Integrin Receptor GPIIb/IIIa Bound State Conformation of the Fibrinogen γ-Chain C-Terminal Peptide 400−411: NMR and Transfer NOE Studies[†]

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Received October 18, 1995; Revised Manuscript Received January 18, 1996[⊗]

ABSTRACT: The C-terminal dodecapeptide from human fibrinogen γ-chain, residues 400−411, HHLGGAKQAGDV (γ12), is known to inhibit fibrinogen-mediated platelet cell aggregation via competitive interactions with platelet glycoprotein integrin receptor GPIIb/IIIa. NMR studies of γ12 in the presence of purified GPIIb/IIIa (230 kDa) demonstrate that two γ12 binding states (γ12-I and γ12-II) are present on the integrin receptor. The N-terminal sequence HHLG is crucial to formation of γ12 state I since in a shorter γ-chain octapeptide, GAKQAGDV, γ12-I is not observed. Addition of the hexapeptide GRGDSP to the γ12-receptor preparation effectively removes the γ12-I population, suggesting either that γ12 and GRGDSP share one binding site or that their binding sites are allosterically linked. Distance geometry calculations using transfer NOEs from γ12-I (γ12-II shows practically no NOEs) indicate the presence of helix conformation when bound to the receptor. Line broadening and chemical shift changes relative to free γ12 suggest that γ12 interacts with GPIIb/IIIa primarily through N-terminal residues H400 to Q407.

Integrins are cell surface glycoprotein (GP)[1] receptors that mediate cell−cell and extracellular matrix−cell adhesion processes. Each heterodimeric integrin is assembled from a pool of α and β subunits which can be combined in various permutations. One α subunit, for example, can form heterodimers with at least five different β subunits (Hynes, 1992). At present, platelets are known to contain five integrins, two of the β_3 class and three of the β_1 class (Ginsberg et al., 1988; Hynes, 1991). Studies with platelets have been instrumental in elucidating various ligand recognition mechanisms of integrins in general. Platelet integrin GP$\alpha_{IIb}\beta_3$ (GPIIb/IIIa) was among the first of the integrins to be identified (Nurden & Caen, 1974) and expressed in completely recombinant form (Poncz et al., 1987; Zimrin et al., 1988; O'Toole et al., 1989).

In the blood clotting cascade [see review by Davie et al. (1991)], 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 (Doolittle, 1984)] binds to GPIIb/IIIa receptors from different platelet cells (Bennett et al., 1982; Savage & Ruggeri, 1991). Three potential bridging sites have been identified in the fibrinogen amino acid sequence: two RGD-containing sites (Rouslahti & Pierschbacher, 1987) located on the α-chain and one non-RGD site located within the γ-chain C-terminal residues 400−411, HHLGGAKQAGDV (γ12) (Kloczewiak et al., 1984). Using electron microscopy, Weisel et al. (1992) observed that the fibrinogen γ-chain C-terminus, but not regions of the α-chain containing RGD sequences, interacts with GPIIb/IIIa. Farrell et al. (1992, 1994) also provided evidence that the fibrinogen γ-chain sequence alone is essential for optimal platelet aggregation, whereas the α-chain RGD sequences are "neither necessary nor sufficient for platelet aggregation."

Integrin GPIIb/IIIa, however, is a promiscuous receptor which will bind to a wide variety of RGD-containing proteins [e.g., fibrinogen (Kloczewiak et al., 1984); fibronectin (Haverstick et al., 1985; Plow et al., 1985); vitronectin (Pytela et al., 1986); and von Willebrand factor (Ruggeri et al., 1983)] 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 (Ruggeri et al., 1986; Takada et al., 1992; Smith & Cheresh, 1988; Bajt et al., 1992a,b) and residues 211−222 (Charo et al., 1991; Bajt et al., 1992a,b; Lanza et al., 1992), and one γ12 binding site on

[†] This work benefitted from research grants from the W. W. Smith Charitable Trust, Philadelphia, PA, and from the R. W. Johnson Pharmaceutical Research Institute, Springhouse, PA.

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[⊗] Abstract published in *Advance ACS Abstracts,* March 15, 1996.

[1] Abbreviations: NMR, nuclear magnetic resonance; 2D-NMR, two-dimensional NMR spectroscopy; HOHAHA, 2D-NMR homonuclear Hartmann−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; γ8, octapeptide GAKQAGDV, derived from the fibrinogen γ-chain C-terminus; RGD, hexapeptide GRGDSP.

0006-2960/96/0435-4434$12.00/0

the α_{IIb}-chain (IIb), residues 294−314 (D'Souza et al., 1990, 1991; Taylor et al., 1992). Other potential RGD interactive sites have been identified by hydropathy complementary approaches and by monoclonal antibody mapping (Calvete et al., 1991a,b; Gartner et al., 1991).

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 (Du et al., 1991). Peptide-induced receptor activation exposes occupancy-dependent antibody binding sites, indicating that *in vitro* activation is associated with ligand-induced conformational changes (Du et al., 1991). 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). The presence of multiple contact points combined with effects of allosteric modulation complicates understanding the fibrinogen-mediated platelet−platelet cell adhesion process.

In the present study, the interaction of fibrinogen γ-chain peptide γ12 with purified platelet integrin receptor GPIIb/IIIa has been followed by using NMR spectroscopy. Data indicate that the γ-chain peptide does interact with GPIIb/IIIb to allow for two peptide−receptor binding states, γ12-I and γ12-II. One of these states, γ12-I, is eliminated by addition of the RGD-containing peptide, GRGDSP. Transfer NOEs are observed only with γ12-I. These NOEs provide the basis for distance geometry calculations of the receptor-bound conformation of γ12-I.

MATERIALS AND METHODS

Peptide Synthesis. Peptides representing amino acid sequences from human fibrinogen γ-chain were synthesized on a Milligen Biosearch 9600 automated peptide synthesizer. The procedures used were based on the Merrifield solid phase system utilizing Fmoc-BOP chemistry (Stewart & Young, 1984). After the sequence had been obtained, the peptide support and side chain protection groups were acid (trifluoroacetic acid and scavanger mixture) cleaved. Crude peptides were anaylzed for purity on a Hewlett-Packard 1090M analytical HPLC using a reverse-phase C18 VyDac column. Peptides generally were about 90% pure. Further purification was done on a preparative reverse-phase HPLC C-18 column using an elution gradient of 0%−60% acetonitrile with 0.1% trifluoroacetic acid in water. Peptides then were analyzed for amino acid composition on a Beckman 6300 amino acid analyzer by total hydrolysis of samples using 6 N HCl at 110 °C for 18−20 h.

GPIIb/IIIa Receptor Purification. Purified GPIIb/IIIa was isolated by a modified method of Pytela et al. (1986). Briefly, outdated human platelets were washed twice with a solution containing 9 parts 0.15 M NaCl and 1 part 3.8% trisodium citrate (pH 6.5). Platelet pellets were resuspended in cold lysis buffer [PBS, pH 7.3, containing 50 mM octyl glucoside (Sigma) and 3 mM phenylmethylsulfonyl fluoride (PMSF)] and incubated at 4 °C for 15 min. The lysate was centrifuged at 30 000*g* for 20 min, and the supernatant was applied to a KYGRGDS-Sepharose affinity column. The extract was incubated overnight at 4 °C with the affinity matrix, and then the column was washed with column buffer (PBS, pH 7.3, containing 25 mM octyl glucoside and 1 mM PMSF). Elution of GPIIb/IIIa was accomplished by washing the column with 10 mM EDTA in the column buffer. The purity of the eluate fractions was determined by SDS−polyacrylamide gel electrophoresis (Laemmli, 1970). Protein bands were stained by Coomassie Blue. Fractions containing GPIIb/IIIa were combined, concentrated, and dialyzed in a 10 mM phosphate buffer containing 1 mM $CaCl_2$, pH 6.0. Protein determination was carried out using a BCA protein assay kit (Pierce Chemical Co., IL) according to the manufacturer's instructions.

GPIIb/IIIa−Fibrinogen Binding Assay. In order to assess the functionality of purified GPIIb/IIIa before and after NMR experiments, an *in vitro* solid phase GPIIb/IIIa binding assay using biotinylated fibrinogen was employed. This rapid and sensitive assay has been fully characterized and documented by Charo et al. (1991). Fibrinogen binding to immobilized GPIIb/IIIa was found to be divalent cation-dependent and was inhibited by peptides and antibodies known to inhibit the binding of adhesive proteins to GPIIb/IIIa on activated platelets. The dissociation constant of fibrinogen to GPIIb/IIIa was similar to that reported by Parise and Phillips (1985) on reconstituted vesicles and higher than that for GPIIb/IIIa on activated platelets. Reported here is a modified procedure as described by Charo et al. (1991). A 96-well Immulon-2 microtiter plate (Dynatech- Immulon) was coated with 50 μL of 1 mM RGD-affinity purified GPIIb/IIIa (effective range 0.5−10 μg/mL) in 10 mM HEPES, 150 mM NaCl, pH 7.4, per well. The plate is covered and incubated overnight at 4 °C. The GPIIb/IIIa solution is discarded, 150 μL of 5% BSA is added, and the resulting mixture is incubated at room temperature for 1−3 h. The plate is washed extensively with modified Tyrodes buffer. Biotinylated fibrinogen (25 μL/well) at 2× final concentration. The plate is covered and incubated at room temperature for 2−4 h. Twenty minutes prior to incubation completion, one drop of Reagent A (Vecta Stain ABC Horse Radish Peroxidase kit, Vector Laboratories, Inc.) and one drop Reagent B are added with mixing to 5 mL of modified Tyrodes buffer mix, and the resulting solution is left to stand. The ligand solution is discarded, and the plate is washed (5 × 200 μL/well) with modified Tyrodes buffer. Vecta Stain HRP-Biotin-Avidin reagent (50 μL/well, as prepared above) is added and incubated at room temperature for 15 min. The Vecta Stain solution is discarded, and the wells are washed (5 × 200 μL/well) with modified Tyrodes buffer. Developing buffer (10 mL of 50 mM citrate/phosphate buffer at pH 5.3, 6 mg of *o*-phenylenediamine, 6 μL of 30% H_2O_2; 50 μL/well) is added and incubated at room temperature for 3−5 min, and then 2 N H_2SO_4 (50 μL/well) is added. The absorbance is read at 490 nM.

This receptor binding assay was used before and after NMR studies to ensure the integrity of the integrin. GPIIb/IIIa was found to bind parent fibrinogen in either case. Prior to NMR studies, fibrinogen−receptor binding was inhibited by 1×10^{-6} M GRGDSP or by 4×10^{-6} M γ12. Following NMR experiments, the receptor was still quite active, but fibrinogen−receptor binding sometimes decreased by about 50%.

NMR Measurements. Freeze-dried samples for NMR measurements were dissolved in H_2O/D_2O (9:1). Peptide concentrations ranged from 0.2 to 10 mM. GPIIb/IIIa

receptor concentration was 0.2 mM. Solution conditions were 50 mM sodium phosphate, 2 mM $CaCl_2$, 10 mM SDS, pH 6. pH was adjusted by adding microliter quantities of NaOD or DCl to the protein sample. For most experiments, the temperature was controlled at 298 K. All NMR spectra were acquired on a Bruker AMX-600 NMR spectrometer.

For sequence-specific assignments, NOESY spectra and HOHAHA spectra obtained by spin-locking with a MLEV-17 sequence (Bax & Davis, 1985) were used. Spectra were acquired in the phase-sensitive mode (States et al., 1982). The water resonance was suppressed by direct irradiation (1 s) at the water frequency during the relaxation delay between scans as well as during the mixing time in NOESY experiments. The majority of the 2D-NMR spectra were collected as 512 t_1 experiments, each with 1K or 2K complex data points over a spectral width of 5 kHz in both dimensions with the carrier placed on the water resonance. 64 or 96 scans were generally time averaged per t_1 experiment. The data were processed directly on the Bruker AMX-600 X-32 or offline on a Bruker Aspect-1 or SGI Indigo workstation with Bruker UXNMR or FELIX programs, respectively. Data sets were multiplied in both dimensions by a 30−60°-shifted sine-bell or Lorentzian to Gaussian transformation function and generally zero-filled to 1K in the t_1 dimension prior to Fourier transformation.

NOESY spectra (mixing times of 50 to 500 ms) were analyzed to identify TRNOE cross-peaks of strong, medium, and weak intensity by analyzing one- dimensional projections of selected spectral regions and by cross-peak volume integration (Wüthrich, 1986). The sizes of some broadened NOE cross-peaks also were taken into account and re-scaled up in magnitude if they were found to be broader than average.

Structural Calculations. All structural calculations were done on a Silicon Graphics (SGI) Indigo Extreme workstation using an R4400 CPU running with 32 MB of RAM. The correct L-amino acid peptide sequence was built using Insight II/Discover (Biosym Technologies, San Diego, CA). To ensure that the starting peptide geometry was correct, initial structures were minimized without NOE-based restraints using the Discover program. A variable-target function distance geometry procedure (DGII in the Biosym suite of programs) was used (Braun & Go, 1985; Ni, 1994). In the absence of stereospecific resonance assignments, pseudoatom corrections were used in these structural calculations. TRNOE-derived distance constraints were incorporated sequentially, starting with one pair at a time from sets of initial random structures. Final iterations incorporated all distance constraints simultaneously. During calculations, the conjugate gradient method was used to minimize the full-matrix error function. Additional NOEs having low intensity or chemical shift degeneracy were then identified from analysis of interim structural models and used iteratively in later calculations. At the final stage, *in vacuo* molecular dynamics (50 ps) and energy minimization were used to relieve structural irregularities, e.g., bad bond lengths and contacts, and to improve structures. Resulting structures were analyzed and compared on an SGI Indigo Extreme workstation using Insight II. Most structures compared favorably with TRNOE-derived distances (less than 0.5 Å deviations) and with energetically favorable ϕ,ψ angles. Structures that did not meet these criteria were discarded.

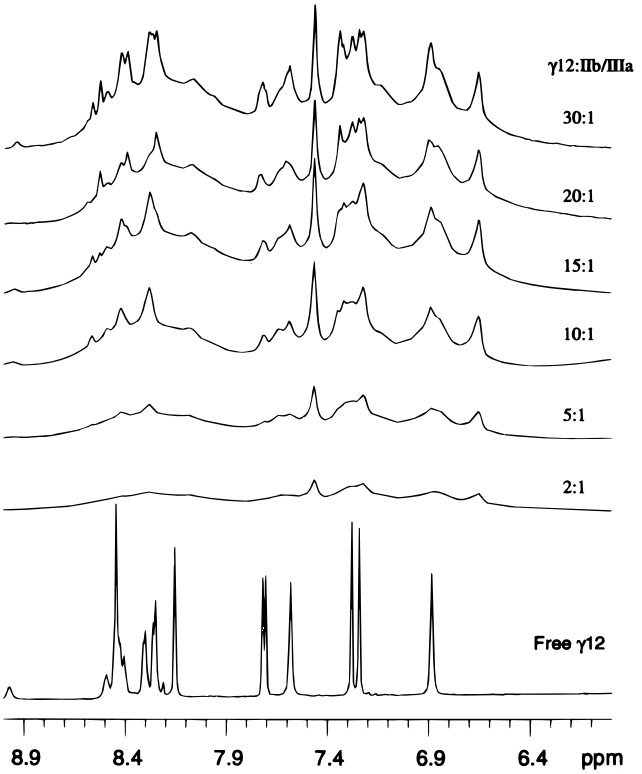

FIGURE 1: NMR spectra for GPIIb/IIIa titration with γ12. A series of ^{1}H NMR spectra are shown for various ratios of γ12:GPIIb/IIIa (indicated at the right). A spectrum of free γ12 peptide in the absence of GPIIb/IIIa is presented at the bottom. All spectra were acquired under the same solution conditions: 50 mM sodium phosphate, 2 mM $CaCl_2$, 10 mM SDS, pH 6, and 298 K. The GPIIb/IIIa protein concentration was 0.2 mM.

RESULTS

Two γ12 Binding States on GPIIb/IIIa. γ12 titration into a solution containing GPIIb/IIIa produces a series of NMR spectra shown in Figure 1. Proton resonances arising from GPIIb/IIIa (220 kDa) are mostly too broad to be observed although some are apparent, suggesting the presence of a few highly mobile residues. Up to a molar ratio of 5:1 (γ12:GPIIb/IIIa), γ12 resonances are broadened compared to those from free γ12 (bottom trace). Moreover, chemical shift patterns are significantly different from those found for free γ12. Increasing the γ12:GPIIb/IIIa ratio causes resonances to sharpen and to be chemically shifted toward free peptide resonances. This behavior is expected for peptide−receptor interactions that fall in the fast to intermediate ligand exchange regime (London et al., 1992; Campbell & Sykes, 1993; Ni, 1994). Due to chemical shift changes and broadening, resonance assignments cannot be made by direct comparison to 1D-NMR spectra of free γ12. Furthermore, at ratios of 15:1 and higher, the resonance envelope changes further as additional resonances appear and/or shift. Although the precise amount of GPIIb/IIIa which can bind γ12 peptide is unknown, the ratio of γ12:GPIIb/IIIa necessary for the observed NMR and TRNOE (see below) effects is consistent with most of the receptor being able to bind peptide (London et al., 1992; Campbell & Sykes, 1993; Ni, 1994).

A HOHAHA spectrum of free γ12 is shown in Figure 2. Sequence-specific resonance assignments (labeled in the figure and listed in Table 1) have been made in the standard way (Wüthrich, 1986) from analysis of HOHAHA and

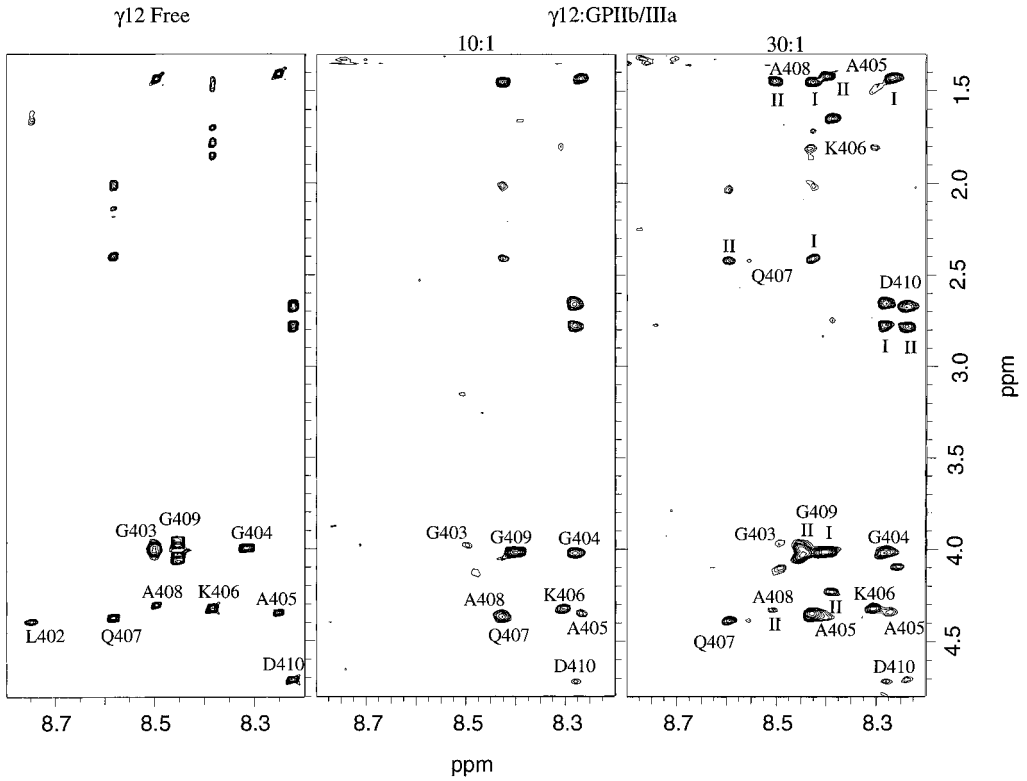

FIGURE 2: HOHAHA spectra of γ12 ± GPIIb/IIIa. Three HOHAHA spectra are presented showing the αH−NH/upfield region for free γ12 (left panel) and for γ12:GPIIb/IIIa molar ratios of 10:1 (middle panel) and 30:1 (right panel). Solution conditions are as described in the legend to Figure 1. Free γ12 peptide concentration was 2 mM, the same concentration as that in the 10:1 ratio spectrum. NMR data were acquired and processed as discussed in Materials and Methods. γ12 states I and II are labeled as discussed in the text.

Table 1: Chemical Shifts (ppm) for γ12 in Free and Receptor "Bound" States I and II

	NH			αH			βH			others		
		bound[a]			bound[a]			bound[a]			bound[a]	
residue	free	I	II	free	I	II	free	I	II	free	I	II
H400				4.18	4.01		3.19					
H401				4.74	4.73		3.2	3.53				
L402	8.75	8.45		4.40	4.47		1.67	1.82	1.82	γH 1.59	γH 1.65	
										δCH$_3$ 0.91	δCH$_3$ 0.99	
G403	8.50	8.39		4.01	4.15							
					3.96							
G404	8.31	8.28	8.30	4.00	4.01	4.01						
A405	8.25	8.27	8.40	4.36	4.35	4.35	1.41	1.44	1.41			
K406	8.38	8.29	8.44	4.33	4.34	4.34	1.87	1.86	1.86	γCH$_2$ 1.71	γCH$_2$ 1.72	γCH$_2$ 1.72
							1.79	1.72	1.72	δCH$_2$ 1.47	δCH$_2$ 1.48	δCH$_2$ 1.48
										ϵCH$_2$ 3.03	ϵCH$_2$ 3.05	ϵCH$_2$ 3.05
Q407	8.58	8.44	8.60	4.39	4.36	4.38	2.15	2.17	2.17	γCH$_2$ 2.40	γCH$_2$ 2.42	γCH$_2$ 2.42
							2.01	2.02	2.02			
A408	8.50	8.43	8.51	4.31	4.33	4.33	1.44	1.47	1.46			
G409	8.45	8.41	8.46	4.06	4.02	4.04						
				3.98	4.02	4.00						
D410	8.22	8.28	8.23	4.72	4.73	4.73	2.79	2.78	2.79			
							2.69	2.65	2.67			
V411	7.75	7.73	7.73	4.08	4.11	4.11	2.13	2.14	2.14	γCH$_3$ 0.93	γCH$_3$ 0.93	γCH$_3$ 0.93

[a] Chemical shifts are taken at a γ12:GPIIb/IIIa ratio of 20:1 and have not been extrapolated to 100% bound peptide.

NOESY spectra. H400 and H401 NH resonances are not observed due to their position at the N-terminus and to their resonating near HDO. Histidine C2, C4, αH, and βH_2 resonances, however, have been resolved in other spectral regions (data not shown). The V411 spin system is observed in HOHAHA spectra but is not shown in Figure 2.

At a γ12:GPIIb/IIIa ratio of 10:1, a HOHAHA spectrum shows significantly shifted and broadened γ12 cross-peaks (Figure 2). Even though only one γ12 binding site on GPIIb/IIIa has so far been reported (D'Souza et al., 1990, 1991; Taylor et al., 1992), we refer to this γ12 resonance population as γ12 binding *state* I (or simply γ12-I) to acknowledge the possibility that more than one γ12 binding *site* may be associated with this binding state. Sequence-specific resonance assignments for γ12-I (Table 1) were made by comparison to chemical shifts for free γ12 and by using the standard assignment approach with αN NOESY connectivities. As with free γ12, H400 and H401 αN HOHAHA and NOESY cross-peaks were absent. In addition, other γ12-I N-terminal resonances, i.e., L402, G403, and G404, were

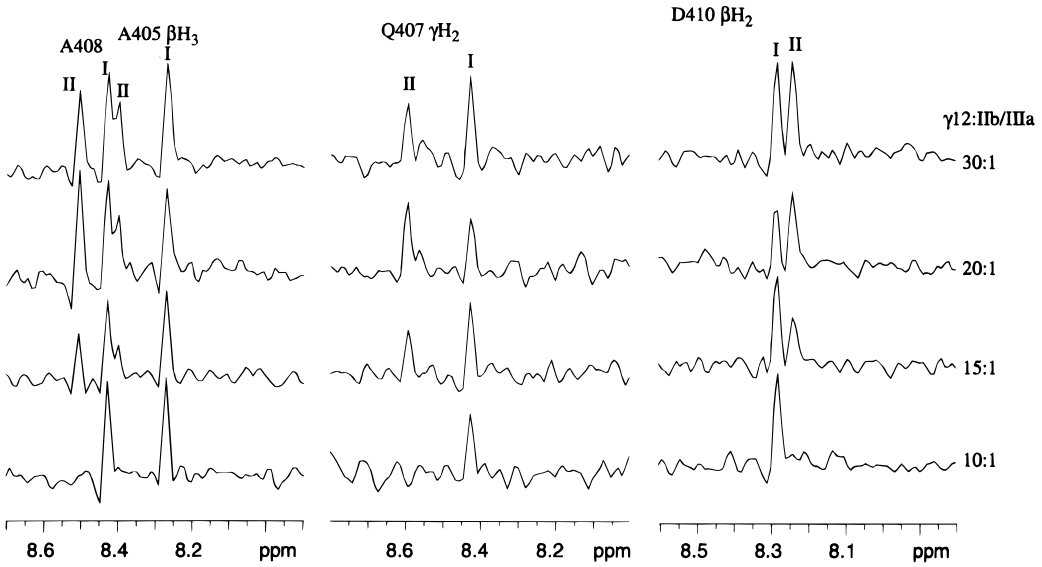

FIGURE 3: 1D HOHAHA slices for γ12 with GPIIb/IIIa. Three series of 1D-NMR spectra are shown for various γ12:GPIIb/IIIa molar ratios (indicated at the right). Spectra were generated as 1D slices through HOHAHA NH side chain cross-peaks as labeled in the figure. γ12 states I and II are indicated as discussed in the text. Solution conditions are as described in the Figure 1 legend.

not observed in HOHAHA spectra but were present in NOESY spectra. This is probably due to the γ12:GPIIb/IIIa ratio used and the relatively increased broadening of N-terminal over C-terminal resonances. γ12 line broadening is the result of dynamic exchange (London et al., 1992; Campbell & Sykes, 1993; Ni, 1994) between free γ12 peptide and GPIIb/IIIa receptor-bound peptide.

At a γ12:GPIIb/IIIa ratio of 30:1, a second γ12 binding state (γ12-II) is present (Figure 2). Since γ12-II gave very weak if any αN NOEs, resonance assignments for the γ12-II state had to be deduced primarily by comparison to chemical shifts for resonances of free γ12 and bound state γ12-I. Cross-peaks are labeled as "I" and "II" for interacting states γ12-I and γ12-II, respectively. Initially, the observation of highly attenuated (mostly absent) NOEs for γ12-II was surprising since even free γ12 gave negative (although small) NOEs. This will be addressed later in this section. Chemical shifts for γ12-II spin systems which could be discerned gave nearly the same chemical shifts as those for free γ12 (see Table 1). Chemical shifts for both γ12-I and γ12-II states listed in Table 1 have been taken from data at a 20:1 γ12:GPIIb/IIIa ratio and do not represent extrapolated 100% bound state shifts. The presence of these two γ12 bound states indicates that at least two γ12 binding sites are present on GPIIb/IIIa.

One γ12 Binding State Induces the Other. The γ12-II state appears at higher γ12:GPIIb/IIIa ratios, after formation of the γ12-I state. In order to illustrate this point, a more complete γ12−GPIIb/IIIa titration is shown in Figure 3, which has been generated by taking 1D slices through HOHAHA cross-peaks for A405 and A408 NH-βH_3, Q407 NH-γH_2, and D410 NH-βH_2. In each case, only γ12-I resonances are apparent up to a γ12:GPIIb/IIIa ratio of 10:1, above which γ12-II resonances are present. In fact, γ12-II resonances appear to increase in intensity between γ12:GPIIb/IIIa ratios of 15:1 and 20:1. Continued addition of γ12 above a ratio of 20:1 produces no further spectral changes. These observations suggest that the γ12-II state may be a direct consequence of the presence of the γ12-I state. In other words, γ12-I may induce an allosteric transition in GPIIb/IIIa which promotes formation of the γ12-II binding state. This is difficult to say for sure since, with an apparent γ12 K_d value of 4×10^{-6} M, receptor binding sites should be saturated at the γ12:GPIIb/IIIa 10:1 molar ratio.

The presence of N-terminal residues HHLG is crucial to the presence of the γ12-I receptor binding state. The absence of these amino acid residues in octapeptide GAKQAGDV (γ8) produces only one NMR observable GPIIb/IIIa interacting state (data not shown) showing spectral characteristics consistent with weaker γ12-receptor interactions. This is corroborated by the γ8 IC_{50} value for inhibition of parent fibrinogen−GPIIb/IIIa binding which is lowered by about 10-fold from 4×10^{-6} M for γ12 to 40×10^{-6} M for γ8. For this octapeptide that lacks the HHLG sequence, the γ12-I state is not observed. In this respect, the N-terminal segment that includes the histidines controls formation of the γ12-I receptor binding state.

Ligand−Receptor Exchange Time Scales. Three observations indicate that free γ12 and receptor-"bound" γ12-I are in the fast [free ⇔ bound] ligand exchange regime: (1) γ12 resonance line widths decrease as the γ12:GPIIb/IIIa ratio is increased (Figure 1), (2) γ12-I resonances are chemically shifted relative to those for free γ12 (Figure 2), demonstrating the presence of chemical shift averaging, and shift toward the free state as the γ12:GPIIb/IIIa ratio is increased, and (3) γ12-I NOE magnitudes are larger in the presence of GPIIb/IIIa [more negative than for free γ12; consistent with the presence of TRNOEs] and decrease as the γ12:GPIIb/IIIa ratio is increased. This indicates that the receptor off-rate is equal to or greater than about 100 s^{-1} (London et al., 1992; Campbell & Sykes, 1993), which is consistent with an apparent equilibrium dissociation constant of 4×10^{-6} M taken from the concentration of γ12 necessary for 50% inhibition of fibrinogen binding to GPIIb/IIIa in the biotinylated fibrinogen binding assay. In this respect, the receptor on-rate would be about 2.5×10^7 M^{-1} s^{-1}, which is within the expected Stokes−Einstein diffusion-limited range for a 1300 dalton peptide.

Assessing the exchange kinetics (and the origin) of the γ12-II state is more complicated, and any interpretation must be approached cautiously. If γ12-II were tightly bound to

GPIIb/IIIa (slow ligand−receptor off-rate), then the bound state NMR signal should disappear for a complex of 220 kDa at a concentration of 0.2 mM, and only free peptide signal would be observed. On the other hand, if there were a rapid equilibrium (fast exchange) between free and bound peptide, then γ12-II state chemical shifts should represent a weighted average between free and bound forms (London et al., 1992; Campbell & Sykes, 1993; Ni, 1994) as observed for γ12-I. γ12-II chemical shifts do vary with respect to those for free γ12 (Table 1). The best explanation for the tendency of γ12-II NOEs to be more positive (and mostly absent) relative to those observed for γ12-I and free γ12 is an overall slower ligand−receptor off-rate (London et al., 1992; Campbell & Sykes, 1993; Ni, 1994). Regardless of the γ12-II (and γ12-I) ligand−receptor exchange time scales, however, free γ12 peptides (released from GPIIb/IIIa from either state I or state II) should exist in solution in fast exchange with each other, and therefore their respective resonance chemical shifts should represent some weighted average. At higher γ12:GPIIb/IIIa ratios, results, however, clearly indicate the presence of two γ12 peptide conformational states (I and II) in slow exchange with each other on the 600 MHz ^{1}H-NMR chemical shift time scale.

Although this is unusual, slowly exchanging multiple γ12 states are not observed in the absence of GPIIb/IIIa or in the presence of SDS alone. Since SDS is known to denature proteins, which could result in artefacts, the conformational integrity of GPIIb/IIIa in the presence of up to 20 mM SDS was checked by both circular dichroism (CD) and fibrinogen binding assay and was found to be unaffected at this SDS:GPIIb/IIIa ratio. GPIIb/IIIa structural/functional integrity was further supported by the ability of GRGDSP to selectively remove the γ12-I state (described below). 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. One possible explanation for this apparent two-state paradox is that ligand binding induces a cis conformation in one or more peptide bonds, resulting in the γ12-II state. This, however, seems unlikely since γ12 contains no proline residues and the trans to cis energy barrier for all other amino acid peptide bonds makes such an interconversion extremely unfavorable under conditions of these experiments. An alternative and perhaps more viable explanation is that GPIIb/IIIa (0.2 mM) in the presence of SDS (10 mM) forms a system which mediates slow exchange among receptor-dissociated peptide conformers. SDS alone is known to form micelles under present conditions (Tanford, 1981) and is most likely associating with the receptor. In this scenario, receptor bound peptides from two binding states are released into an SDS/receptor environment which effectively acts to slow down the normally fast conformational exchange among peptides free in solution. In any event, the origin of the γ12-II state remains unclear.

RGD Displaces One γ12 Binding State. Addition of the hexapeptide GRGDSP at the appropriate γ12:GPIIb/IIIa ratio effectively eliminates γ12-I resonances. This is best observed in one-dimensional traces taken from HOHAHA spectra as cross-sections through A405 and A408 NH−βH$_3$ and D410 NH−βH$_2$ cross-peaks as shown in Figure 4. In the absence of GRGDSP, γ12-I and γ12-II resonance populations are about equal. At a 1:1 RGD:γ12 ratio, the γ12-I resonance population is decreased considerably, while

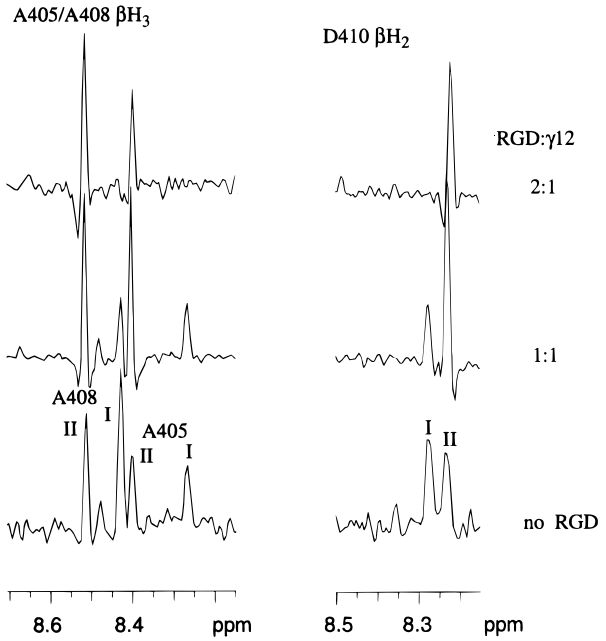

FIGURE 4: NMR spectra for titration with GRGDSP. Two series of 1D-NMR spectra are shown for γ12 in the presence of GPIIb/IIIa (γ12:GPIIb/IIIa ratio of 30:1) and at RGD:γ12 molar ratios of 1:1 and 2:1 as indicated at the right in the figure. Spectra were generated as 1D slices through HOHAHA NH side chain cross-peaks as labeled in the figure. γ12 states I and II are indicated as discussed in the text. Solution conditions are as described in the Figure 1 legend.

at a 2:1 ratio, γ12-I resonances are not observed. Depletion of γ12-I resonance intensities at the 2:1 RGD:γ12 ratio is consistent with stronger GRGDSP−receptor binding. In the biotinylated fibrinogen−receptor binding assay, a smaller IC_{50} value for GRGDSP relative to that for γ12 was measured. The most probable interpretations for these results are either that γ12-I and GRGDSP directly compete for one (or part of one) binding site on GPIIb/IIIa or that their binding sites are allosterically linked. The former explanation is perhaps the more likely since it has been reported (Lam et al., 1987; Ginsberg et al., 1993) that γ12 and RGD share one (or part of one) binding site on GPIIb/IIIa. On the basis of observations of unaltered γ12-II NOE magnitudes, binding state γ12-II appears not to be affected by the presence of GRGDSP. γ12-II resonances may be shifted slightly due to the γ12-I and γ12-II exchange process.

Transferred NOEs and GPIIb/IIIa Bound State Conformation. While obviating derivation of the γ12-II receptor bound state conformation, the near absence of γ12-II NOEs simplifies NOESY spectral analysis for the γ12-I state. Aside from γ12-I *i,i* and *i,i+1* αN cross-peaks identified in Figure 5, a number of conformationally constraining NOEs are labeled in Figures 5−7. Free γ12 NOEs are either smaller or absent relative to those for γ12-I, and analysis of NOE buildup curves for γ12-I indicates that maximum NOEs occur at shorter mixing times than for free γ12. These observations indicate the presence of TRNOEs from γ12-I due to populations of receptor "bound" peptide in dynamic exchange with free peptide (London et al., 1992; Campbell & Sykes, 1993; Ni, 1994), in agreement with results based on differential line broadening of peptide resonances (Figure 1). Apart from an indication of GPIIb/IIIa binding, the observation of TRNOEs also suggests that the range of conformations accessible to the bound peptide is restricted by the geometry of the binding site on the integrin receptor.

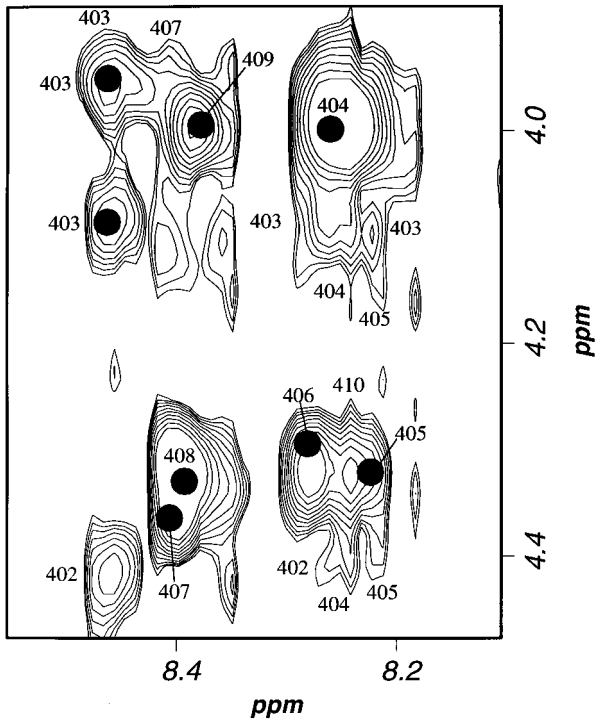

FIGURE 5: NOESY spectrum for γ12-I receptor bound state. The αH−NH region from a NOESY spectrum (200 ms mixing time) is shown. Solution conditions are as described in the legend to Figure 1. NMR data were acquired and processed as discussed in Materials and Methods. γ12-I state HOHAHA cross-peaks are indicated as filled-in circles and labeled as discussed in the text. Various TRNOEs have been identified.

NOESY spectra (50, 100, 200, and 400 ms) were analyzed to identify TRNOEs and to check for possible spin diffusion. Figure 7 identifies three sequential NH−NH TRNOEs: 403−404, 406−407, and 409−410. By varying solution conditions, we can also observe 405−406 and 407−408 NH−NH TRNOEs (data not shown). At the very least, this series of NH−NH TRNOEs indicates some type of multiple turn or helical conformation in the integrin bound state. Some *i,i+2* and *i,i+3* TRNOEs which support the presence of helical conformation are identified in Figures 5 and 6. Table 2 lists all γ12-I TRNOEs. Identification of some potentially informative TRNOEs was precluded by resonance overlap, particularly of αH resonances. Using DG-generated structures described below, NOE data were reanalyzed to help identify additional TRNOEs.

TRNOEs have been used to determine the bound structure of γ12-I. Derivation of TRNOEs can be complicated if the free peptide shows some kind of NOE cross-peaks, especially for sequential and intraresidue interactions (Ni, 1994). In this study, analysis of TRNOEs was simplified considerably since in free γ12, there were no NOEs greater than *i,i+1* and those NOEs which were present were smaller than for the "bound" state. When necessary, corrections were made by subtracting the free γ12 NOE from that of the γ12 bound state NOE. At lower mixing times, free γ12 NOEs respresented at most 20% of the uncorrected NOE.

TRNOE cross-peak intensities were classified as strong, medium, weak, or very weak by analyzing one-dimensional F1 and F2 slices from selected NOESY spectral regions and by counting NOESY cross-peak contours (Wüthrich, 1986). The sizes of some broadened NOE cross-peaks also were taken into account and re-scaled up in magnitude if they were

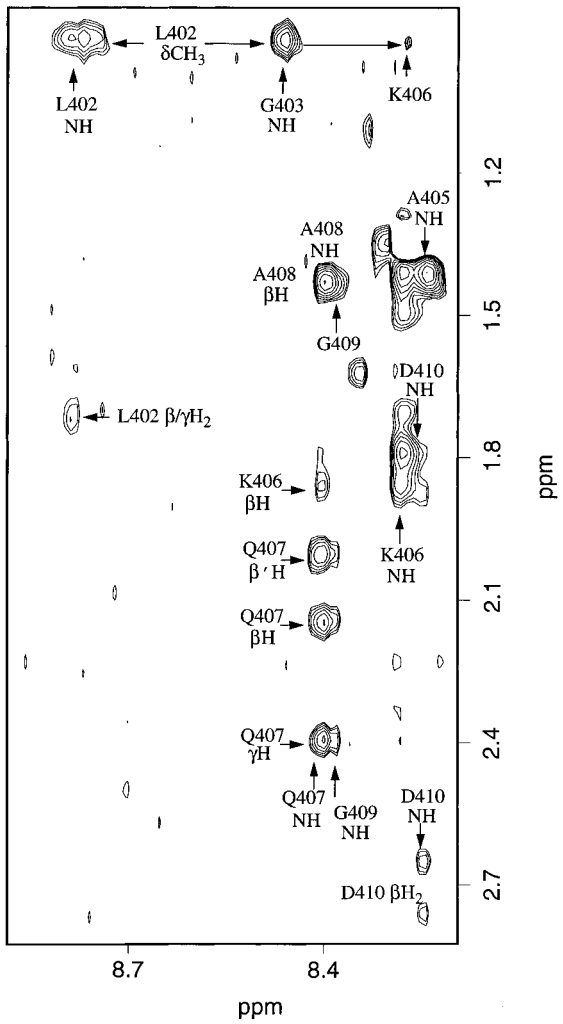

FIGURE 6: NOESY spectrum for γ12I receptor bound state. The NH−βH/upfield region from a NOESY spectrum (200 ms mixing time) is shown. Solution conditions are as described in the legend to Figure 1. NMR data were acquired and processed as discussed in Materials and Methods. Various TRNOEs have been identified.

found to be broader than average. Increased lower bounds between some sequential αH−NH protons also were identified for a few proton pairs which gave smaller NOEs than for free γ12. Distance cutoffs were then assigned: strong NOEs, 1.8−2.8 Å; medium NOEs, 1.8−3.3 Å; weak NOEs, 1.8−4.0 Å, and very weak NOEs, 1.8−5.0 Å. 0.5 Å was added to the upper limit for each interproton distance involving a side chain. These interproton distances are listed in Table 2. A more quantitative approach was not used primarily because no good distance calibration was available. γ12 contains as aromatics only two histidines whose resonances are not observed, and alanine αH−βH$_3$ NOEs could not be determined accurately due to overlap of A405 and A408 αH resonances. Although the G403 αH−αH NOE remained as a potential calibration factor, inaccuracies in TRNOE magnitudes can easily arise with short internuclear distances (Campbell & Sykes, 1993; London et al., 1992; Ni, 1994) as exist between gylcine methylene protons [1.8 Å].

On the basis of interproton distances listed in Table 2, a distance geometry (DG) minimization protocol described in the Materials and Methods was used to determine the most probable bound state ensemble of conformations for γ12-I. Of 50 final structures calculated, the best 35 had favorable

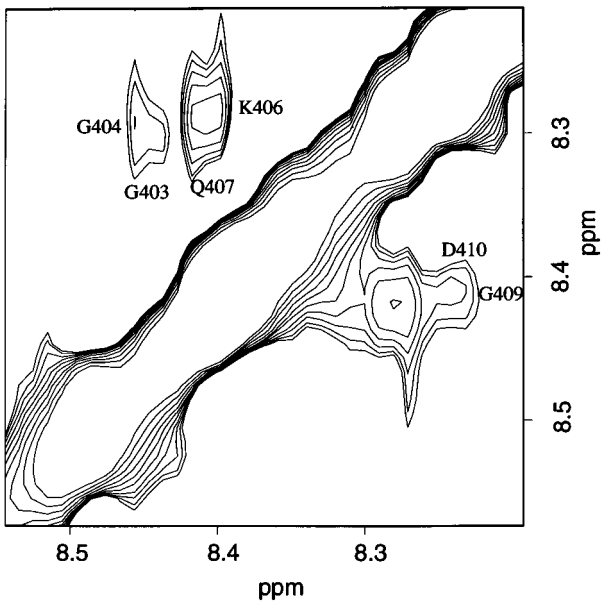

FIGURE 7: NOESY spectrum for γ12-I receptor bound state. The NH−NH region from a NOESY spectrum (200 ms mixing time) is shown. Solution conditions are as described in the legend to Figure 1. NMR data were acquired and processed as discussed in Materials and Methods. Various TRNOEs have been identified.

Table 2: γ12−GPIIb/IIIa Transferred NOEs

NOE[a]			NOE[a]		
from	to	size[b]	from	to	size[b]
402 αH	404 NH	w	407 NH	408 NH	m
402 NH	402 βH_2	m		407 βH^1	w
	402 δH_2	m		407 γH_2	m
403 NH	404 NH	m		403 αH	w
	402 δH_3	w	408 NH	407 βH_2	w
403 αH^2	405 NH	w		407 γH_2	w
	406 δH_2	vw		408 βH_3	s
404 αH	406 NH	w		406 βH_2	w
404 NH	402 δH_3	vw	409 NH	408 βH_3	m
	406 βH_2	w		407 βH_2	vw
405 NH	406 NH	m		407 γH_2	w
	403 αH	w		410 NH	m
	402 αH	w		411 γH_3	vw
406 NH	406 βH_2	m	410 NH	408 αH	w
	406 γH_2	m		410 βH^1	m
	406 δH_2	w		407 βH_2	vw
	405 βH_3	m		407 γH_2	w
	407 NH	m			
	402 δH_3	vw			

[a] NOEs have been tabulated from 100 and 200 ms NOESY spectra accumulated at 25 °C and under conditions as described in the text. αN *i,i+1* NOEs have not been listed since these were not used in structural calculations. [b] Relative NOE magnitudes have been derived from volume integration and/or contour level counting. Lower−upper bound distances used in structural calculations are as follows: strong (s) NOEs, 1.8−2.8 Å; medium (m) NOEs, 1.8−3.3 Å; weak (w) NOEs, 1.8−4.0 Å, and very weak (vw) NOEs, 1.8−5.0 Å. 0.5 Å was added to the upper limit for each interproton distance involving a side chain.

ϕ,ψ angles (Richardson, 1981; Iijima et al., 1987) and have been superimposed in Figure 8 by using the backbone heavy atoms of residues G403−G409. Not surprisingly, the N- and C-termini are least defined due to a paucity of NOEs to/from the more terminal residues. Of these 35 structures, 22 superimposed very well with average backbone RMSD values of 0.56 Å (G403−A408). A second subfamily composed of eight other structures also gave good backbone RMSD values (0.68 Å for G403−A408). Another five

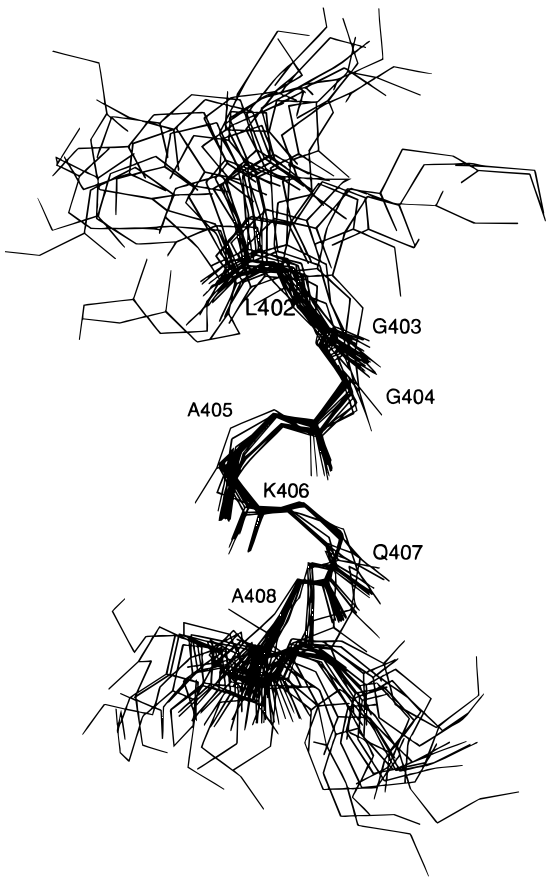

FIGURE 8: Distance geometry calculated structures for γ12-I. 35 distance geometry calculated structures for the γ12-I GPIIb/IIIa receptor bound state are overlayed in this figure. DG calculations were performed as discussed in the text. Only backbone heavy atoms have been shown. Residues are labeled close to their C_α atoms.

Table 3: Structural Statistics and Atomic RMSD Values

NOE-Derived Distance Restraint Violations (Residues 402−411)[a]			
	no. of restraints	av RMS (Å)	av max (Å)[b]
all[c]	36	0.054	0.28
intraresidue (*i,i*)	9	0.048	0.22
sequential (*i,i+1*)	10	0.032	0.16
other (≥*i,i+2*)	17	0.071	0.32

Atomic RMSD Values (Å)		
residues	backbone atoms	all heavy atoms
402−409	0.79	1.93

[a] No violations greater than 0.4 Å. [b] For residues showing violations; mostly with restraints to side chains. [c] Does not include $\alpha N_{(i,i+1)}$ restraints which were not used in structural calculations. See Table 2.

structures fell close to either subfamily. Table 3 lists some pertinent structural statistics. Among all structures, the conformation from G403 to D410 is best defined as helical.

DISCUSSION

Understanding ligand−integrin interactions (not to mention integrin-mediated cell adhesion processes) is complicated by the promiscuous nature of these receptors in binding many different ligands, the presence of multiple binding sites, and ligand-induced conformational transitions (Ginsberg et al., 1993). Even working with purified receptor, γ12 peptide interactions with purified GPIIb/IIIa appear to be no less complicated.

Only one γ12 binding site on the integrin (α_{IIb} chain) so far had been reported (D'Souza et al., 1990, 1991; Taylor et al., 1992). The present NMR data indicate that γ12 interacts with GPIIb/IIIa on at least two binding sites or states, γ12-I and γ12-II. The γ12-II interaction state appears to be induced by the presence of the γ12-I state. This may explain why fibrinogen binding to GPIIb/IIIa also can be activated by the presence of peptide ligands (Du et al., 1991). It has been hypothesized that fibrinogen binding to GPIIb/IIIa may proceed by initial recognition of an RGD-like sequence, recognition-induced receptor conformational change(s), and then additional high-affinity ligand−receptor interaction(s) (Du et al., 1993; Ginsberg et al., 1993). Du et al. (1991) found that when soluble purified GPIIb/IIIa is pretreated with γ12 or GRGDSP, the receptor binds fibrinogen with 1:1 stoichiometry and high affinity. This is consistent with the present findings and supports the idea that binding of γ12 to an initial site on GPIIb/IIIa induces an allosteric transition in the receptor which promotes binding at an additional site(s).

The presence of hexapeptide GRGDSP at the appropriate γ12:GPIIb/IIIa ratio, effectively removes the γ12-I state without affecting the γ12-II state. This suggests that the RGD ligand either competes directly with the γ12-I state or induces a conformational change in the receptor to obviate the γ12-I interaction state. The former explanation may be more likely. It should be noted that, at higher RGD:γ12 ratios, RGD induces formation of up to at least four or five γ12 binding sites on GPIIb/IIIa (Mayo et al., 1996). Lam et al. (1987) have provided evidence that fibrinogen γ-chain C-terminal peptides and RGD-containing peptides do share a common binding site on GPIIb/IIIa. Furthermore, Abrams et al. (1992) found that certain monoclonal antibodies to the idiotypic determinant of PAC1 (which binds to GPIIb/IIIa in ways similar to fibrinogen) bind to the γ-chain C-terminus as well as to both α-chain regions containing RGD sequences. This being the case, one might conclude that in the absence of RGD we are dealing only with two γ12 binding sites on GPIIb/IIIa. In any event, RGD apparently can replace γ12-I in its role to induce the γ12-II state. This is consistent with results of Timmons et al. (1989), who reported that platelet receptors interacting with γ- and α-chains of fibrinogen can be blocked interchangeably by synthetic γ12 and RGD-containing peptides representing sequences on either chain and supports the findings of Weisel et al. (1992) and Farrell et al. (1992) that the γ-chain C-terminal segment alone is essential for optimal fibrinogen-mediated platelet aggregation.

Two RGD ligand binding sequences have been identified on GPIIb/IIIa by chemical/genetic means, and other potential RGD interactive sites have been identified by hydropathic complementary approaches and by monoclonal antibody mapping (Ginsberg et al., 1993). It is unknown, however, if any of these sequences are spatially related, forming fewer actual RGD binding sites. The GPIIb/IIIa γ12/RGD ligand binding sequences which so far have been identified suggest the importance of oxygenated residues like aspartic acid and serine in the ligand−receptor binding process (Ginsberg et al., 1993). For example, the GPIIIa (β3) integrin subunit amino acid sequence D^{119}LSYSMKDDLWS^{130} is highly conserved among known β subunits (Loftus et al., 1990). Moreover, D^{119} is absolutely conserved and is proximal to the residue proposed to be cross-linked to the bound RGD peptide at K^{125} (D'Souza et al., 1988). It has been proposed that the clustering of oxygenated residues could provide coordination sites for various ligands and divalent cations (Kretsinger, 1980; Frelinger et al., 1988). Ginsberg et al. (1993) have hypothesized that oxygenated residues from both the receptor and the ligand are bridged, on interacting, via a divalent cation. The aspartic acid residues in GRGDSP (Ginsberg et al., 1985) and γ12 (D410) (Ruggeri et al., 1986) are given central roles in receptor binding. Alternatively, these are not the only residues found to be crucial to GPIIb/IIIa receptor binding. It has long been held that several other residues in γ12 are crucial for fibrinogen binding to GPIIb/IIIa (Kloczewiak et al., 1989). Substitutions of K406 and V411 are particularly detrimental to bioactivity. Furthermore, Yao and Mayo (1996) recently found that arginines and histidines along with hydrophobic residues on GPIIb/IIIa most probably also mediate fibrinogen binding.

Kloczewiak et al. (1984, 1989) also have noted that even the pentapeptide QAGDV derived from γ12 is only six times less reactive than γ12 in inhibiting fibrinogen-mediated platelet aggregation, while Plow et al. (1984) claim that γ12 without its N-terminal histidines is as reactive as parent γ12 and that shorter peptides derived from γ12 are inactive. While we cannot resolve this controversy, these present NMR studies indicate that the histidines do indeed play a role in γ12 interactions with GPIIb/IIIa. Resonance line broadening and chemical shift changes at any γ12:GPIIb/IIIa ratio are greatest for γ12 N-terminal residues indicating stronger interactions with GPIIb/IIIa through the N-terminal sequence. Chemical shift differences relative to those of free γ12, for example, become greater as one moves from the C- to the N-terminus, and G403 αH_2 resonances, in fact, become non-degenerate in the bound state. Furthermore, removal of the first four γ12 N-terminal residues (HHLG) produces an eight-residue C-terminal peptide which shows only one GPIIb/IIIa interacting state; the γ12-I state is apparently eliminated. In this respect, the N-terminal segment that includes the histidines apparently controls formation of the γ12-I receptor binding state.

TRNOE-derived DG calculated structures for the γ12-I GPIIb/IIIa bound state can be classified as helical. Earlier NMR studies on a longer γ-chain C-terminal peptide 385−411 (Mayo et al., 1990) provided evidence for helix-like conformation running from 390 through 402, with the C-terminal segment residues 403−411 being less defined due to its high internal mobility. One exception within the flexible C-terminal segment was a turn/loop within the 406−410 sequence. This observation was generally supported by Blumenstein et al. (1993) who found a relatively stable turn centered at residues 408−409 in γ-chain peptide 392−411. This C-terminal segment in γ-chain peptide 385−411 does form additional helix structure that includes the C-terminal segment when the dielectric of the medium is reduced in solutions containing trifluoroethanol (Fan & Mayo, 1995).

Hoekstra et al. (1994, 1995) based the design of a peptidemimetic GPIIb/IIIa antagonist on the NMR-derived conformation of γ-chain C-terminal residues 385−411 (Mayo et al., 1990). Since the γ-chain 400−411 sequence is required for γ-chain bioactivity and is a unique recognition sequence among ligands for integrins, vis-à-vis other RGD-presenting proteins, these γ12 mimetics may represent a new, selective approach to inhibition of fibrinogen-mediated platelet aggregation. For the rational design phase, the K406, G409

$C_\alpha-C_\alpha$ distance was maintained between 4.5 and 5 Å with a charge separation of less than 10 Å between the two crucial residues K406 and D410. Although in the present study the receptor-bound conformation for this C-terminal segment 400−411 differs from that for peptide 385−411 (Mayo et al., 1990; Hoekstra et al., 1994, 1995), the K406 to D410 inter-residue distances are essentially the same due to the presence of helical structure. Apparently, electrostatic interactions between K406 and D410 (and/or V411 carboxy terminus) stabilize the observed turn conformation in peptide 385−411 (Mayo et al., 1990). The differences between these structures result from a lack of NOE-derived distance constraints due to the high flexibility of this γ-chain segment noted above. In the receptor bound state, the conformation is "locked-in."

The γ12-I receptor bound state conformation defines a surface composed of residues L402−G403, K406−Q407, and D410−V411. The latter four residues were previously identified as being crucial to fibrinogen-mediated platelet−platelet cell adhesion. On the basis of line broadening and chemical shift changes due to the dynamic exchange process between free and bound peptide it is apparent that γ12 N-terminal residues up to Q407 interact more strongly with GPIIb/IIIa than do the C-terminal residues. These data, therefore, suggest that designing a mimetic that includes elements of this entire surface may make an effective, γ-chain-specific antithrombotic agent.

CONCLUSIONS

γ12 interacts at two sites on GPIIb/IIIa. Binding at one site apparently induces an allosteric change in GPIIb/IIIa which exposes the second binding site. The presence of the N-terminal histidines is crucial to interactions at the first GPIIb/IIIa binding site. The first occupied site can be competed off with RGD, indicating either that this site is shared with RGD or that RGD induces an allosteric transition to effectively remove this site. The bound state conformation of γ12 in the first site is helical, placing the important C-terminal lysine, glutamine, aspartic acid, and valine residues on the same surface for interactions with the receptor.

ACKNOWLEDGMENT

We are very grateful to Eric Eccleston and Denisha Walik of the Department of Human Genetics Microchemical Facility, University of Minnesota, for the synthesis of peptides used in this study. The authors also thank Jeff Press for his support for this project.

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BI952485N