpiece/Fab crystals (Xiao et al., 2004). Examination of fibrinogen γ subunit sequences in a diverse range of vertebrates (fibrinogen is found only in vertebrates) revealed that RGD is found in γC in frog and lamprey (Fig. 1 b). This observation, which, to our knowledge, is previously unremarked and therefore was a surprise to us, suggests an obvious evolutionary pathway from promiscuous integrin recognition of RGD in the primordial jawless vertebrate the lamprey to monospecific integrin recognition of non-RGD sequences in most higher vertebrates. Therefore, for comparison to human γC, we also soaked crystals with chimeric deca- or dodecapeptides containing the RGD sequence present in lamprey (Fig. 1 b). Structures were determined at 2.4–2.8-Å resolution (Table I), and previous structures with antagonists were re-refined to lower Rfree (Table II). The structures contain the β propeller domain in the α1β3 subunit; the I, hybrid, plexin-semaphorin integrin, and integrin EGF-like (I-EGF) domain 1 in the β1 subunit; 15 carbohydrate residues; and bound ligand and Fab. The structures all have an open headpiece (i.e., with the hybrid domain swung out) and the β I domain in the high affinity state (Xiao et al., 2004).

The γC peptide binds at the interface between the α1β3 β propeller and β1 I domains (Fig. 2 a). The binding site overlaps with, but is more extensive on both the α and β subunits, than previously described for cyclic RGD peptides (Xiong et al., 2002; Xiao et al., 2004). The contacts on the β3 subunit include not only MIDAS but also a novel water-mediated coordination of the γC C terminus with ADMIDAS, as described in more detail in the next section. The γC peptide contacts with α1β3 lie in a groove between two long loops that connect blades (β sheets of the α1β3 β propeller) 2 and 3 and blades 3 and 4. The cap subdomain of the β propeller (Xiao et al., 2004) forms one side of this groove (Fig. 2 b). In the region of the γC peptide N terminal to residue 404, extension along the groove...
is blocked by contact of γC residue Leu-402 with a neighboring molecule in the crystal lattice, and residues 400–403 have weak electron density and extend toward solvent. Therefore, we limit our structural analysis to γC octapeptide residues 404–411, which show strong electron density in omit maps, intimately contact αββ3 (Fig. 2 b, and Fig. 3 a), and have essentially identical conformations in crystals soaked with γC peptides 400–411 and 402–411 (Table I).

Surface representations of the binding site show not only the groove in which the γC peptide lies but two deeper pockets within this groove (Fig. 2 b). The penultimate residue in the γC peptide, Asp-410, buries its side chain in a pocket at the MIDAS Mg2+ ion in the β3 domain. The surface at the MIDAS Mg2+ ion is not visible in this view, which is in contrast to the surface at the ADMIDAS Ca2+ ion (Fig. 2 b, purple), which is much more solvent exposed. The other deep pocket, on the αββ3 side of the interface, is occupied by Lys-406 of the γC peptide (Fig. 2 b). This is the same pocket that is occupied by the Arg side chain of RGD peptides.

Fig. 3 compares the binding modality of the γC peptide (Fig. 3 a) to that of RGD-like peptides (Fig. 3 b–d). We first will describe the RGD peptide-binding modality and then return to γC and compare it with RGD. The A408R substitution yielding the lamprey-like RGD motif in the chimera peptide γC (Fig. 1 b) results in retention of αββ3 antagonist activity (Kloczewiak et al., 1989). In the chimera γC peptide, the Arg-408 side chain extends directly into the αββ3-binding pocket (Fig. 3 b) and displaces Lys-406, which binds in this pocket in the γC peptide (Fig. 3 a).

In contrast to previous integrin structures with cyclized peptides (Xiong et al., 2002; Xiao et al., 2004), the αββ3 complexes with the chimera peptides show how an RGD peptide unconstrained by cyclization binds to an integrin and provide the best comparison with the γC peptide. The RGD tripeptide adopts a highly extended conformation across the integrin αβ3 intersubunit interface (Fig. 3 b). The Arg and Asp side chains extend in opposite directions, and the backbone in between is also extended. The conformation of the ligand backbone is stabilized as it crosses the interface between the αββ3 and β3 subunits by two previously unremarked water molecules with strong density that are held in place by hydrogen bonds to the side chain of αββ3 Asp-232 and backbone of β3 Ala-218 and that hydrogen bond to the carbonyl oxygen of the Arg of RGD (Fig. 3 b). The Arg side chain of RGD forms a charged hydrogen bond to αββ3 residue Asp-224 and also forms a hydrogen bond to an αββ3 backbone carboxyl. The Asp side chain of RGD directly coordinates to the MIDAS Mg2+ cation and is further satisfied by three hydrogen bonds to the backbone nitrogens of the β3 Asn-215. The same geometry at the Asp is seen for all αββ3 complexes, which are in the high affinity state (Fig. 3, a–c). Only one of the four Asp hydrogen bonds are seen at the Asp of the αββ3 complex (Fig. 3 d) because it has a closed headpiece, and the two backbone nitrogens of the β3-α loop have not moved within hydrogen bonding distance; furthermore, the side chain of Asn-215 should probably be flipped.

By reducing the loss of peptide entropy upon binding, cyclization can increase affinity and is therefore widely adopted in drug development. Eptifibatide is a seven-residue L peptide that is cyclized with a disulfide bond, contains homoarginine-Gly-Asp in place of RGD, and is in clinical use for prevention
Asp moiety of γC has a conformation identical to that of Arg-Gly-Asp, and the carbonyl oxygen of Ala-408 hydrogen bonds to the two water molecules held in place by αIIb Asp-232 at the interface with the β3 subunit in a geometry identical to that seen for the Arg and homoarginine carbonyl oxygens of RGD and eptifibatide (Fig. 3, a–c). Indeed, the Cβ atoms of Ala-408 of γC and the Arg of RGD occupy identical positions (Fig. 3, a and b). The critical difference between the γC peptide– and RGD peptide–binding modalities is the backbone turn at γC Gln-407, which orients the N-terminal portion of the γC peptide into the groove formed by the long loops that connect αIIb propeller blades 2 and 3 and blades 3 and 4 (Fig. 3 a). The backbone turn at γC Gln-407 enables the γC Lys-406 side chain to enter the αIIb binding pocket from a markedly different location than the Arg side chain of RGD (Fig. 3, a and b). The last few atoms of the Lys-406 side chain turn to approach αIIb Asp-224 from a similar direction as Arg (Fig. 3, a and b). A 15-fold loss of potency upon substitution of Lys-406 with Arg (Kloczewiak et al., 1989) is explicable by the inability of the planar guanido group of Arg to similarly turn. Hydrophobic αIIb residues Tyr-190, Leu-192, and Phe-231 line the pocket and contact the aliphatic portion of the γC Lys-406 side chain and the Ala-408 side chain.

Cilengetide, an αvβ3 antagonist, is a five-residue peptide that contains RGD, N-methyl-Val, and d-Phe (Fig. 3 d). The Arg of cilengitide forms a charged hydrogen bond to αv residue Asp-218. Cilengetide is cyclized through its peptide backbone in a considerably smaller ring than eptifibatide. Tight cyclization, which is important to prevent binding to αIIbβ3 (Scarborough and Gretler, 2000; Gottschalk and Kessler, 2002), pulls the Arg backbone back away from the αv subunit and helps place the Arg side chain further above the subunit than the Arg or homoarginine of αIIb ligands, as appropriate for the shallower αv binding pocket (Fig. 3 d).

The fibrinogen γC peptide complex with αIIbβ3 reveals that γC residue Lys-406 forms a charged hydrogen bond to αIIb residue Asp-224 (Fig. 3 a). Therefore, γC Lys-406 is functionally equivalent to the Arg of RGD (Fig. 3 b) and the homoarginine of eptifibatide (Fig. 3 c). Furthermore, the Ala-Gly-Asp moiety of γC has a conformation identical to that of Arg-Gly-Asp, and the carbonyl oxygen of its homoarginine residue similarly hydrogen bonds to the waters held in place by αIIb Asp-232 (Fig. 3 c). However, cyclization pins back the homoarginine Cα and N atoms so that the Cγ atom of homoarginine is in a similar position to the Cβ atom of Arg, explaining the requirement for the one-carbon longer homoarginine side chain (Fig. 3, b and c).

Eptifibatide binds similarly to RGD, and the carbonyl oxygen of its homoarginine residue similarly hydrogen bonds to the waters held in place by αIIb Asp-232 (Fig. 3 c). However, cyclization pins back the homoarginine Cα and N atoms so that the Cγ atom of homoarginine is in a similar position to the Cβ atom of Arg, explaining the requirement for the one-carbon longer homoarginine side chain (Fig. 3, b and c).
\( \gamma C \) residues Gly-404, Ala-405, and Lys-406 fill the groove between adjacent \( \beta \) propeller blades and form backbone–backbone and backbone–side chain hydrogen bonds to \( \alpha_m \) residues Asp-159 and Ser-226 (Fig. 3 a). \( \gamma C \) residues Gly-404 and Ala-405 also seal the binding pocket for the Lys-406 side chain, with \( \gamma C \) residue Gly-404 forming a backbone hydrogen bond to the \( \gamma C \) Lys-406 side chain.

**ADMIDAS coordination**

An important and unexpected observation is that the free carboxyl group of the \( \gamma C \) C-terminal residue Val-411 coordinates the \( \beta_3 \) I domain ADMIDAS calcium ion through an intermediate water molecule with strong density (Fig. 3 a). The contribution of this coordination to ligand binding is demonstrated by the finding that amidation of the \( \alpha \)-carboxyl group decreases by sixfold the potency of \( \gamma C \) peptides in inhibiting fibrinogen binding to \( \alpha_m \beta_3 \) (Kloczewiak et al., 1989). An identical ADMIDAS coordination is seen with the chimera QRGDV peptide (Fig. 3 b). The significance of this observation, the notable similarity in backbone positions of Val-411 of the \( \gamma C \) and chimera peptides, and the equivalent residue in eptifibatide, is discussed in the next section.

**Discussion**

**Specific recognition of the fibrinogen \( \gamma C \) peptide by \( \alpha_m \beta_3 \)**

Our structures of \( \gamma C \) peptides bound to \( \alpha_m \beta_3 \) reveal the interface that is biologically important for the binding of fibrinogen to activated integrin \( \alpha_m \beta_3 \) on platelets. This binding event, in turn, leads to cross-linking of platelets by fibrinogen and the formation of platelet plugs in hemostasis and thrombosis. Whereas Arg-Gly-Asp binds to eight different integrins, the fibrinogen \( \gamma C \) peptide binds only to \( \alpha_m \beta_3 \).

Our \( \gamma C \) peptide complex with \( \alpha_m \beta_3 \) reveals the basis for binding of platelet \( \alpha_m \beta_3 \) and endothelial \( \alpha_\mathbf{\nu} \beta_3 \) to distinct sites in fibrinogen, enabling these integrins to have complementary rather than competitive functions in hemostasis (Fig. 1 a; Cheresh et al., 1989; Smith et al., 1990). The interface on the integrin \( \alpha \) subunit occupied by the \( \gamma C \) peptide is much larger than that occupied by RGD. \( \gamma C \) residues Gly-404, Ala-405, Lys-406, and Ala-408 all interact with \( \alpha_m \). In contrast, in the linear RGD chimera peptide, only Arg-408 binds to the \( \alpha_m \) subunit. This much more extensive interface with \( \gamma C \) enables specific differences between \( \alpha_m \) and other integrin \( \alpha \) subunits to be recognized as good contacts made by \( \alpha_m \) and clashes made by \( \alpha_\mathbf{\nu} \). The shallower and narrower binding site in the \( \alpha_m \) subunit precludes binding of the KQAGDV moiety using the \( \alpha_m \beta_3 \)-bound conformation. For example, \( \alpha_\mathbf{\nu} \) Ala-215 and Asp-218 would both clash with KQAGDV. These results show why the \( \gamma C \) peptide does not bind to \( \alpha_\mathbf{\nu} \).

In contrast, RGD does bind to \( \alpha_m \beta_3 \), and, therefore, the selectivity of \( \alpha_m \beta_3 \) for the \( \gamma C \) site over RGD in fibrinogen (Cheresh et al., 1989; Smith et al., 1990) must reflect a higher affinity for \( \gamma C \). Fibrinogen lacking the \( \gamma C \) QAGDV pentapeptide fails to bind \( \alpha_m \beta_3 \) and results in bleeding disorders in mice (Farrell et al., 1992; Holmback et al., 1996). This emphasizes
the absolutely critical role of the γC peptide in recognition by αΙβ3; however, it does not mean that the γC peptide is responsible for all of αΙβ3’s affinity for fibrinogen. Indeed, αΙβ3 has a 100-fold higher affinity for fibrinogen than for the γC octapeptide (Kloczewiak et al., 1983, 1984). Mutagenesis studies show that the binding site on the αΙβ3 β propeller domain includes, but is larger than, the γC octapeptide–binding site defined here (Kamata et al., 2001; Xiao et al., 2004). The 10E5 Fab binds within this region of the αΙβ3 β propeller that is important in fibrinogen binding and blocks binding of fibrinogen but not the octapeptide to αΙβ3β. Because the N terminus of the octapeptide extends toward the 10E5 Fab–binding site (Fig. 2), it is tempting to speculate that regions N terminal to the γC peptide, including well-folded γC module residues 144–392, also bind to αΙβ3β and, together with the γC peptide, account for the higher biological affinity of αΙβ3β for fibrinogen than for RGD peptides. Such other regions of the γC domain may also account for the retention of clot-retraction activity by fibrinogen deleted in the QAGDV pentapeptide (Holmback et al., 1996). Integrin binding or inhibition of clot retraction has been demonstrated using portions of γC N terminal to QAGDV made in Escherichia coli (Medved et al., 1997; Yokoyama et al., 1999; Podolnikova et al., 2003); however, E. coli γC fragments also express integrin-binding sites that are not present on native fibrinogen (Akakura et al., 2006).

Multiple crystal structures of fibrinogen and its fragments show that the γC chain residues 404–411 are disordered and that residues 393–403, when ordered, differ in conformation from one crystal lattice to another (Pratt et al., 1997; Spraggon et al., 1997; Yee et al., 1997; Yang et al., 2001; Kostelansky et al., 2002). Previous intense and creative efforts using carrier protein-driven crystallization (Donahue et al., 1994; Ware et al., 1999) failed to reveal the biologically relevant conformation of the γC peptide.

Our structures reveal the conformation that γC residues 404–411 adopt when bound to αΙβ3β. Although not inconsistent with the previous conclusion that QAGDV represents a minimal recognition unit in γC, our structure resolves the conundrum that QAGDV lacks a basic residue by demonstrating that Lys-406 fulfills a function similar to the Arg in RGD and that the KQAGDV motif in γC occupies the same site as RGD. Residues Gly-404 and Ala-405 also appear to have an important role in sealing the binding pocket for Lys-406 in the αΙβ3 groove.

In the absence of information on how the γC peptide binds αΙβ3β, development of the two currently approved small molecule αΙβ3β therapeutics, tirofiban and epifibatide, proceeded from RGD and barbourin, respectively (Scarborough and Gretler, 2000). Barbourin is a disintegrin with two highly unusual properties for a disintegrin: specificity for αΙβ3β and KGD in place of the RGD motif (Scarborough et al., 1991). Lysine has a side chain with Cβ, Cγ, Cε, and Nɛ atoms. The Arg side chain has Cβ, Cγ, and Cε atoms and a guanido group with Nɛ, Nη1, Nη2, and Cζ atoms. The three N atoms of Arg give it versatility for hydrogen bonding. Although the Arg side chain readily forms charged hydrogen bonds through different guanido nitrogens to αΙβ1–Asp-224 in Fig. 3 b and αγ–Asp-218 in Fig. 3 d, it is easy to imagine how a Lys in a similar position might better form a hydrogen bond to αΙβ1–Asp-224 than to αγ–Asp-218. The final drug developed from barbourin, epifibatide, has a homoarginine side chain in place of lysine (Fig. 3 c). This drug binds very similarly to RGD (Fig. 3 b) and quite differently from the γC peptide, in which the Lys side chain enters the αΙβ3β binding pocket from a completely different direction (Fig. 3 a). Thus, drug design starting from KGD ironically resulted in an RGD-like antagonist with a binding modality very different from KQAGDV.

The γC complex structure provides new insights for the development of second generation integrin antagonists. Mimicking the novel route into the αΙβ3β binding pocket adopted by the Lys of the KQAGDV motif should enable development of new classes of highly specific αΙβ3β antithrombotics. Furthermore, mimicking the novel interaction with the ADMIDAS metal ion is attractive for improving the specificity and affinity of antagonists to a wide range of integrins.

The finding that the γC peptide–binding site completely overlaps the RGD peptide–binding site definitively resolves the long-standing controversy about whether these peptides compete for binding to the same site or bind to distinct sites (Lam et al., 1987; Santoro and Lawing, 1987; D’Souza et al., 1990; Hu et al., 1999; Bennett, 2001). Furthermore, our studies explain previous observations of photo–cross-linking of the γC dodecapeptide to only the αΙβ3 subunit and of an RGD hexapeptide to both the αΙβ3 and β3 subunits, with cross-competition by RGD and γC peptides, respectively (Santoro and Lawing, 1987). Both peptides appeared to have the radiolabeled, photoactivatable reagent attached to their N-terminal α-amino groups. The exclusive labeling of αΙβ3 by the reagent attached to γC His-400 is consistent with the position of Gly-404 in our structures in the αΙβ3 β propeller groove and also with further extension in the N-terminal direction in this groove. In contrast, labeling of both the αΙβ3 and β3 subunits by the reagent attached to the residue before the Arg of RGD is consistent with the position of the corresponding Gln-407 residue at the interface between the αΙβ3 and β3 subunits in the chimera deca- and dodecamer structures.

A role for ADMIDAS in ligand binding

Previous crystal structures and mutation of MIDAS-coordinating residues have shown that the MIDAS metal ion has a direct role in ligand binding (Luo et al., 2007). In contrast, the ADMIDAS metal ion has been shown to have a regulatory role in ligand binding. During conversion from the low affinity, closed conformation to the high affinity, open conformation of the integrin headpiece, remodeling of the β3-α1 loop that coordinates to the ADMIDAS metal ion shifts the position of this metal ion by 3 Å (Xiao et al., 2004). Ca2+ and Mn2+ compete for binding to ADMIDAS, resulting in inhibition or stimulation of ligand binding, respectively (Chen et al., 2003). We note that physiologically and in the current structures, Ca2+ is present at ADMIDAS (Xiao et al., 2004) and that coordination is pentagonal bipyramidal, with seven oxygen ligands as typically seen for Ca2+.

ADMIDAS mutations augment ligand binding by integrins αβ3 and αβ2 (Chen et al., 2003, 2006) and, in contrast, inhibit ligand binding by αΙβ3β and αβ2 (Bajt and Loftus, 1994; Mould et al., 2003). In this study, we find that in addition to its regulatory role, ADMIDAS can also directly contribute to ligand
binding. The γC C-terminal COOH group coordinates the ADMIDAS Ca\(^{2+}\) ion through water with strong electron density. The importance of this interaction is demonstrated by the sixfold loss in inhibitory potency of γC peptides when the C terminus is amidated.

It should be noted that the amidation experiment only demonstrates that coordination to a charged carboxyl oxygen is stronger than to a carbonyl oxygen. Both types of oxygens form direct and indirect water-mediated coordinations to Ca\(^{2+}\) (Harding, 2001). Therefore, the importance of ADMIDAS coordination may extend beyond fibrinogen to include ligands with RGD sequences that are not followed by the C-terminal residue. In epifibatide, the Arg-Gly-Asp-Trp-Pro-Cys sequence places a Pro in the same position as in the Arg-Gly-Asp-Ser-Pro sequence of fibronectin; this Pro, the bulky Trp, and cyclization place constraints on backbone conformation. Nonetheless, the Trp carbonyl oxygen in epifibatide is in a position very similar to that of one of the two α-carboxyl oxygens of the γC and chimera peptide Val-411 residues (Fig. 3, a–c). In the absence of the constraints in the cyclic peptide, this carbonyl could move slightly and form a water-mediated coordination to the ADMIDAS similarly to the γC and RGD chimera peptide Val-411 carboxyl group. Water with weak density may be in a position to make this coordination in the epifibatide complex structure (Fig. 3 c). Furthermore, the carbonyl oxygen of the amidated Cys two residues after the Trp forms a water-mediated coordination to the ADMIDAS (Fig. 3 c).

Water-mediated ADMIDAS coordination to RGD ligands provides a plausible explanation for the finding that mutation of ADMIDAS-coordinating residues inhibits RGD-dependent binding of α\(\beta_2\) to fibronectin (Mould et al., 2003) and binding of α\(\mu\)β\(\beta_2\) to the peptide GRGDSP (Bajt and Loftus, 1994), although ADMIDAS mutations also appear to favor the inactive conformation in the epifibatide complex structure (Fig. 3 c). Furthermore, the carbonyl oxygen of the amidated Cys two residues after the Trp forms a water-mediated coordination to the ADMIDAS (Fig. 3 c).

Evolution of the KQAGDV motif from an RGD motif in fibrinogen

Fibrinogen first evolved in vertebrates (Jiang and Doolittle, 2003). The significance of ADMIDAS coordination by the C-terminal carboxyl group of the fibrinogen γ subunit is emphasized by the fact that in vertebrates, the position of the C-terminal carboxyl group relative to the penultimate Asp residue has been invariant for the last 450 million years (Fig. 1 b; Strong et al., 1985). Thus, the interaction with ADMIDAS of the C-terminal carboxyl group first evolved in jawless vertebrates in the context of an RGD motif and was maintained when warm-blooded vertebrates evolved the KQ(A/V)GD motif and bony fishes evolved the KQFGG(II)GD motif in place of the RGD motif (Fig. 1 b). Our structures also explain why in fibrinogen the residue that takes the place of the Arg in RGD is conserved as a hydrophobic residue and suggest that the three-residue insertion in bony fishes can be accommodated as a longer turn between the Lys and this hydrophobic residue.

The presence of an RGD motif in both lamprey and frog and the presence of a KQ(A/V)GDX motif in warm-blooded vertebrates and a KQFGG(II)GD motif in bony fishes (Fig. 1 b) raises the possibility that evolution from the recognition of Arg to Lys occurred more than once. A plausible stepping stone in this evolutionary process is provided by the function of Lys in fibrinogen cross-linking. In lamprey as well as in higher vertebrates, fibrinogen is cross-linked within the γC peptide by the factor XIIIa transglutaminase (Strong et al., 1985). Lys-406 is cross-linked to Gln-398 or Gln-399 in human, and the Lys N-terminal to the RGD in lamprey is cross-linked to one of the more N-terminal Gln residues (Strong et al., 1985). Xenopus laevis appears similar to lamprey, with Lys and Gln residues N-terminal to the RGD. In contrast, humans, other warm-blooded vertebrates, and bony fishes resemble one another by all having the lysine that is known (human) or implicated (other species) in binding to α\(\mu\)β\(\beta_2\) as the same lysine that is known (human) or implicated (other species) in cross-linking. In γC peptides with RGD motifs, the Lys that functions in cross-linking could later have evolved a second function in specific recognition of a platelet integrin, providing an evolutionary stepping stone from RGD-based recognition of fibrinogen by multiple integrins to Lys-based recognition of fibrinogen by a specific integrin on platelets or thrombocytes. Whether there is any more significance to the dual function of Lys-406 in human fibrinogen γ is unclear. However, there would be little, if any, competition between these two functions of Lys-406 because fibrinogen binding to platelets occurs much earlier in the clotting cascade than fibrin formation, and the fibrinogen that contributes to fibrin formation is in great excess over that bound to platelet integrin α\(\mu\)β\(\beta_2\).

Materials and methods

The γC dodecapeptide was obtained from the American Peptide Co., and other peptides were synthesized and verified by mass spectrometry by the biopolymer facility at the Department of Biological Chemistry and Molecular Pharmacology at Harvard Medical School (Boston, MA). Crystals of the α\(\mu\)β\(\beta_2\) headpiece bound to 10E5 Fab in 11% polyethylene glycol 3350, 0.7 M magnesium acetate, and 0.1 M cacodylate, pH 6.5 (Xiao et al., 2004), were cryoprotected with a solution containing 15% polyethylene glycol 3350, 0.7 M magnesium acetate, and 0.1 M imidazole, pH 6.5, plus 5% step increases of glycerol concentration to a final 20% glycerol, and then 50 μM of synthetic peptides was added in the final cocktail. Crystals were soaked for 4 d at 4 °C before freezing in liquid nitrogen. Diffraction data were collected with beamline 19ID (Advanced Photon Source) and processed with program suite HKL2000 (Otwinowski and Minor, 1997). As there is little difference in the dimensions of the unit cell compared with nonpeptide-soaked crystals, the previously determined structure of the α\(\mu\)β\(\beta_2\) headpiece complexed with 10E5 Fab [1TXV; 2.7 Å] was directly subjected to rigid body refinement program CNS (Brunger et al., 1998) using the diffraction data. Electron density calculated using phases from the refined structure clearly showed the presence of the peptides. The peptide structures were built with program O and subjected to iterative cycles of model rebuilding in O and refinement using CNS (version 1.1) as previously described (Xiao et al., 2004).

Subsequent rebuilding with Coot and refinement with REFMAC5 (Murshudov et al., 1997) resulted in structures with unusually low Rfree (Tables I and II), which may be attributed to multiple factors. Regions for rebuilding were identified using multiple verification tools in Coot to detect structural defects supplemented with improved definition of Ramachandran outliers, clashes, and improbable rotamers using MolProbity (Davis et al., 2007). Rebuilding focused on the higher resolution 2.4 and 2.6 Å γC.
chimera deco- and dodecadipeptide structures. These structures were refined in parallel so that rebuilding could focus on problems identified by verification tools or discrepancies between the model and electron density that were shared between two independent datasets. One of the shared discrepancies was a clash at αββ6 residue 2β2 accompanied by a lack of density for the putative Ala-2β6 Cβ atom; checking of the nucleotide sequence used for αββ6 expression and RefSeq entry gi|87585615 reveals Gly at this position compared with Ala in P08514. Rebuilding of the Fab was aided by comparison with multiple high resolution Vβ, Vγ, and Cγ domains that were found by Blast search of the protein database and were individually superimposed on the 10E5 Fab. In the four γC and chimeric γC peptide complexes, Fab residue Asn-157 in the light chain appears to have largely undergone degradation with conversion to aspartic acid. This residue is in the Asn-Gly region, the sequence known to be most susceptible to deamidation (Aswad et al., 2000), and previous protein chemistry on the mouse light chain peptide containing this sequence revealed heterogeneity, suggesting chemical rearrangement (Savitski and Milstein, 1972). The previously determined headpiece structures (Table I) did not show evidence for aspartic acid, which is consistent with deamidation during prolonged storage of the Fab before preparation of the more recent crystals (Table I). Although aspartic acid has been built in one case (Esposito et al., 2000), we did not do so because of the difficulty of implementing the correct restraints for an isopeptide bond in the peptide backbone and the mixture of products, including v and d-aspartic acid, that result from deamidation (Aswad et al., 2000). The Xpleo server was used to guide rebuilding of several loops that were difficult to build manually (van den Bedem et al., 2004). Although isoaspartic acid has been built in one case (Esposito et al., 2000), carbohydrate linkages were corrected to those known to occur in high mannose N-linked glycans; T.A. Springer.

This work is supported by National Institutes of Health grant HL48675 to T.A. Springer.

Submitted: 22 January 2008
Accepted: 24 June 2008

References


