Molecular Determinants of Arg-Gly-Asp Ligand Specificity for β3 Integrins

(Received for publication, September 4, 1996, and in revised form, November 19, 1996)

Thomas J. Kunicki, Douglas S. Annis, and Brunhilde Felding-Habermann
From the Room Research Center for Arteriosclerosis and Thrombosis, Division of Experimental Hemostasis and Thrombosis, Department of Molecular and Experimental Medicine, and the Department of Vascular Biology, The Scripps Research Institute, La Jolla, California 92037

The Arg-Tyr-Asp (RYD) and Arg-Gly-Asp (RGD) sequences within the third complementarity-determining region of the heavy chain (H3) of murine recombinant Fab molecules OPG2 and AP7, respectively, are responsible for their specific binding to the platelet integrin αIIbβ3. In this study, we evaluated the influence of divalent cation composition and single amino acid substitutions at key positions within H3 on the selectivity of these Fab molecules for integrin αIIbβ3 versus the vitronectin receptor αVβ3. The parent Fab molecule OPG2 (H3 sequence, HPFYRYDGNN) binds selectively to αIIbβ3 and not at all to any other RGD-cognitive integrin, particularly αVβ3, under any divalent cation conditions. The binding of the AP7 Fab molecule (HPFYRGDGNN) to αIIbβ3 is not affected by the relative composition of calcium, magnesium or manganese. However, AP7 binding to αVβ3, either expressed by M21 cells or as the purified integrin, is supported by manganese and inhibited by calcium. If the flanking asparagine 108 residue within the AP7 H3 loop is replaced by alanine (HPFYRGDGGGA), the resulting Fab molecule AP7.4 binds selectively to αVβ3 in a cation-dependent manner, but does not bind at all to αIIbβ3 under any conditions. AP7.4 binding to αVβ3 is supported by manganese, completely inhibited by calcium, and largely unaffected by magnesium. This behavior mimics that of the adhesive protein, osteopontin, another ligand that binds preferentially to αVβ3. Despite these differences in specificity for αIIbβ3 and αVβ3, AP7 and AP7.4 remain selective for the β3 integrins and do not bind to cell lines that express the RGD-cognitive integrins αVβ3 or αVβ1. These results confirm that subtle changes in the amino acid composition immediately flanking the RGD or RYD motifs can have a profound effect on β3 integrin specificity, most likely because they influence the juxtaposition of the arginine and aspartate side chains within the extended RGD loop sequence.

The two members of the β3 integrin subgroup are αIIbβ3, which is required for platelet cohesion mediated by the binding of fibrinogen or von Willebrand factor (1–3); and αVβ3, the ubiquitous vitronectin receptor that mediates a variety of cellular processes, including migration, tumor cell metastasis, and angiogenesis (4–7). The distinctive α subunits, αIIb and αV, are relatively unique within the integrin family and exhibit only 36% sequence identity (8).

Although both β3 integrins recognize the RGD motif, each exhibits a preference for certain RGD-containing ligands. An example is the snake venom disintegrin barbourin (9), containing the RGD sequence, which binds with greater affinity to the platelet integrin αIIbβ3 Smith et al. (10) have exploited this fact to alter the specificity of an engineered RGD-containing Fab molecule and increase its selectivity for αIIbβ3 over αVβ3. In addition, this fundamental observation has led to the synthesis of a cyclic homoarginine-Gly-Asp (cHarGD)1 peptide that has one of the highest differential affinities for αIIbβ3 versus αVβ3 (11). Nonetheless, each of these ligands, including this peptide, retain some affinity for αVβ3 so that they still inhibit cell adhesion mediated by αVβ3 (11). The relative specificity of ligands for αIIbβ3 versus αVβ3 is also markedly influenced by divalent cations. For example, fibrinogen binds to αVβ3 in the presence of Mn2+ but not in the presence of Ca2+ (12).

To address these factors, Suehiro et al. (13) compared carefully the binding of various RGD ligands and peptides to the β3 integrins as a function of divalent cation composition, and grouped ligands into four classes. Class I, represented by RGD peptides and vitronectin, bind equivalently to αIIbβ3 and αVβ3. Class II, represented by cHarGD, fibrinogen, or fibrinogen γ-chain peptides, bind to both integrins in the presence of Mn2+, but only to αIIbβ3 in the presence of Ca2+. Class III, such as barbourin, bind exclusively to αIIbβ3 under any condition. Class IV, represented by osteopontin, bind primarily to αVβ3.

To gain further insight into the molecular basis for differences in ligand specificity, we exploited our well characterized, recombinant RGD-containing Fab molecule AP7, and its RYD-containing progenitor OPG2 (14). By the criteria of Suehiro et al. (13), OPG2 is a Class III ligand, binding only to αIIbβ3 under any condition, while AP7 is a Class II ligand, binding to αVβ3 in Mn2+ but not Ca2+ and to αIIbβ3 in either cation. The single amino acid replacement Asn → Ala within the RGD-containing loop of AP7 further changes the specificity of the mutagenized Fab molecule. The new Fab molecule, which we designate AP7.4, binds exclusively to αVβ3 and belongs to the Class IV RGD ligand group. These findings confirm that selectivity for the β3 integrins is determined by precise juxtapositions of the Arg and Asp side chains that are significantly affected by flanking amino acid sequences.

**MATERIALS AND METHODS**

Synthesis of Recombinant OPG2 Fd and κ Chain cDNA—The term Fd denotes the segment of the Ig heavy chain that includes the VH

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1 The abbreviations used are: cHar, cyclic homoarginine; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.
Integrin $\beta_3$ Ligand Specificity

domain, the C1 domain, and a portion of the hinge region up to and including the cysteine residue, which participates in a disulfide bond with the carboxyl-terminal cysteine residue of the light chain (14). Fab molecules represent disulfide-linked heterodimers composed of Fd plus $\kappa$ chains. Hexahistidine-tagged Fd cDNAs and $\kappa$ chain cDNA were expressed individually as described previously (14) in Sf9 insect cells. An oligonucleotide fragment of 1.4.

The 5'-CATCAC-3' was inserted upstream from a domain, the C1 domain, and a portion of the hinge region up to and including the cysteine residue, which participates in a disulfide bond with the carboxyl-terminal cysteine residue of the light chain (14). Fab molecules represent disulfide-linked heterodimers composed of Fd plus $\kappa$ chains. Hexahistidine-tagged Fd cDNAs and $\kappa$ chain cDNA were expressed individually as described previously (14) in Sf9 insect cells. An oligonucleotide fragment of 1.4.

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Binding of Recombinant Fab Molecules to Purified αIIBβ3—The selectivity of each Fab molecule for αIIB3 or αVβ3 and the dependence of binding on the RGD sequence were further investigated using the purified integrins in an ELISA (Fig. 3).

In the presence of 1 mM each of Ca²⁺ and Mg²⁺, AP7 or OPG2 Fab molecules exhibit a strong affinity for αIIB3 (Fig. 3A) but fail to bind to αVβ3 (Fig. 3B). Binding of either Fab molecule is completely inhibited by 1 mM EDTA or ≥10 μM RGEW (data not shown). Under the same divalent cation conditions, both AP7.4 and AP7.7 Fab molecules fail to bind to either αIIB3 (Fig. 3A) or αVβ3 (Fig. 3B).

In the presence of ≥10 mM Mn²⁺, the binding of each Fab molecule to purified αIIB3 is unchanged (Fig. 3C). However, AP7 and AP7.4 Fab molecules now bind strongly to αIIB3 in the presence of Mn²⁺, while OPG2 Fab molecules fail to bind (Fig. 3D). The binding of AP7 or AP7.4 Fab molecules to αIIB3 in this cell-free system is completely inhibited by 1 mM EDTA or ≥10 μM RGEW, but not at all by up to 2 mM RG EW (data not shown).

As a negative control, AP7.7 Fab molecules (50 μg/ml) fail to bind to either integrin under any conditions (Fig. 3, A–D).

Discussion

Using the recombinant murine Fab molecule OPG2 as a versatile framework, our results validate the hypothesis that the amino acid composition immediately flanking an RGD tripeptide can profoundly influence the specificity and divalent cation modulation of ligand binding to β3 integrins. The relevant sequences of the recombinant Fab molecules, OPG2, AP7, AP7.4, and AP7.7, and their comparative specificities, as determined by this study, are summarized in Table I.

The model that we have developed using OPG2 and the AP7 series of recombinant Fab molecules provides a unique opportunity to compare both RGD and RYD analogs of the same ligand and to predict the impact of single amino acid substitutions on specificity based upon known side chain interactions defined by x-ray crystallography of the parent Fab molecule (28). In the case of each Fab molecule in our series, a single amino acid substitution within the third complementarity-determining region of the heavy chain results in a profound
change in specificity. For example, there is the complete loss of binding to either β₃ integrin that is characteristic of AP7.7 created by the replacement of Asn₁₀₈ by an Alanine within the OPG2 H₃ sequence. The major reason for our selection of Asn₁₀₈ as a target for substitution is the fact that, in the crystal structure of the OPG2 Fab molecule (28), the side chain of Asn₁₀₈ is in a position to form a hydrogen bond with that of Asp₁₀₅, i.e. the distance between Asp₁₀₅-OD₁ and Asn₁₀₈-ND₁ is 2.8 or 3.2 Å in each of two alternate conformers of the H₃ loop. We reasoned that disruption of such a side chain interaction would likely influence the juxtaposition of the remaining side chains, particularly those of Arg₁₀₃ and Asp₁₀₅. This hypothesis is borne out by our experimental evidence, and our results provide strong support for the presence of a hydrogen bond between these side chains. It follows that this side chain interaction probably holds the Asp¹⁰⁵ carboxyl group in a unique orientation with respect to the amino group of Arg¹⁰³ such that OPG2 is recognized exclusively by αIIbβ₃.

The most dramatic and novel finding of our study is the change in specificity created by the engineering of the AP7.4 Fab molecule. The sole difference between the Fab molecule AP7, which binds preferentially to αIIbβ₃ in the presence of calcium ions, and AP7.4, which binds solely to αⅤβ₃ in the presence of manganese ions, is a single amino acid substitution adjacent to the RGD motif within the H₃ loop (HPFYRGDGNA in AP7 versus HPFYRGDGGA in AP7.4). To our knowledge, this is the first published report of a complete change in specificity of an RGD ligand from αIIbβ₃ to αⅤβ₃ as a result of a single amino acid substitution. While others have shown that single amino acid differences in RGD peptides or an Fab mol-

**Table I**

| Fab     | H3 sequence | αIIβ3 | αⅤβ3 | Platelets | M21 αIIβ3 | αⅤβ3 | Platelets | M21 |
|---------|-------------|-------|------|-----------|-----------|------|-----------|-----|
| OPG2    | HPFYR[DGGN| Yes   | No   | Yes       | No        | Yes  | No        | Yes |
| AP7     | HPFYRGDGGA| No    | No   | No        | Yes       | Yes  | Yes       | Yes |
| AP7.4   | HPFYRGDGGA| No    | No   | No        | No        | No   | No        | No  |
| AP7.7   | HPFYRGDGGA| No    | No   | No        | No        | No   | No        | No  |
ecule can increase their relative affinities for $\alpha_{III}\beta_3$ (10, 11, 29, 30), the engineered AP7.4 molecule represents the first instance in which a dramatic decrease in affinity for $\alpha_{III}\beta_3$ and reciprocal increase in affinity for $\alpha_v\beta_3$ has been produced. This change in relative affinities is so extreme that the binding of the Fab molecule to $\alpha_{III}\beta_3$ has fallen below the level of detection. Apparently, because Tyr104 of OPG2 has been replaced by the Fab molecule to change in relative affinities is so extreme that the binding of one hand, integrin conformation is likely influenced by cations, and the accommodation of this change by the mutated H3 loop must increase the flexibility of the Arg103 and Asp105 side chains and facilitate the increased selectivity of AP7.4 for the integrin $\alpha_v\beta_3$. Our results would argue that both $\alpha_{III}\beta_3$ and $\alpha_v\beta_3$ are highly restrictive with respect to the Arg and Asp side chain orientations that each will recognize.

There are at least two mechanisms that may be involved in the divalent cation regulation of ligand binding to integrins. On one hand, integrin conformation is likely influenced by cations, particularly Mn$^{2+}$. As an example, the monoclonal antibody 9EG7 binds to a Mn$^{2+}$-induced epitope and stimulates $\beta_3$ integrin functions (31). On the other hand, there is substantial evidence that divalent cations support an initial ternary complex with integrin and ligand. As the ligand-integrin binding becomes stabilized, the divalent cation is displaced (32). Replacement of Asn$^{108}$ with Ala may eliminate the ability of AP7.4 to disrupt cation coordination upon contact with an integrin. This would not explain, however, why AP7.4 then binds selectively to $\alpha_v\beta_3$, an integrin whose recognition of RGD ligands is equally regulated by divalent cations.

Osteopontin was the first RGD ligand identified that has a substantial preference for $\alpha_v\beta_3$ relative to $\alpha_{III}\beta_3$ (33). It is particularly relevant that Ca$^{2+}$ is a strong inhibitor of osteopontin binding to $\alpha_v\beta_3$, while Mn$^{2+}$ enhances this interaction (33). In the microenvironment of bone tissue, osteoclasts liberate Ca$^{2+}$ from demineralized bone during resorption such that levels of free Ca$^{2+}$ increase locally. This would then favor detachment of osteoclasts from bone by inhibition of the osteopontin binding to $\alpha_v\beta_3$. Conversely, increases in the relative level of Mg$^{2+}$ compared to Ca$^{2+}$ in areas of bone growth would favor osteopontin-mediated cell attachment. Micromolar levels of Mn$^{2+}$ found in many tissues, including bone and liver, would support cell attachment via $\alpha_v\beta_3$ (12). Clearly, the engineered recombinant Fab molecule AP7.4 behaves precisely as does osteopontin with respect to its selectivity for the integrin $\alpha_v\beta_3$ and the influence of divalent cations on its binding properties. Thus, AP7.4 is a powerful new tool to investigate the role of the integrin $\alpha_v\beta_3$ in various biological processes, including bone resorption.

Acknowledgments—We thank Dr. E. Wayner (University of Minnesota, St. Paul, MN) for monoclonal antibody P1D6 and Dr. D. Cheresh (The Scripps Research Institute) for monoclonal antibody LM609.