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Glycoprotein IIb peptide 656–667 mimics the fibrinogen γ chain 402–411 binding site on platelet integrin GPIIb/IIIa

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The human integrin glycoprotein IIb/IIIa complex plays a central role in haemostasis as an inducible receptor for fibrinogen and other adhesive proteins at the platelet plasma membrane. Current evidence indicates that the ligand-binding domain of GPIIb/IIIa is discontinuous and placed at the subunit interface. Here we show that a synthetic peptide containing the polypeptide stretch GPIIb 656–667, which is hidden within the resting platelet GPIIb/IIIa heterodimer but becomes exposed following platelet activation with thrombin, binds to soluble fibrinogen ($n = 2.3 \pm 1.3$; $K_d = 2 \pm 0.8 \times 10^{-5}$ M). This interaction is Ca^{2+}-independent and can be partially inhibited with synthetic fibrinogen γ-chain peptide 400–411 but not with GRGDS. In addition, peptide GPIIb 656–667 inhibits in a dose-dependent manner the aggregation of activated platelets ($IC_{50} = 170\ \mu M$). Altogether, our results indicate that the GPIIb 656–667 region may form part of the inducible fibrinogen binding site and may not overlap with the integrin RGD-recognition domain.

Platelet; GPIIb/IIIa; Platelet aggregation; Fibrinogen binding site; Peptide synthesis; Equilibrium sedimentation

1. INTRODUCTION

Human glycoprotein IIb/IIIa, a Ca^{2+}-dependent heterodimer, is the major integrin on the platelet plasma membrane where it serves as an inducible receptor for plasma fibrinogen (Fb) and other adhesive proteins, including fibronectin (Fn), vitronectin (Vn) and von Willebrand factor (vWF) (reviewed in [1]). Dissection of the fibrinogen molecule led to the identification of several putative receptor-recognition sequences within its dimeric structure (reviewed in [2]). Notably, small synthetic peptides containing the amino acid sequence Arg-Gly-Asp (RGD), which is present within the primary structure of Fb, Fn, Vn, and vWF, inhibited the binding of each of these adhesive proteins to GPIIb/IIIa. In addition, a dodecapeptide only found at the C-termini of the fibrinogen γ-chains (^{400}HHLGGAKQAGDV^{411}) also inhibits the binding of each of the adhesive proteins to activated GPIIb/IIIa [2]. Although the binding of RGD and γ-chain peptides is mutually exclusive (reviewed in [3,4]), immunological data [5], studies on recombinant human fibrinogen [6], and examination by electron microscopy of the interaction of isolated GPIIb/IIIa with fibrinogen [7], all suggest that the C-terminus of the γ-chain contains the essential information for receptor binding.

On the other hand, human platelet GPIIb/IIIa is the most thoroughly studied integrin [3]. However, despite the wealth of information regarding structure-function relationships of GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$), the precise structure of the heterodimer, and consequently the relationship between the γ-chain- and the RGD-binding site(s), remain obscure. In addition, the identification of a variety of GPIIb and GPIIIa domains putatively involved in ligand recognition (reviewed in [3,8]) indicates that the integrin's receptor surface may be made up of regions from both subunits which, although distantly located within the linear primary structure, are brought into juxtaposition by the spatial folding of the polypeptide chains [9]. Here, we have focused on the GPIIb 656–667 polypeptide stretch because this region (a) contains the epitope for the platelet aggregation inhibitory monoclonal antibody M6, whose surface expression on GPIIb/IIIa is platelet activation-dependent, and (b) shows a high hydropathic complementary to the fibrinogen γ402–411 GPIIb/IIIa recognition sequence [10]. Our results show that synthetic GPIIb 656–667 binds to soluble human fibrinogen with a dissociation constant of $2 \pm 0.8 \times 10^{-5}$ M in a Ca^{2+}- and RGD-independent manner. In addition, GPIIb 656–667 inhibits ADP-induced platelet activation with an IC_{50} of around 170 μM. Taken together, these data indicate that the GPIIb

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656–667 domain fulfills the requirements expected for an inducible fibrinogen-specific recognition domain.

2. MATERIALS AND METHODS

2.1. *Peptide synthesis*

Peptides GRGDS, GPIIb$^{656-667}$ (GAHYMRALSNVE) [21], CGAHYMRALSNVE (Cys-GPIIb$^{656-667}$), and HHLGGAKQAGDV ($\gamma^{400-411}$) were synthesized using a semi-automated peptide synthesizer (Labortec SP 640) as described [10,11]. The molecular masses of the peptides were measured by fast atom bombardment mass spectrometry using a MAT 900 instrument (Finnigan MAT, Bremen) equipped with a liquid secondary-ion ionization system. Quantification of peptides was done by amino acid analysis after hydrolysis with 6 N HCl for 24 h at 110°C.

2.2. *Selective labeling and purification*

For selective labeling of the N-terminal cysteine residue with eosin-5′ iodoacetamide (Molecular Probes, Eugene, Oregon), 100 mg of side chain-protected resin-attached peptide Cys-GPIIb$^{656-667}$ were swollen in 1 ml dichloromethane for 30 min, washed twice with dimethylformamide (DMF), and incubated with 100 μl (DMF), 10 μl deionized water, 23 μl tributylphosphine, and 5 μl triethylamine, for 2 h at room temperature with gentle stirring. This treatment selectively releases the *S*-tert-butyl group from the N-terminal cysteine. After exhaustive washing with DMF, the peptide was incubated with a 1.5 molar excess of eosin-5′iodoacetamide in DMF/100 mM phosphate pH 8.0 (80:20, v/v) for 4 h at room temperature, in the dark, and under continuous stirring. Thereafter, the excess of dye was extracted with DMF (until the eluent did not absorb at 530 nm), and the labeled peptide was deprotected and released from the polymeric resin by treatment with 85% (v/v) trifluoroacetic acid and 5% (v/v) thioanisol for 6 h at room temperature with gentle stirring. The peptide was precipitated with diethylether and purified by reverse-phase HPLC using a Lichrospher RP-100 C18 column (Merck, Darmstadt) (25 × 0.4 cm, 5 μm particle size) eluted at 1 ml/min with a gradient of 0.1% (v/v) trifluoroacetic acid in water (A) and acetonitrile (B) as follows: isocratically (15% B) for 5 min, followed by 15–60% B for 90 min. The eluate was monitored at both 220 and 490 nm.

2.3. *Platelet aggregation inhibition experiments*

Platelet aggregation inhibition experiments were performed as described [12]. Briefly, 400 μl platelet suspension aliquots (3×10^8 platelets/ml in Tyrode's buffer) were activated at 37°C with 10 μl ADP (60 μM, final concentration) and mixed with 40 μl of fibrinogen solutions (0.6 mg/ml) which had been preincubated at 37°C with the GPIIb$^{656-667}$ peptide (final concentration ranging from 30 μM to 10 mM).

2.4. *Equilibrium sedimentation*

The interaction of soluble human fibrinogen (6 μM in 10 mM Tris-HCl, 150 mM NaCl, pH 7.4, containing either 1 mM $CaCl_2$ or 1 mM EGTA) with different amounts of eosin-labeled peptide Cys-GPIIb$^{656-667}$ (10^{-6} to 10^{-3} M) was analyzed by short column sedimentation equilibrium experiments, performed essentially as described [13,14], using an XLA Optima analytical ultracentrifuge (Beckman Instruments Inc., Palo Alto) run at 10,000 rpm and 20°C. The equilibrium distributions of human fibrinogen and eosin-peptide were determined by radial scanning at 300 nm and 530 nm, respectively. Similar experiments were performed in the presence of 1 mM (final concentration) of either peptide GRGDS or GAHYMRALSNVE.

3. RESULTS AND DISCUSSION

If GPIIb 656–667 constitutes an element of the fibrinogen-recognition domain in GPIIb/IIIa, then it may interact directly with fibrinogen inhibiting thereby platelet aggregation. Fig. 1 shows a typical curve of the concentration-dependent ability of synthetic GPIIb 656–667 peptide to inhibit the fibrinogen-mediated aggregation of human gel-filtered platelets activated with ADP. Inhibition reached 93% when the final concentration of peptide was 1 mM. The calculated IC_{50} from two experiments was 173 ± 10 μM.

Solid-phase (ELISA) analysis using eosin-labeled GPIIb peptide showed that it does not interact with isolated GPIIb/IIIa but binds in a concentration-dependent manner to immobilized human fibrinogen (not shown). The possibility that the eosin moiety might play a role in the GPIIb peptide-fibrinogen interaction was ruled out because the unlabeled peptide competes quantitatively with the labeled one for binding to immobilized fibrinogen. We then studied the interaction of eosin-labeled GPIIb 656–667 peptide with human fibrinogen in solution (TBS buffer, pH 7.4, 20°C) by equilibrium sedimentation. Fig. 2 shows the equilibrium sedimentation distribution of fibrinogen (6 μM) in the presence of 20 μM peptide recorded at 300 nm (A), a wavelength where the peptide but not eosin absorb, and at 530 nm (Fig. 2B ○–○), where only eosin absorbs. This indicates that the synthetic peptide cosedimentes with fibrinogen. The interaction was apparently specific for fibrinogen, since the synthetic peptide did not bind to other proteins, like catalase (Fig. 2B, ◆–◆), and followed the equation $[L_o] = [L] + B[P]$ (Fig. 3), where $[L_o]$ is the total peptide concentration, [L] is the concentration of free peptide (Y-axis intercept), and [P] is the concentration of fibrinogen. Assuming a single class of equivalent and independent binding sites, B (the slope) can be defined as $n\,K\,[L]/(1 + K[L])$ [15], where K is the association constant, and n the number of binding sites. Chi-square was minimized using a non-linear least square procedure based on the modified Nelder–Mead simplex algorithm [16]. Representation of B versus log[L] (Fig. 4) allowed us to determine the following parameters: $n = 2.3 \pm 1.3$ and $K = 2 \pm 0.8 \times 10^{-5}$ M. Interestingly, the GPIIb 656–667 synthetic peptide-fibrin-

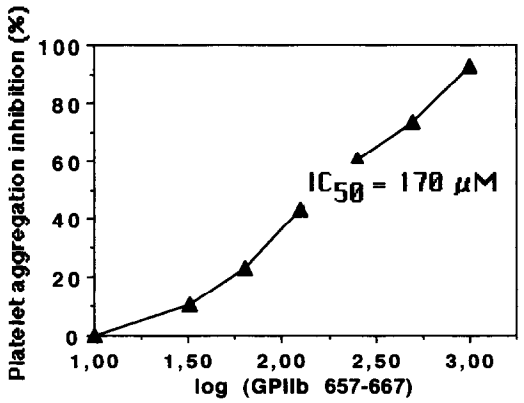

Fig. 1. Dose-dependent inhibition of the fibrinogen-mediated aggregation of washed platelets activated with ADP by synthetic peptide GAHYMRALSNVE (GPIIb$^{656-667}$). IC_{50} is the final concentration of peptide causing 50% of maximal inhibition.

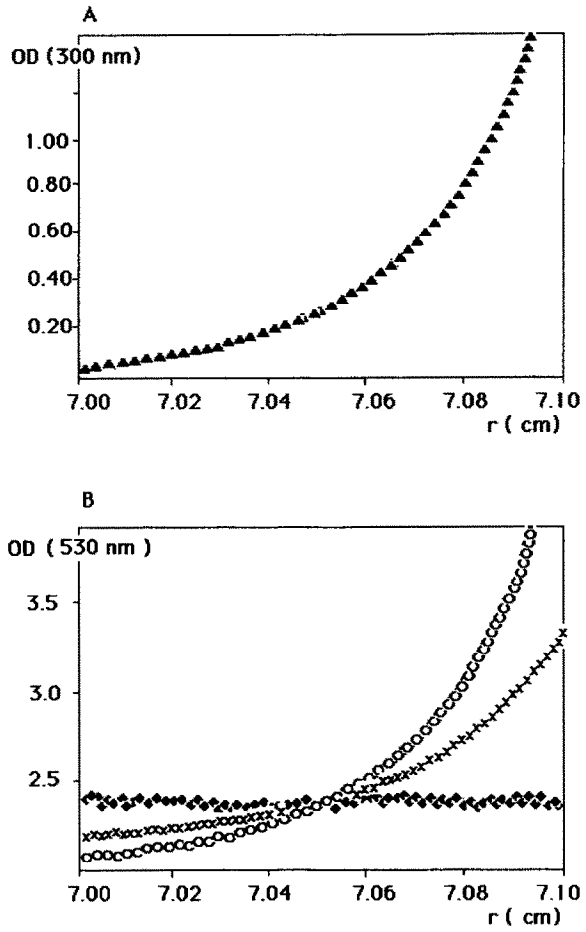

Fig. 2. (A) Equilibrium sedimentation profile of human fibrinogen (6 μM in TBS pH 7.4) at 10,000 rpm, 20°C, detected at 300 nm; r, radius. (B) Equilibrium sedimentation profile (as in A) at 530 nm of eosin-labeled peptide Cys-GPIIb 656–667 (20 μM) in the presence of 5 μM catalase (◆–◆), 6 μM fibrinogen (○–○), and 6 μM fibrinogen + 1 mM g-chain 400–411 peptide (×–×). Each curve is the average of 2–4 experiments.

ogen binding could be partially (around 30%) reverted with 1 mM fibrinogen γ-chain 400–411 peptide (Fig. 2B, ×-×), but not with 1 mM GRGDS peptide (curve not shown), and was not affected by either 1 mM $CaCl_2$ or 1 mM EGTA.

The low inhibition of GPIIb 656–667 (20 μM) binding to soluble fibrinogen (6 μM) exert by 1 mM synthetic γ-chain 400–411 peptide (Fig. 2B) suggests that only a small population of the isolated synthetic fibrinogen peptide adopts an active conformation in solution, or that the binding of the GPIIb peptide to fibrinogen stabilizes a preferred conformer. The first possibly would be in accordance with a recent two-dimensional ^{1}H-NMR study of the conformation of the 392–411 segment of human fibrinogen γ-chain in aqueous solution [17].

Both its ability for binding fibrinogen in solution and its selectivity for the γ-chain make GPIIb 656–667 unique among the peptides derived from GPIIb [7,18] or GPIIIa [19,20] reported to bind to fibrinogen. Our data favor the hypothesis that γ-chain- and RGD-peptides bind to different sites in GPIIb/IIIa. On the other hand, the fact that (a) the peptide-fibrinogen interaction is Ca^{2+}-independent, whereas the binding of fibrinogen to activated platelets requires Ca^{2+} [21,22], and (b) the affinity of the peptide-fibrinogen binding is two orders of magnitude lower that that of fibrinogen for its activated receptor (K_d ~0.1×10^{-6} M) [23], indicate that GPIIb 656–667 mimics to a certain extent but does not completely represent the fibrinogen γ chain recognition site of GPIIb/IIIa. This is not surprising, however, as the whole fibrinogen distal (D) domain (90 kDa, which includes mainly the C-terminal domains of the β and γ chains) is 70-fold more active than the synthetic γ400–411 peptide in inhibiting ADP-induced platelet aggregation [24]. This clearly indicates that a much larger surface than that provided by the C-terminal γ-chain peptide must be involved in receptor–ligand interaction. In addition, receptor-induced binding sites (RIBS) within the fibrinogen structure, which are elicited upon interaction with placelet membrane GPIIb/IIIa [25,26], might be involved in further interactions confering high-affinity to the fibrinogen-GPIIb/IIIa association.

As several integrin receptors which share the same β-subunit (homologous to GPIIIa) recognize different ligands [27,28], the distinct α-subunits (homologous to GPIIb) must impart ligand specificity. Thus, it is noteworthy that the GPIIb 656–667 region has been poorly conserved within the primary structure of integrin α-chains. In particular, this GPIIb sequence (GAHYMRALSNVE) corresponds to QADFIGVVRNNE of the vitronectin receptor α-subunit (α_V) [29]. The vitronectin receptor (VnR) shares with GPIIb/IIIa the β-subunit and the overall amino acid sequence identity between α_V and GPIIb is 60%. Interestingly, although both VnR and GPIIb/IIIa are promiscuous receptors which bind fibrinogen, the former only recognizes an

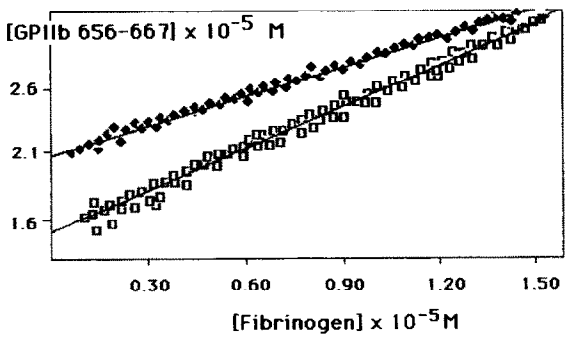

Fig. 3. Representation of the linear relationship between the concentrations of eosin-labeled peptide Cys-GPIIb 656–667 and fibrinogen obtained by analysis of experiments done as in Fig. 1 when [L] = 1.57×10^{-5} M, and B = 1.00 mol GPIIb peptide/mol fibrinogen (□–□); and [L] = 2.07×10^{-5} M, and B = 0.68 mol GPIIb peptide/mol fibrinogen (◆–◆).

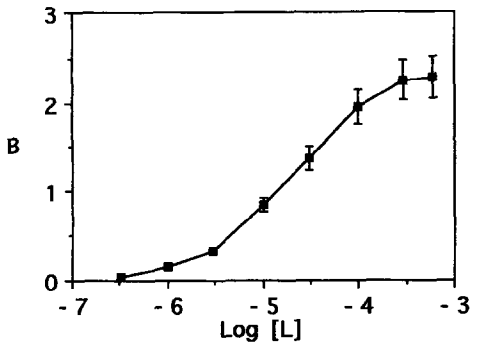

Fig. 4. Graphical relationship between the logarithm of the concentration of free peptide eosin-Cys-GPIIb 656–667 (log [L]) and B (average of 3 different experiments). At saturation, B tends to $n = 2.3 \pm 1.3$.

epitope containing the RGD sequence at position 572–574 of the Fb Aα chain, whereas GPIIb/IIIa binds preferentially to the Fb D-domain including the γ-chain 400–411 site [6,7,30,31]. These observations together with our present results suggest that the GPIIb 656–667 amino acid sequence may contribute to the binding specificity of GPIIb/IIIa. On the other hand, since the interaction of the GPIIb peptides 296–306 and 300–312, and the GPIIIa sequences 211–222 and 217–231) with fibrinogen can be inhibited by both RGD and gamma-chain peptides [7,18–20], it is tempting to speculate that these receptor regions, which are highly conserved between different integrins, may be important for recognition of RGD-containing ligands, i.e. fibronectin, vitronectin, von Willebrand factor, and fibrinogen antagonists like disintegrins [32,33], ornatins [34], mambin [35], and decorsin [36].

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