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Characterization of the β-chain N-terminus heterogeneity and the α-chain C-terminus of human platelet GPIIb

Posttranslational cleavage sites

J.J. Calvete[1,2], W. Schäfer[1], A. Henschen[1] and J. González-Rodríguez[2]

[1] *Max Planck Institut für Biochemie, D-8033 Martinsried, FRG* and [2] *Instituto de Química Física, CSIC, Serrano 119, 28006 Madrid, Spain*

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Human platelet glycoprotein IIb (GPIIb) and IIIa (GPIIIa) form a Ca^{2+}-dependent heterodimer, the integrin GPIIb/IIIa, which functions as the fibrinogen receptor at the surface of activated platelets. GPIIB and GPIIIa are synthesized as single polypeptides from single messages and their amino acid sequences were derived from their cDNAs. The GPIIb precursor is proteolytically processed to yield the known disulphide-bonded two-chain (GPIIbα and GPIIbβ) covalent structure found in mature GPIIb. Our present protein chemical and mass spectrometric analyses indicate that the GPIIb precursor is proteolytically cleaved at two or three sites, to give rise to an homogeneous α-chain (GPIIb 1–856) single disulphide-bonded to one of the two β-chains, which are present in a nearly 1:1 ratio: GPIIbβ1 (860–1008), with pyroglutamic acid as its blocked N-terminal residue; and GPIIbβ2 (872–1008), with the already known N-terminal sequence. These results satisfy the previously observed electrophoretic size-heterogeneity of the β-chain, confirmed the potential cleavage sites in the junction region, and indicate a probable dual proteolytic processing of GPIIb, which may be relevant to the rest of the two-chain α-subunits of the integrin family.

Platelet glycoprotein IIb; α-Chain C-terminus; β-Chain N-terminus heterogeneity; Posttranslational proteolytic processing

1. INTRODUCTION

Glycoprotein IIb (GPIIb) of the human platelet plasma membrane is a 136 kDa two-chain bitopic protein [1–3], which together with glycoprotein IIIa (GPIIIa), a 91 kDa bitopic membrane protein [1,2], forms a Ca^{2+}-dependent heterodimer [4], the integrin GPIIb/IIIa [5,6], which serves as the receptor for fibrinogen and other adhesive proteins upon platelet activation [7]. The GPIIb precursor gene spans about 17 kb on the long arm of chromosome 17 [8], and contains 30 exons [9]. Biosynthetic studies in human megakaryocyte [10] and HEL cell [11] confirmed the observation that GPIIb is synthesized as a single-chain precursor [12], whose amino acid sequence was cDNA-derived [9,13]. Like other integrin α-subunits, GPIIb is posttranslationally cleaved after being assembled with GPIIIa in the GPIIb/IIIa heterodimer [11]. In mature GPIIb the heavy-chain (GPIIbα, 114 kDa) is single disulphide-bonded to the light-chain (GPIIbβ, 22 kDa) between the last cysteine of GPIIbα and the first of GPIIbβ [14]. Although the disulphide bond and glycosylation patterns of GPIIb and the C-terminal of GPIIbβ have already been chemically determined [14–16], the present study on the structural consequences of the posttranslational cleavage of GPIIb was begun to clarify the electrophoretic size-heterogeneity of GPIIbβ [3] and some apparent discrepancies on the N-terminal sequence of GPIIbβ. While N-terminal sequence analysis indicated that GPIIbβ begins at position 872 (LQDPV...) [14,17], Loftus et al. [18] suggested that the GPIIbβ N-terminus is blocked. They also raised an antiserum against a synthetic peptide analogous to the predicted 859–871 peptide stretch of GPIIb, which recognized GPIIbβ by immunoelectroblotting analysis. Here we have characterized the C-terminus of the α-chain and the heterogeneity of the N-terminus of the β-chain, which indicate a probable dual proteolytic processing of GPIIb.

Correspondence address: J. González-Rodríguez, Instituto de Química Física, CSIC, Serrano 119, 28006 Madrid, Spain

Abbreviations: GPIIb, GPIIIa, and GPIIb/IIIa, glycoproteins IIb, IIIa, and the heterodimer formed by them, respectively; CM-CPIIbα and CM-GPIIbβ, the α- and β-chains of GPIIb, after full reduction and alkylation of GPIIb

2. MATERIALS AND METHODS

2.1. *Materials*

TPCK-trypsin and endoprotease Lys-C were from Sigma and Boehringer-Mannheim, respectively, and the porous glass beads (CPG-10, 200 mesh, 375 Å pore diameter) from Corning. GPIIb and its fully reduced and alkylated chains, CM-GPIIbα and CM-GPIIbβ, were isolated as previously described [3,19]. The C-terminal 19 kDa CNBr product of cleavage of CM-GPIIbα was prepared as in [14] and isolated by reverse-phase HPLC on a C4 Vydac column (25 ×

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0.4 cm, pore size 30 μm, particle size 10 μm) equilibrated in a mixture of 0.1% trifluoroacetic acid in water (A) and 0.1% trifluoroacetic acid in acetonitrile (B) (90% A/10% B), and eluted at 1 ml/min first isocratically for 5 min, followed by a linear gradient up to 50% B in 90 min.

2.2. *Methods*

Protein assay was done by amino acid analysis on a Biotronik analyzer after hydrolysis at 110°C in 6 N HCl for 24 h. N-terminal sequence analyses were carried out using either a Beckman 890C spinning cup or an Applied Biosystems 470 gas-phase sequencer [20]. Electroblotting of SDS/PAGE peptide bands into polyvinylidene difluoride membranes was performed following [21].

CM-GPIIbβ (10 mg/ml) in 50 mM ammonium bicarbonate, 0.1% (v/v) *N*-ethylmorpholine, pH 8.0, was digested with trypsin at an enzyme/substrate ratio 1:100 (w/w) for 18 h at 37°C. The 19 kDa CNBr product of GPIIbα (5 mg/ml) in 0.1 M Tris-HCl, pH 8.6, was digested with endoprotease Lys-C at an enzyme/peptide ratio 1:25 (w/w) for 24 h at 37°C. Both tryptic and endoLys-C digestion products were isolated by reverse-phase HPLC, as indicated above.

Peptide C-terminal sequence determination was carried out according to [22] with minor modifications described in [16]. Mass spectra were recorded with a mass spectrometer HSQ 30 (Finnigan MAT, Bremen) equipped with an atom gun (Ion Tech. Ltd., Teddington, UK). Peptides were dissolved in acetonitrile/acetic acid/water (50:10:40 v/v) and mixed with glycerol [23].

3. RESULTS AND DISCUSSION

3.1. *The GPIIbβ N-terminal heterogeneity*

To find out the structural bases of the size-heterogeneity of the β-chain of GPIIb, CM-GPIIbβ was subjected to SDS/12% polyacrylamide gel electrophoresis (Fig. 1) where it splits in two separate bands, about 40% of β1, the larger, and 60% of β2, as determined by densitometry. These bands were subsequently electroblotted into polyvinylidene difluoride membranes and subjected to gas-phase N-terminal sequence analysis, where β2 yielded the already chemically determined N-terminal sequence of GPIIbβ (872 LQDPV...), whereas the β1 N-terminus was found blocked. To determine this blocked N-terminus, CM-GPIIbβ was digested with trypsin and the tryptic products, after separation by reverse-phase HPLC (Fig. 2), were identified by N-terminal sequence and amino acid analyses. All the obtained fractions had sequenceable material, except for fraction 2, whose amino acid analysis clearly let us assign it to the peptide stretch 860 QIFLPEPEQPSR 871 in the predicted sequence of GPIIb [13]. Mass spectrometric analysis of fraction 2 (Fig. 3), besides confirming this assignment, also showed that the blocked N-terminus of GPIIbβ1 is a pyroglutamic acid residue ($M+H^+$ calculated 1422.6, found 1422.9). These results explain the previously found heterogeneity of GPIIbβ [3] and confirm the suggestion that the N-terminus of GPIIbβ is readily blocked [18]. However, our data differ quantitatively from those of Loftus et al. [18]. While they estimated that β2 represents as little as 3% of mature GPIIbβ, we have always found a β1/β2 ratio of nearly 1:1 in our preparations from outdated platelet concentrates. Further experiments will have to be designed to ascertain whether β2 is formed at the same stage as β1, or whether it is formed at later stages during thrombogenesis, in the blood stream or during blood bank storage.

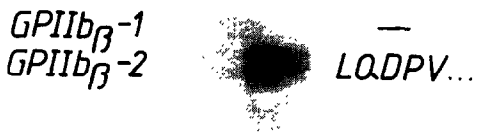

Fig. 1. Analysis of the size-heterogeneity of the fully reduced and alkylated β chain of GPIIb by SDS-12% polyacrylamide gel electrophoresis and Coomassie blue staining. The blocked N-terminus of the β1 chain and the chemically determined N-terminal sequence of the β2 chain are indicated on the right-hand side.

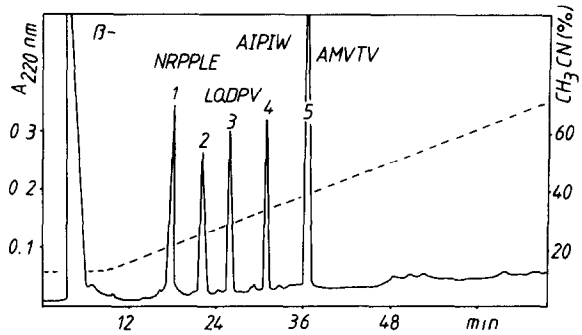

Fig. 2. Isolation by reverse-phase HPLC of the tryptic peptide carrying the blocked N-terminal sequence of GPIIbβ1. The tryptic products of digestion of CM-GPIIbβ were isolated on a C4 Vydac column equilibrated in a mixture of 0.1% trifluoroacetic acid in water (v/v) (A) and 0.1% trifluoroacetic acid in acetonitrile (B) (90% A/10% B) and eluted at 1 ml/min, first isocratically for 5 min, followed by a linear gradient up to 70% of B in 60 min. The chemically determined N-terminal sequence for each fraction is indicated, except that for fraction 2, which contains the peptide carrying the blocked N-terminus of the β1 chain.

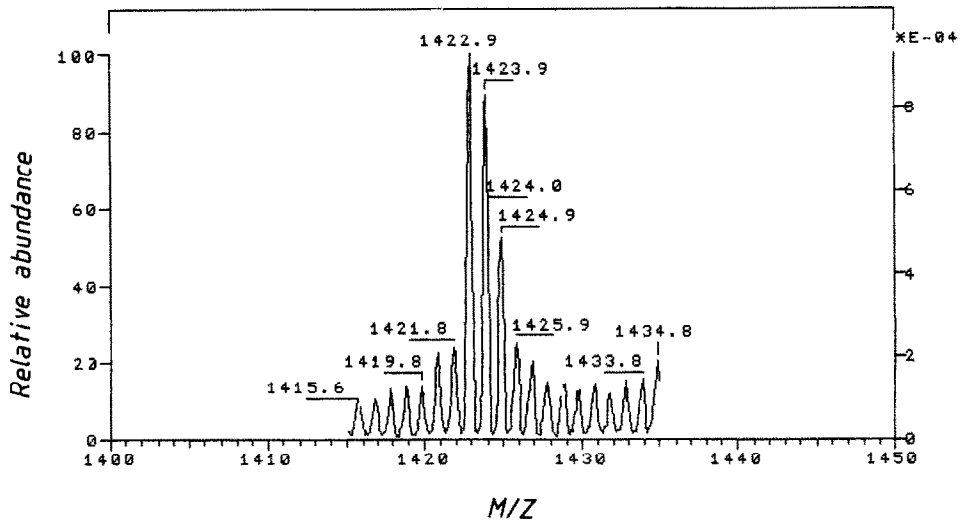

Fig. 3. Fast atom bombardment mass spectrometry of the tryptic peptide of CM-GPIIbβ from fraction 2 in Fig. 2, which confirms the chemically determined sequence of this peptide (β1 860–871) and identifies the blocked N-terminus of GPIIbβ1 as a pyroglutamic acid residue.

3.2. *The localization of the C-terminus of GPIIbα*

The above results raised the question of the actual location of the C-terminus of GPIIbα within the GPIIb precursor sequence [9,13]. When CM-GPIIbα was digested with trypsin, and the tryptic products were isolated by reverse-phase HPLC (not shown) and subjected to amino acid and N-terminal sequence analyses, all the possible peptides around the α–β chain junction were obtained, except those upstream Lys-855, which let us deduce that the α chain C-terminus must be somewhere between Lys-855 and Arg-859, because residues upstream Arg-859 are already found in the β1 chain. To locate the exact position of this C-terminus, the C-terminal 19 kDa CNBr-product of cleavage of CM-GPIIbα was prepared and isolated, and its C-terminal sequence chemically determined, as described in section 2.2. Two degradation cycles were successfully performed (Fig. 4), which allowed us to identify Lys-855 and Arg-856 as the last two residues of GPIIbα, and to locate a third proteolytic cleavage site, besides the two already found (Fig. 5).

Our results confirm the current hypothesis [24–27] that the occurrence of integrin two-chain or single-chain α subunits is related with the conservation of the sequence of the heavy–light chain junction region (between residues 855 and 871 in the cDNA-derived GPIIb sequence), which contains the two consensus pairs of basic residues and the single basic residue preceded by a number of prolines and acid residues, which determine the presence of the three proteolytic cleavage sites. The abnormal GPIIb found by Jung et al. [28] in the platelets of a thrombasthenic patient, which does

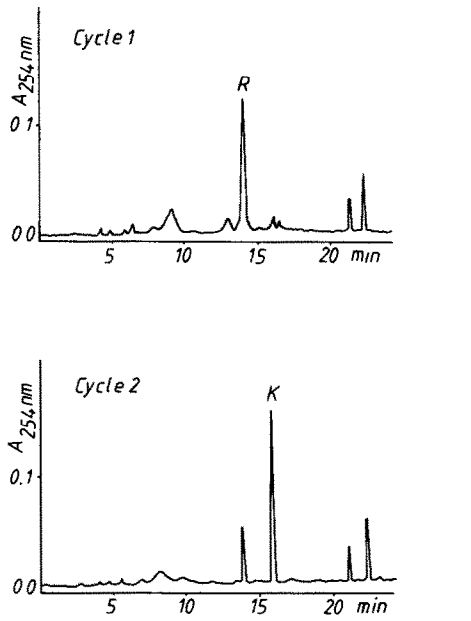

Fig. 4. Chemical determination of the C-terminal sequence of the GPIIbα chain. HPLC analysis of the thiohydantoin-amino acid derivatives from the first two cycles of C-terminal sequential degradation of the C-terminal 19 kDa CNBr-product of cleavage of CM-GPIIbα, as described in section 2.2. Peaks are labelled using the one-letter symbols.

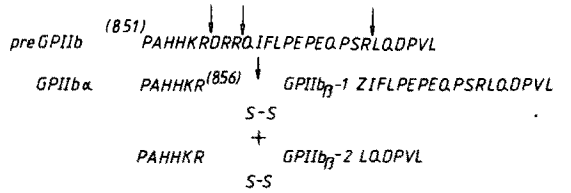

Fig. 5. Localization of the three proteolytic sites in the cDNA-derived amino acid sequence of the light-heavy chain junction region in the GPIIb precursor [13], together with the C-terminal sequence of GPIIbα and the two N-terminal sequences of GPIIbβ (β1 and β2). S–S represents the single interchain disulphide bond [3], and Z in β1 is the symbol for pyroglutamic acid.

not release the β chain upon reduction, could be related to a lack or anomaly in exon 26, which codes for the α–β chain junction region, although other genetic defects must be involved, because this abnormal GPIIb does not assemble in the GPIIb/IIIa heterodimer, which occurs at an earlier stage in the biosynthesis than the proteolytic processing [11]. Finally, Bray et al. [29] have suggested that the size-heterogeneity of GPIIbβ that we found [3] could be due to the splicing out of exon 28, corresponding to 34 amino acids of the extracellular domain of GPIIbβ, among them ASN 60 (in β2): the single glycosylation point of GPIIbβ [15]. This does not seem to be the case, because both β1 and β2 are glycosylated, and therefore, none of them can be the products of the transcript due to the splicing out of exon 28.

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