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

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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 αβ3 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 (ADMIDAS) Ca2+ ion binds the γC C terminus, revealing a contribution for ADMIDAS 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 β 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 (ADMIDAS). Structures of αIβ3 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).

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Abbreviations used in this paper: ADMIDAS, adjacent to MIDAS; I-EGF, integrin EGF-like; MIDAS, metal ion–dependent adhesion site.

Early in hemostasis and thrombosis, the integrin αIIIbβ3 on platelets is activated and binds to its ligand fibrinogen. Fibrinogen is a dumbbell-shaped molecule (Fig. 1 a). Integrin αIIIbβ3 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 αIIIbβ3-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 αIIIbβ3 and αIVβ3, 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 αvβ3 (Cheresh et al., 1989; Smith et al., 1990).

Whether RGD and γC peptides bind to distinct or similar sites on αιιβ3 is controversial. Several studies show competition for the same binding site (Lam et al., 1987; Santoro and Lawing, 1987; Bennett, 2001). Interestingly, although γC and RGD peptides were found to cross-compete, a γC dodecapeptide was photo–cross-linked only to the αιιβ subunit, whereas a RGD hexapeptide was cross-linked to both the αιιβ and αιιβ subunits (Santoro and Lawing, 1987). Binding has been described of RGD and γC 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 αιιβ (Cierniewski et al., 1999). Furthermore, γC peptide was reported to cross-link to an αιιβ 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 γC peptide in hemostasis and thrombosis, there has been great interest in determining its biologically relevant integrin-bound
features important for specific recognition of (Mayo and Fan, 1996). In the absence of appreciation of the headpiece/Fab crystals (Xiao et al., 2004). Examination of and screening then focused on obtaining compounds that were compounds with RGD or KGD motifs. Subsequent synthesis pharmaceutical development of antithrombotics began with the disordered (Pratt et al., 1997; Spraggon et al., 1997; Yee et al., conformation. However, in multiple crystal structures of fibrinogen and its C-terminal γ subunit fragment, the γC peptide is disordered (Pratt et al., 1997; Spraggon et al., 1997; Yee et al., 1997; Yang et al., 2001; Kostelanska et al., 2002). Crystals of the γC peptide fused to lysozyme or glutathione S-transferase reveal different conformations (Donahue et al., 1994; Ware et al., 1999) that are unrelated to the integrin-bound conformation described here. Nuclear magnetic resonance measurements of γC interaction with α3β3 provided limited information (Mayo and Fan, 1996). In the absence of appreciation of the features important for specific recognition of γC by α3β3, pharmaceutical development of antithrombotics began with compounds with RGD or KGD motifs. Subsequent synthesis and screening then focused on obtaining compounds that were selective for α3β3 compared with other integrins that recognize RGD, such as α5β1 and α2β1 (Scarborough and Gretler, 2000). Two RGD-based drugs, a small molecule, tirofiban, and a cyclic peptide, eptifibatide, are currently used clinically to prevent thrombosis, and their complex structures with α3β3 have been determined (Xiao et al., 2004).

In this study, we investigate how the γC peptide and a related peptide containing the RGD sequence present in lamprey γC bind to α3β3. The results reveal the biologically important interface between γC peptide and α3β3, and unexpected features of the integrin recognition of ligands, including a direct role for ADMIDAS in ligand recognition.

### Results

#### Overall complex structure

We soaked human γC deca- and dodecapeptides into α3β3 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. 1b). 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 γC (Fig. 1b). Structures were determined at 2.4–2.8-Å resolution (Table I), and previous structures with antagonists were re-refined to lower R_free (Table II). The structures contain the β propeller domain in the α3β3 subunit; the I, hybrid, plexin-semaphorin integrin, and integrin EGF-like (I-EGF) domain in the β3 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 α3β3 propeller and β3 I domains (Fig. 2a). 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 α3β3 lie in a groove between two long loops that connect blades (β sheets of the α3β3 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. 2b). In the region of the γC peptide N terminal to residue 404, extension along the groove

### Table I. α3β3 γC peptide complex x-ray diffraction and refinement data

| Peptide sequence | HHLGGAKQAGDV | LGGAKQAGDV | HHLGGAKQRGDV | LGGAKQRGDV |
|------------------|--------------|------------|--------------|------------|
| Space group      | P3,21        | P3,21      | P3,21        | P3,21      |
| Unit cell (a, b, c) (Å) | 148.3, 148.3, 176.6 | 148.4, 148.4, 177.2 | 148.5, 148.5, 176.4 | 148.3, 148.3, 176.8 |
| α, β, γ (degree) | 90, 90, 120   | 90, 90, 120 | 90, 90, 120  | 90, 90, 120 |
| Wavelength (Å)   | 1.07223      | 1.07223    | 1.07223      | 1.07223    |
| Resolution (Å)   | 50-2.5       | 50-2.8     | 50-2.6       | 50-2.4     |
| Number of reflections (total/unique) | 557,015/77,023 | 362,650/55,536 | 416,764/68,878 | 609,049/87,704 |
| Completeness (%) | 99.8/100b    | 99.4/99.6b | 98.5/87.4    | 99.9/99.0  |
| Rmerge (%)       | 17.6/3.5b    | 16.2/2.8b  | 19.1/2.2b    | 17.3/2.3b  |
| Rfree (%)        | 8.6/55.2b    | 10.5/62.3b | 8.5/43.3     | 9.0/56.9b  |
| Number of atoms  | 10,538/1,043/205 | 10,464/557/205 | 10,515/1,332/205 | 10,537/1,142/205 |
| Ramachandran statistics | 96.9/3.0/0.1 | 96.5/3.4/0.1 | 97.1/2.6/0.2 | 97.1/2.7/0.2 |
| PDB code        | 2VD          | 2VDQ       | 2VDQ         | 2VDR       |

*Rmerge = ΣI/Σ(h) − <I(h)>/ΣI/Σ(h), where I(h) and <I(h)> are the ith and mean measurement of the intensity of reflection, h. Rwork = ΣI/Σ(h) − |Fobs(h)|/Σ|Fcalc(h)|, where Fobs(h) and Fcalc(h) are the observed and calculated structure factors, respectively. No I/σ cut off was applied. Rfree is the R value obtained for a set of reflections consisting of a randomly selected 5% subset of the data set excluded from refinement.

These numbers correspond to the last resolution shell.
404 – 411, which show strong electron density in omit maps, in-
weak electron density and extend toward solvent. Therefore,
ing molecule in the crystal lattice, and residues 400 – 403 have
more solvent exposed. The other deep pocket, on the
at the ADMIDAS Ca 2+ ion (Fig. 2 b, purple), which is much
ion is not visible in this view, which is in contrast to the surface

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, in-
timately contact αIAβ3 (Fig. 2 a 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
Mg 2+ ion in the β1 domain. The surface at the MIDAS Mg 2+
ion is not visible in this view, which is in contrast to the surface
at the ADMIDAS Ca 2+ ion (Fig. 2 b, purple), which is much more
solvent exposed. The other deep pocket, on the α96 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 αIAβ3 antagonist activity (Kloczewiak
et al., 1989). In the chimera γC peptide, the Arg-408 side chain
extends directly into the αIA-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 αIAβ3
complexes with the chimera peptides show how an RGD pep-
tide 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 back-
bone in between is also extended. The conformation of the li-
gand backbone is stabilized as it crosses the interface between
the αIA and β3 subunits by two previously unremarked water
molecules with strong density that are held in place by hydro-
gen bonds to the side chain of Asn-215 should probably be fl ipped.

Ramachandran statistics

Table II. Refined αIAβ3 complex x-ray diffraction and refinement data

| Ligand             | Native1 | Native2 | Tirofibate | Epitifibate | L-739758 |
|--------------------|---------|---------|------------|-------------|----------|
| Space group        | P3 2 1  | P3 2 1  | P3 2 1     | P3 2 1      | P3 2 1   |
| Unit cell (Å)      | 148.9, 148.9, 176.1 | 148.9, 148.9, 176.4 | 148.6, 148.6, 177.2 | 149.6, 149.6, 175.7 | 149.2, 149.2, 176.2 |
| α, β, γ [degree]   | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 |
| Wavelength [Å]     | 0.9793 | 0.9793 | 0.9760 | 0.9760 | 0.9793 |
| Resolution [Å]     | 50–2.8 | 50–2.75 | 50–2.9 | 50–2.9 | 50–3.1 |
| Number of reflections | 400,460/55,875 | 442,121/62,512 | 330,745/50,662 | 357,439/50,647 | 263,308/41,586 |
| completeness [%]   | 99.9/99.8 | 99.9/100 | 99.7/98.2 | 100/100 | 99.8/99.7 |
| I/σ (l)           | 14.0/3.1 | 20.0/3.0 | 12.1/2.0 | 14.9/2.7 | 12.1/2.7 |
| Rmerge (%)        | 13.0/68.1 | 9.6/59.8 | 13.9/57.8 | 11.7/61.5 | 14.0/55.8 |
| Number of atoms   | 10,404/612/210 | 10,461/1,129/210 | 10,363/355/235 | 10,385/364/223 | 10,348/98/240 |
| Rwork [%]         | 16.1 | 16.1 | 16.3 | 16.3 | 18.1 |
| Rfree [%]         | 20.4 | 19.1 | 21.3 | 21.3 | 22.0 |
| Ramachandran statistics
| 97.3/2.5/0.2 | 97.2/2.5/0.3 | 96.5/3.3/0.2 | 96.8/2.9/0.2 | 96.5/3.3/0.2 |
| Obsolete PDB code | 1TY3 | 1TXV | 1TY5 | 1TY6 | 1TY7 |
| New PDB code      | 2VDK | 2VDL | 2VDM | 2VDN | 2VC2 |

Rmerge = Σ Ii( h )/Σ Ii( h )/| Ii( h ) | - | Fcalc( h ) |/| Fobs( h ) |
Rwork = Σ | Ii( h ) | - | Fcalc( h ) |
Rfree = Σ | Ii( h ) |

Table is determined with RAMPAGE (Cowell et al., 2003).

These numbers correspond to the last resolution shell.
Asp moiety of $\gamma$C 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 $\alpha_{\text{th}}$ Asp-232 at the interface with the $\beta_3$ 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 $C$ atoms of Ala-408 of $\gamma$C and the Arg of RGD occupy identical positions (Fig. 3, a and b). The critical difference between the $\gamma$C peptide– and RGD peptide–binding modalities is the backbone turn at $\gamma$C Gln-407, which orients the N-terminal portion of the $\gamma$C peptide into the groove formed by the long loops that connect $\alpha_{\text{IIb}}$ propeller blades 2 and 3 and blades 3 and 4 (Fig. 3, a). The backbone turn at $\gamma$C Gln-407 enables the $\gamma$C Lys-406 side chain to enter the $\alpha_{\text{IIb}}$ 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 $\alpha_{\text{IIb}}$ 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 $\alpha_{\text{th}}$ residues Tyr-190, Leu-192, and Phe-231 line the pocket and contact the aliphatic portion of the $\gamma$C Lys-406 side chain and the Ala-408 side chain.

Cilengetide, an $\alpha_{\text{V}}\beta_3$ antagonist, is a five-residue peptide that contains RGD, $N$-methyl-Val, and $d$-Phe (Fig. 3 d). The Arg of cilengetide forms a charged hydrogen bond to $\alpha_{\text{V}}$ 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 $\alpha_{\text{th}}$ (Scarborough and Greter, 2000; Gottschalk and Kessler, 2002), pulls the Arg backbone back away from the $\alpha_{\text{V}}$ subunit and helps place the Arg side chain further above the subunit than the Arg or homoarginine of $\alpha_{\text{th}}$ ligands, as appropriate for the shallower $\alpha_{\text{V}}$ binding pocket (Fig. 3 d).

The fibrinogen $\gamma$C peptide complex with $\alpha_{\text{th}}\beta_3$ reveals that $\gamma$C residue Lys-406 forms a charged hydrogen bond to $\alpha_{\text{th}}$ residue Asp-224 (Fig. 3 a). Therefore, $\gamma$C 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 $\gamma$C 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 $\alpha_{\text{th}}$ Asp-232 at the interface with the $\beta_3$ 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 $C$ atoms of Ala-408 of $\gamma$C and the Arg of RGD occupy identical positions (Fig. 3, a and b). The critical difference between the $\gamma$C peptide– and RGD peptide–binding modalities is the backbone turn at $\gamma$C Gln-407, which orients the N-terminal portion of the $\gamma$C peptide into the groove formed by the long loops that connect $\alpha_{\text{th}}$ propeller blades 2 and 3 and blades 3 and 4 (Fig. 3 a). The backbone turn at $\gamma$C Gln-407 enables the $\gamma$C Lys-406 side chain to enter the $\alpha_{\text{th}}$ 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 $\alpha_{\text{th}}$ 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 $\alpha_{\text{th}}$ residues Tyr-190, Leu-192, and Phe-231 line the pocket and contact the aliphatic portion of the $\gamma$C Lys-406 side chain and the Ala-408 side chain.
γC residues Gly-404, Ala-405, and Lys-406 fill the groove between adjacent β propeller blades and form backbone–backbone and backbone–side chain hydrogen bonds to αmβ3 residues Asp-159 and Ser-226 (Fig. 3 a). γC residues Gly-404 and Ala-405 also seal the binding pocket for the Lys-406 side chain, with γC residue Gly-404 forming a backbone hydrogen bond to the γC Lys-406 side chain.

**ADMIDAS coordination**

An important and unexpected observation is that the free carboxyl group of the γC C-terminal residue Val-411 coordinates the β3 1 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 α-carboxyl group decreases by sixfold the potency of γC peptides in inhibiting fibrinogen binding to αmβ3 (Kloczewiak et al., 1989). An identical ADMIDAS coordination is seen with the chimera RGDV peptide (Fig. 3 b). The significance of this observation, the notable similarity in backbone positions of Val-411 of the γC and chimera peptides, and the equivalent residue in epifibatidate, Trp-5 (Fig. 3, a–c), is discussed in the next section.

**Discussion**

**Specific recognition of the fibrinogen γC peptide by αmβ3**

Our structures of γC peptides bound to αmβ3 reveal the interface that is biologically important for the binding of fibrinogen to activated integrin αmβ3 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 γC peptide binds only to αmβ3.

Our γC peptide complex with αmβ3, reveals the basis for binding of platelet αmβ3 and endothelial αvβ3 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 α subunit occupied by the γC peptide is much larger than that occupied by RGD. γC residues Gly-404, Ala-405, Lys-406, and Ala-408 all interact with αmβ3. In contrast, in the linear RGD chimera peptide, only Arg-408 binds to the αmβ3 subunit. This much more extensive interface with γC enables specific differences between αmβ3 and other integrin α subunits to be recognized as good contacts made by αmβ3 and clashes made by αv. The shallower and narrower binding site in the αv subunit precludes binding of the KQAGDV moiety using the αmβ3-bound conformation. For example, αv Alu-215 and Asp-218 would both clash with KQAGDV. These results show why the γC peptide does not bind to αv.

In contrast, RGD does bind to αmβ3, and, therefore, the selectivity of αmβ3 for the γC site over RGD in fibrinogen (Cheresh et al., 1989; Smith et al., 1990) must reflect a higher affinity for γC. Fibrinogen lacking the γC QAGDV pentapeptide fails to bind αmβ3 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 αmβ3; however, it does not mean that the γC peptide is responsible for all of αmβ3’s affinity for fibrinogen. Indeed, αmβ3 has 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 αmβ 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 αmβ propeller that is important in fibrinogen binding and blocks binding of fibrinogen but not the octapeptide to αmβ. 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 αmβ and, together with the γC peptide, account for the higher biological affinity of αmβ3 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 γ chain residues 404–411 are disordered and that residues 393–403, when ordered, completely 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 αmβ3. 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 αmβ groove.

In the absence of information on how the γC peptide binds αmβ3, development of the two currently approved small molecule αmβ3 therapeutics, tirofiban and epifibatide, proceeded from RGD and barbourin, respectively (Scarborough and Gretter, 2000). Barbourin is a disintegrin with two highly unusual properties for a disintegrin: specificity for the RGD motif (Scarborough et al., 1991). Lysine has a side chain with Cβ, Cγ, Cε, and Nε atoms. The Arg side chain has Cβ, Cγ, and Cε atoms and a guanido group with Nε, 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 αmAsp–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 αmAsp–224 than to αγAsp–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 αmβ3 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 αmβ3 binding pocket adopted by the Lys of the KQAGDV motif should enable development of new classes of highly specific αmβ3, antithrombotics. Furthermore, mimicking the novel interaction with the ADIMDAS 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 αmβ subunit and of an RGD hexapeptide to both the αmβ 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 αmβ by the reagent attached to γC His–400 is consistent with the position of Gly–404 in our structures in the αmβ propeller groove and also with further extension in the N—terminal direction in this groove. In contrast, labeling of both the αmβ 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 αmβ and β3 subunits in the chimera deca— and dodecamer structures.

A role for ADIMDAS 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 ADIMDAS 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 ADIMDAS metal ion shifts the position of this metal ion by 3 Å (Xiao et al., 2004). Ca2+ and Mn2+ compete for binding to ADIMDAS, 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 ADIMDAS (Xiao et al., 2004) and that coordination is pentagonal bipyramidal, with seven oxygen ligands as typically seen for Ca2+. ADIMDAS mutations augment ligand binding by integrins αβ3 and αβ2 (Chen et al., 2003, 2006) and, in contrast, inhibit ligand binding by αmβ3 and αβ3 (Bajt and Loftus, 1994; Mould et al., 2003). In this study, we find that in addition to its regulatory role, ADIMDAS 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 epftibatide, 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 epftibatide 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 epftibatide 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\(_{IIb}\)β\(_{3}\) to fibronectin (Mould et al., 2003), and binding of α\(_{IIIb}\)β\(_{3}\) to the peptide GRGDSP (Bajt and Loftus, 1994), although ADMIDAS mutations also appear to favor the inactive conformation of α\(_{IIb}\)β\(_{3}\) (Mould et al., 2003). We propose that the stimulatory effect of ADMIDAS mutations on ligand binding by integrins that do not recognize RGD, α\(_{IIb}\)β\(_{3}\) (Chen et al., 2003), and α\(_{IIIb}\)β\(_{3}\) (Chen et al., 2006) reflects the regulatory role of ADMIDAS and that the inhibitory effect of ADMIDAS mutations on ligand binding by integrins that recognize RGD (or KQAGDV), α\(_{IIIb}\)β\(_{3}\) (Bajt and Loftus, 1994), and α\(_{IIb}\)β\(_{3}\) (Mould et al., 2003) reflects a direct role of ADMIDAS in ligand recognition, in addition to a regulatory role. This proposal requires testing with further integrin–ligand crystal structures.

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(IL)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 KQA/VGDX motif in warm-blooded vertebrates and a KQFGG(IL)GD1 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\(_{IIIb}\)β\(_{3}\) 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\(_{IIIb}\)β\(_{3}\).
chimera deco- and dodecapeptide 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 α8β strand 282 accompanied by a lack of density for the putative Ala-282 Cβ atom; checking of the nucleotide sequence used for α8β expression and RefSeq entry gi|88758615 reveals Gly at this position compared with Ala in our experimental 288–282. Rebuilding of the Fab was aided by comparison with multiple high resolution structures. Vβ, Vµ, and Cλ domains that were found by BlastP search of the protein database and were individually superimposed on the 10E5 Fab. In the four γC and chimera γC peptide complex, Fab residue Asn-157 in the light chain appears to have largely undergone deamidation with conversion to aspartic acid. This residue is in the Asn-Gly sequence; 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 (Savist and Milstein, 1972). The previously determined headpiece structures (Table II) 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 α- and β-aspartic acid, that result from deamidation (Aswad et al., 2000). The Xpleo server was used to guide rebuilding of several regions that were difficult to build manually (van den Bedem et al., 2005). Although hot spots in our previous headpiece structures (Xiao et al., 2004), IEGF domain 1 residues 436–446 are clearly present, although they are less well ordered than other domains. The last disulfide-bonded loop of IEGF1, residues 462–472, either was disordered or was removed by carboxypeptidase A, which stopped at Arg-461. Arg is known to be resistant to digestion by carboxypeptidase A (Ambler, 1967). Compared with our previous headpiece structures (Xiao et al., 2004), carbohydrate linkages were corrected to those known to occur in high mannose N-linked glycans; these cis-prolines were added, and one was removed. TLS refinement in REFMAC5 used groups determined with the TLS Motion Determination procedure. The 100th percentile for all nine structures (where 100th percentile is the best) compared with other deposited structures at similar resolutions. The unusually low R-free of the five previously deposited structures is decreased by 2.1–5.1%.

The γC dodecamer and decamer structures and the five previously deposited α8βδ3 headpiece 10E5 Fab complex structures (Xiao et al., 2004) were then refined starting with the ligands in the previous models and the headpiece-Fab moiety from the refined chimera γC decamer complex. 2.4-A-structure described in Table I. Ligand topology and parameter cif files were created by the PRODRG2 server (Schuttelkopf and van Aalten, 2004). The cacodylate and epiphatidate ligands are unchanged. Use of correct chirality and β of the peptide bond relative to the thiophenothiophene moiety in the L739758 ligand and rotations around the sulfonamide N at-tom; checking of the nucleotide sequence used for α8β expression and RefSeq entry gi|88758615 reveals Gly at this position compared with Ala in our experimental 288–282. Rebuilding of the Fab was aided by comparison with multiple high resolution structures. Vβ, Vµ, and Cλ domains that were found by BlastP search of the protein database and were individually superimposed on the 10E5 Fab. In the four γC and chimera γC peptide complex, Fab residue Asn-157 in the light chain appears to have largely undergone deamidation with conversion to aspartic acid. This residue is in the Asn-Gly sequence; 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 (Savist and Milstein, 1972). The previously determined headpiece structures (Table II) 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 α- and β-aspartic acid, that result from deamidation (Aswad et al., 2000). The Xpleo server was used to guide rebuilding of several regions that were difficult to build manually (van den Bedem et al., 2005). Although hot spots in our previous headpiece structures (Xiao et al., 2004), IEGF domain 1 residues 436–446 are clearly present, although they are less well ordered than other domains. The last disulfide-bonded loop of IEGF1, residues 462–472, either was disordered or was removed by carboxypeptidase A, which stopped at Arg-461. Arg is known to be resistant to digestion by carboxypeptidase A (Ambler, 1967). Compared with our previous headpiece structures (Xiao et al., 2004), carbohydrate linkages were corrected to those known to occur in high mannose N-linked glycans; these cis-prolines were added, and one was removed. TLS refinement in REFMAC5 used groups determined with the TLS Motion Determination server (Painter and Merritt, 2006) and contributed a 2–3% drop in Rfree.

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