RGD induces conformational transition in purified platelet integrin GPIIb/IIIa–SDS system yielding multiple binding states for fibrinogen γ-chain C-terminal peptide

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Abstract Fibrinogen γ-chain C-terminal peptide HHLGGAKQAGDV (γ12) and α-chain peptide GRGDSP are known to inhibit fibrinogen-mediated platelet cell aggregation via competitive interactions with platelet integrin receptor GPIIb/IIIa. NMR studies of γ12 in the presence of purified GPIIb/IIIa in SDS/water solution have demonstrated the presence of two γ12 binding states, one of which is eliminated by GRGDSP (RGD) up to a RGD:γ12 ratio of 2:1. RGD:γ12 ratios greater than 2:1 produce multiple sets of γ12 NMR signals in TOCSY spectra. At a ratio of 4:1, two to four such resonance sets can be resolved for A405, Q407, A408, G409, D410 and V411 spin systems. The number of multiple resonances remains unchanged at ratios of 6:1 and 8:1. Addition of γ12 to reverse the ratio to 8:8 (1:1) has no apparent effect on the RGD-induced distribution. Results suggest that RGD irreversibly induces a conformational transition(s) in GPIIb/IIIa to produce multiple γ12 binding sites on the receptor.

Key words: Fibrinogen; Peptide; Platelet; Integrin GPIIb/IIIa; NMR spectroscopy

1. Introduction

Integrins are cell surface glycoprotein (GP) receptors that mediate cell–cell and extracellular matrix–cell adhesion processes [17]. Platelets are known to contain five integrins of which $GP\alpha_{IIb}\beta_3$ (GPIIb/IIIa) is the major constituent [17]. In the blood clotting cascade (see review [8]), activated platelets adhere to each other via a bridge which is formed when plasma fibrinogen (a 330,000 dalton dimer of three polypeptide chains, i.e. α, β, and γ [9]) binds to GPIIb/IIIa receptors from different platelet cells [3,30]. In fibrinogen, two RGD-containing binding sites [29] are located on the α-chain, and one non-RGD site is located within the γ-chain C-terminal residues 400–411, HHLGGAKQAGDV (γ12) [18,19].

Integrin GPIIb/IIIa, however, is a promiscuous receptor which will bind to a wide variety of RGD containing proteins (e.g. fibrinogen [18], fibronectin [16,25], vitronectin [26], and von Willebrand factor [27]) as well as to the fibrinogen γ-chain C-terminal sequence. Three ligand binding sites so far have been identified on GPIIb/IIIa: two RGD binding sites on the β_3-chain (IIIa): residues 119–130 [1,28,31,32] and residues 211–222 [2,7,20] and one γ12 binding site on the α_{IIb}-chain (IIb): residues 294–314 [10,11,12,34]. Other potential RGD interactive sites have been identified by hydropathy complimentary approaches and by monoclonal antibody mapping [4,5,14].

Short, linear RGD-containing peptides as well as the fibrinogen γ-chain C-terminal dodecapeptide γ12, can compete with parent fibrinogen for GPIIb/IIIa binding and, depending on the peptide concentration, even activate GPIIb/IIIa-fibrinogen binding [13]. Peptide-induced receptor in vitro activation is associated with conformational changes on GPIIb/IIIa [13]. In several studies, it has been hypothesized that fibrinogen binding to GPIIb/IIIa may proceed by initial recognition of an RGD-like sequence, recognition-induced conformational change, and then additional high affinity ligand–receptor interaction(s).

Previous NMR studies of γ12 interactions with GPIIb/IIIa showed that γ12 alone can induce at least two γ12 binding sites on the receptor, one of which is eliminated by GRGDSP [23]. In the present NMR study, the interaction of fibrinogen γ-chain peptide γ12 with purified GPIIb/IIIa has been followed in the presence of SDS and increasing concentrations of hexapeptide GRGDSP. Data indicate that GRGDSP induces a conformational transition(s) in GPIIb/IIIa–SDS system to generate multiple γ12 ligand binding states and to modify peptide binding affinities.

2. Methods and materials

2.1. Peptide synthesis

Peptides representing amino acid sequences from human fibrinogen gamma chain were synthesized as described in ref. [23].

2.2. GPIIb/IIIa-fibrinogen binding assay

In order to assess the functionality of purified GPIIb/IIIa [26] before and after NMR experiments, an in vitro solid phase purified GPIIb/IIIa binding assay using biotinylated fibrinogen was employed as previously described [23]. GPIIb/IIIa was found to bind parent fibrinogen before and after NMR studies.

2.3. NMR measurements

Freeze-dried samples for NMR measurements were dissolved in H_2O/D_2O (9:1). Peptide concentration ranged from 3 mM to 24 mM. GPIIb/IIIa receptor concentration was 0.1 mM. pH was adjusted by

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Abbreviations: NMR, nuclear magnetic resonance; 2D-NMR, two-dimensional NMR spectroscopy; HOHAHA, 2D-NMR homonuclear Hartman-Hahn spectroscopy; NOE, nuclear Overhauser effect; NOESY, 2D-NMR nuclear Overhauser effect spectroscopy; rf, radio frequency; FID, free induction decay; GP, glycoprotein; GPIIb/IIIa, integrin receptor $GP\alpha_{IIb}\beta_3$; SDS, sodium dodecyl sulfate; γ12, dodecapeptide HHLGGAKQAGDV, derived from the fibrinogen γ-chain C-terminus; RGD, hexapeptide GRGDSP.

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adding μl quantities of NaOD or DCl to the protein sample. Solution conditions were 50 mM sodium phosphate, 2 mM $CaCl_2$, 10 mM SDS, pH 6 and 298 K. NOESY and TOCSY (HOHAHA) spectra were obtained as described in ref. [23]. All NMR spectra were acquired on a Bruker AMX-600 NMR spectrometer.

3. Results and discussion

A TOCSY spectrum of γ12 in the presence of purified GPIIb/IIIa and a 2-fold molar excess of GRGDSP (RGD) is shown in Fig. 1. For GRGDSP, sequence-specific assignments indicated in this figure were made in the standard way [35] from analysis of NOESY and TOCSY spectra. *Cis–trans* proline isomerization [35] of the S5-P6 peptide bond in GRGDSP gives rise to two sets of slowly exchanging (600 MHz NMR chemical shift time scale) resonances for D4 and S5 as labeled in the figure. At this RGD:γ12 ratio, only one set from an original two sets of γ12 resonances is observed since RGD effectively competes with one γ12 receptor binding species [23]. Resonance assignments for γ12 are taken from [23]. Due to differential line broadening from dynamic exchange between free and bound γ12 peptide [23], not all γ12 resonances are observed, particularly those belonging to N-terminal residues H400 through G404.

Increasing the RGD:γ12 ratio modifies TOCSY spectra significantly, causing additional resonances to appear and original resonances to shift, broaden or disappear. This is exemplified at ratios of 4:1 (Fig. 1) and 8:1 (Fig. 2). Since NOEs were mostly absent in NOESY spectra, resonance assignments of new cross-peaks could not be made in the standard way [35]. Assignments, therefore, were made by spectral comparison and spin system identification. Since L402, K406, Q407, and V411 present unique γ12 spin systems, their identification is unambiguous. For RGD, R2, S5 and P6 are unique. In any event, the attempt here is not to make complete resonance assignments, but rather to report the effect of RGD on γ12 interactions with a GPIIb/IIIa–SDS system. Based on these unique spin systems, resonances can be assigned to γ12 and RGD protons as indicated in Figs. 1 and 2. γ12 D410 assignments are also based on knowledge of RGD D3 chemical shifts. At an RGD:γ12 ratio of 4:1 (Fig. 1), multiple γ12 spin systems have appeared. These are most clearly observed for V411 where about four valine spin systems can be followed from NH through γH_3 resonances. At higher ratios (see Fig. 2), these same γ12 states are still present but with near equal αN cross-peak intensities. Notice also that as the RGD:γ12 ratio is increased to 8:1, RGD cross-peaks have significantly decreased in intensity even though the RGD concentration has been increased. This is especially evident with the G3 cross-peak. Furthermore, what appears to be three RGD S5 spin systems are also indicated by arrows in Fig. 2, suggesting the presence of multiple RGD binding states.

The reversibility of this RGD effect was tested by increasing the γ12 concentration to return to RGD:γ12 ratios of 2:1 and 1:1. Fig. 2 presents a TOCSY spectrum at a ratio of 8:8 (1:1)

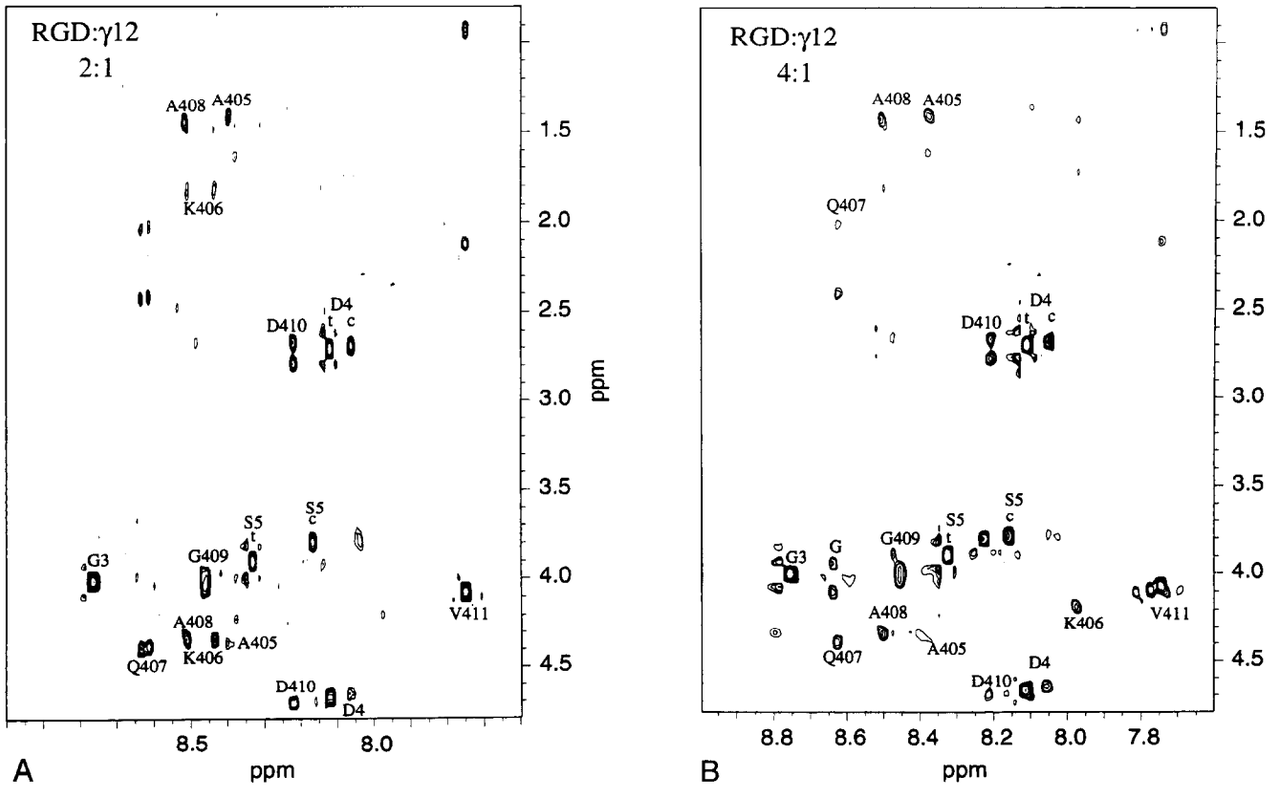

Fig. 1. HOHAHA spectra of γ12 with GPIIb/IIIa ± GRGDSP. Two HOHAHA spectra are presented showing the αH-NH/upfield region for RGD:γ12 molar ratios of 2:1 (left panel) and 4:1 (right panel). The γ12:GPIIb/IIIa molar ratio is 30:1 with a GPIIb/IIIa receptor protein concentration of 0.1 mM. Spectra were acquired under the same solution conditions: 50 mM sodium phosphate, 2 mM $CaCl_2$, 10 mM SDS, pH 6 and 298 K. NMR data were acquired and processed as discussed in section 2. Multiple γ12 resonance are labeled as discussed in the text.

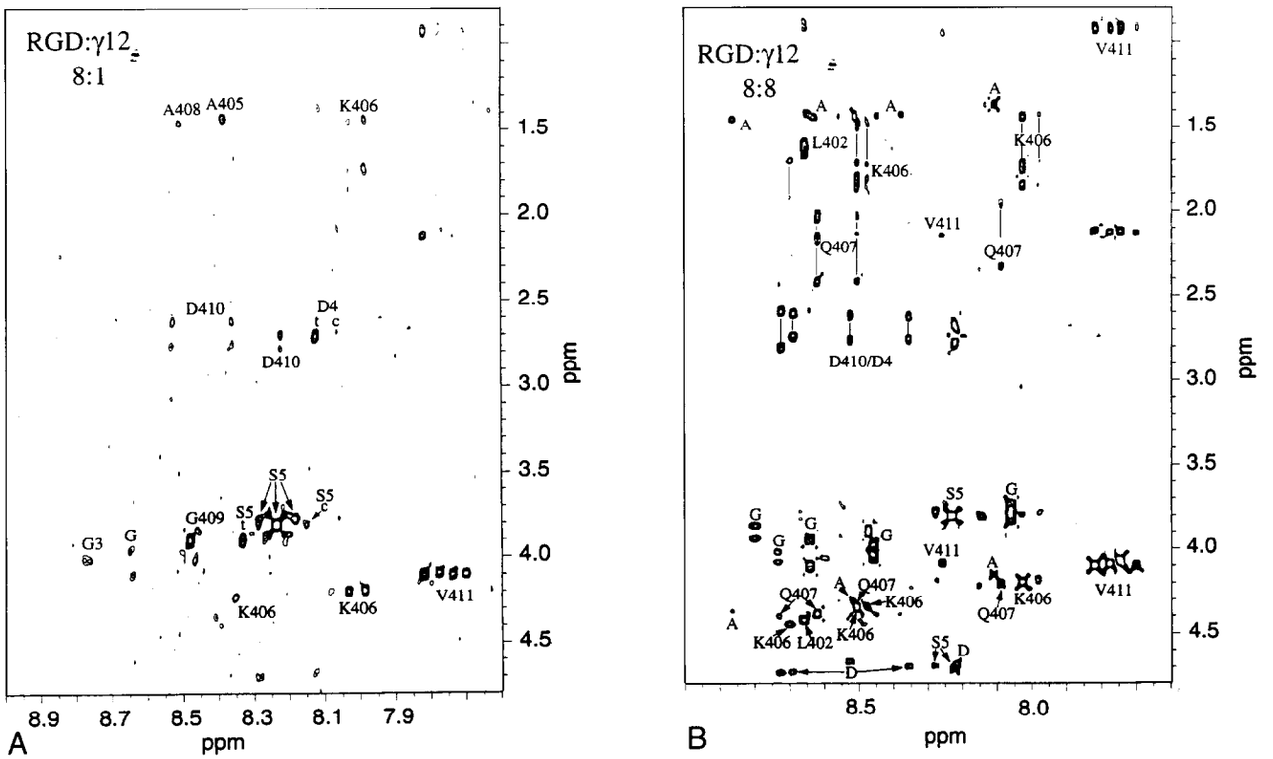

Fig. 2. 1D HOHAHA slices for ratios of RGD:γ12. Two HOHAHA spectra are presented showing the αH-NH/upfield region for RGD:γ12 molar ratios of 8:1 (left panel) and 8:8 (right panel). The γ12:GPIIb/ IIIa molar ratio is 30:1 with a GPIIb/IIIa receptor protein concentration of 0.1 mM. Spectra were acquired under the same solution conditions: 50 mM sodium phosphate, 2 mM $CaCl_2$, 10 mM SDS, pH 6 and 298 K. NMR data were acquired and processed as discussed in section 2. Multiple γ12 resonance are labeled as discussed in the text.

showing that the effect of RGD on GPIIb/IIIa γ12 binding is not reversed. No reduction in the number of γ12 spin systems occurs, and no RGD cross-peaks can be found at their previously observed (2:1 ratio) chemical shift positions. In fact, if anything additional γ12 spin systems are observed. This is most likely due to increasing γ12 peptide concentration as opposed to the creation of new states. Five K406, Q407 and V411 spin systems are now identifiable (Figure 2), and although seven aspartic acid, eight alanine and five glycine spin systems also can be observed, these cannot be unambiguously assigned.

Since these studies have been done with purified GPIIb/IIIa (0.1 mM) in the presence of SDS (10 mM), interpretation must be approached cautiously. Results, however, do clearly indicate the presence of multiple peptide conformational states in slow exchange on the NMR chemical shift time scale. Moreover, the appearance of these multiple states is a direct consequence of the presence of RGD. The crucial question then is the origin of these states. Given the high ratio of peptide to receptor that is used, experimental artifacts arising from some type of non-specific binding are a possibility. However, five observations underline the probable specific nature of at least some of these interactions: (1) a discrete number of states is observed even when the peptide concentration is increased; (2) multiple states are *not* present in the absence of GPIIb/IIIa, i.e. in the presence of SDS alone; (3) multiple states are not observed when an eight residue fragment of γ12 consisting of the last eight C-terminal residues is used; (4) the conformational integrity of the receptor checked by both circular dichroism (CD) and fibrinogen binding assay is unaffected at this SDS:GP IIb/IIIa ratio. SDS had to be used since GPIIb/IIIa, a membrane protein, is relatively insoluble at concentrations required for these experiments without some detergent being present. (5) From other studies (see section 1), both RGD and γ12 are known to bind the receptor at multiple sites.

If peptide were tightly bound to GPIIb/IIIa with a slow off rate, then its NMR signal should disappear for a complex of 220 kDa at a concentration of 0.1 mM. On the other hand, if there were a rapid equilibrium between free and bound peptide, then chemical shifts should represent a weighted average between free and bound forms [6,21,24]. Neither scenario precisely explains the NMR observations. At higher RGD concentrations, RGD cross-peak behavior may fit the first scenario since resonances appear to be highly broaden and disappear. For γ12, however, the only viable explanation for the appearance of multiple binding states appears to be that GPIIb/IIIa in the presence of SDS forms a system which mediates slow exchange among multiple peptide conformational states. SDS is known to form micelles under present conditions [33] and is most likely associating with the receptor, prehaps like an artificial membrane. It is conceivable that peptides interact with GPIIb/IIIa through an SDS interface which acts to slow down exchange among receptor-dissociated peptide states.

Since RGD only acts this way in the presence of GPIIb/IIIa whose integrity is maintained at this SDS concentration, RGD must be inducing changes in the receptor. Du et al. [13] have stated that RGD/γ12 peptide ligands can activate receptor–

ligand binding by inducing conformational changes in GPIIb/IIIa, and in several studies, it has been hypothesized that fibrinogen binding to GPIIb/IIIa may proceed by initial recognition of an RGD-like sequence, recognition-induced conformational change, and then additional high affinity ligand–receptor interaction(s) [15]. At this level of analysis, the precise nature of these changes observed in our system remains unknown. Possibilities include an increased number of γ12 bound state at a single or a few binding sites, a combination of multiple bound states at multiple sites, or multiple independent binding sites. Some non-specific binding also can not be excluded. Furthermore, although the meaning of these results in terms of in vivo γ12/RGD GPIIb/IIIa integrin receptor–fibrinogen interactions and platelet cell adhesion also remains unclear, the creation of multiple γ12 binding sites on GPIIb/IIIa may facilitate fibrinogen γ-chain interactions with the platelet cell surface receptor. The C-terminal segment in a longer γ-chain peptide 385–411 has been found to be highly flexible [22] allowing for considerable sampling of conformational space such that any number of structures may be selected on binding to the receptor.

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