C-terminal amino acid determination of the transmembrane subunits of the human platelet fibrinogen receptor, the GPIIb/IIIa complex

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Glycoproteins IIb (GPIIb) and IIIa (GPIIIa) form the Ca^{2+}-dependent GPIIb/IIIa complex, which acts as the fibrinogen receptor on activated platelets. GPIIb and GPIIIa are synthesized as single peptide chains. The GPIIb precursor is processed proteolytically to yield two disulphide-bonded chains, GPIIbα and GPIIbβ. The GPIIb/IIIa complex has two membrane attachment sites located at the C-termini of GPIIbβ and GPIIIa. The short cytoplasmic tails of GPIIbβ and/or GPIIIa become most likely associated to the cytoskeleton of activated platelets. In the present work the C-terminal amino acid residues of platelet GPIIbβ and GPIIIa have been analyzed by protein-chemical methods and compared with those predicted from cDNA analysis. We were able to confirm the positions of the C-termini in both glycoproteins and the identity of the C-terminus predicted for GPIIIa, i.e. threonine. However, glutamine, not glutamic acid as predicted for GPIIbβ from the human erythroleukemic cell line and megakaryocyte cells, was found to be the C-terminal amino acid of GPIIbβ. This indicates that the glutamic acid in the GPIIb precursor is posttranslationally modified to glutamine.

Platelet; GPIIb/IIIa; C-terminal sequencing; Mass spectrometry; Posttranslational modification

1. INTRODUCTION

GPIIb and GPIIIa form a surface-oriented, Ca^{2+}-dependent heterodimer which serves as the fibrinogen receptor at the surface of activated platelets [1]. GPIIb (136.5 kDa) is made up of two chains, GPIIbα (114 kDa) and GPIIbβ (22.5 kDa) [2], joined by a single disulphide bond [3] between α Cys-826 and β Cys-9 [4]. GPIIIa (91.5 kDa) [2] is a single-chain, highly disulphide-bonded protein [5]. The cDNA sequences of GPIIb- and GPIIIa-like glycoproteins from HEL, megakaryocytes and endothelial cells suggest that GPIIbα does not possess any transmembrane segment and, therefore, is fully extracellular, and that GPIIbβ and GPIIIa each possess a single transmembrane segment and a short cytoplasmic tail [6–9], so that most of their mass is extracellular and involved in the recognition of adhesive proteins. The cytoplasmic domains of GPIIbβ and/or GPIIIa would most likely be responsible for the association of the platelet fibrinogen receptor to the cytoskeleton of activated platelets [10,11]. Therefore, a detailed structural knowledge of the cytoplasmic moieties of GPIIb and GPIIIa is necessary to understand the molecular steps of the platelet response to aggregation agonists involving the cytoplasmic domains of the GPIIb/IIIa complex. In the present work the cytoplasmic ends of the GPIIb/IIIa complex are characterized by isolation and amino acid sequence analysis of C-terminal fragments.

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Abbreviations: GPIIb, glycoprotein IIb; GPIIIa, glycoprotein IIIa; CM-GPIIIa, CM-GPIIbβ, fully reduced and carboxymethylated glycoprotein IIIa and IIbβ, respectively; HEL, human erythroleukemic cell line

2. MATERIALS AND METHODS

2.1. *Materials*

TPCK-trypsin was from Sigma; porous glass beads (CPG-10, 200 mesh, 37.5 nm pore diameter) were from Corning. Chromatography columns and buffers were as previously described [3,5].

2.2. *Methods*

Preparation of human platelets, platelet plasma membranes, and the isolation of GPIIb, GPIIIa and CM-GPIIbβ were described previously [3,5]. Fully reduced and alkylated GPIIIa was prepared by reduction of pure GPIIIa (5 mg/ml in 50 mM Tris-HCl, pH 8.0) with 1% (v/v) 2-mercaptoethanol (2 min, 100°C), and alkylation with 1.5 molar excess of iodoacetate over reducing agent.

Protein assay was performed according to [13]. Amino acid analyses were performed with a Biotronik amino acid analyzer after hydrolysis at 110°C in 6 N HCl for 24 h. N-terminal sequence analysis was carried out as described in [14].

Aminopropyl glass beads were prepared according to [15], and the aminopropyl side chains converted into isothiocyanate side chains according to [16]. Protein (5 nmol) was coupled to the derivatized glass beads (25 mg) in 200 μl of 1% SDS, 0.1 M sodium bicarbonate, pH 9.0 at 45°C for 3 h, and the reaction stopped with a molar excess of *n*-propylamine over reactive side chains. The coupling yield was 80–90% as estimated by amino acid analysis.

C-terminal sequence determination was carried out according to [17], except that the hydrolysis was done in 0.2 M acetohydroxamate,

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0.5 M sodium phosphate, pH 8.3 at 55°C for 30 min [18]. The extracted 2-thiohydantoin derivatives were analyzed by reverse-phase HPLC [17] using a Lichrospher 100 RP-18 (5 μm) column (Merck, Darmstadt). Standard 2-thiohydantoin derivatives were prepared as described [19].

Isolation of the CNBr-degradation fragment containing the C-terminal segment of GPIIbβ was performed as described [20]. Tryptic digestion of this fragment (10 mg/ml in 50 mM ammonium bicarbonate, 0.1% (v/v) N-ethylmorpholine, pH 8.0) was done at an enzyme/substrate ratio of 1:100 (w/w) for 3 h at 37°C. The tryptic peptides were separated by reverse-phase HPLC using a Vydac C_4 (10 μm, 30.0 nm) column, and acetonitrile gradient elution.

Mass spectra were recorded with a mass spectrometer HSQ 30 (Finnigan MAT, Bremen) equipped with an atom gun (Ion Tech Ltd, Teddington, England). Peptides were dissolved in acetonitrile/acetic acid/water (50:10:40, v/v/v) and mixed with glycerol [21]. Esterification of peptides was carried out by treating 10 nmol of freeze-dried peptide with 50 μl of 1 M isopropanol in 12 N HCl overnight at room temperature.

3. RESULTS AND DISCUSSION

Fully reduced and carboxymethylated GPIIbβ was cleaved with CNBr, and the peptide containing the C-terminus of GPIIbβ (β(117–137)) was isolated by reverse-phase HPLC as previously described [20]. This peptide was sequenced up to glycine 136, i.e. the penultimate residue. In order to identify the C-terminal residue of platelet GPIIbβ, CM-GPIIbβ was chemically sequenced from its C-terminus (see section 2). Two cleavage cycles were successfully effected, the amino acids recovered being glutamine and glycine, respectively (Fig. 1B). When the same chemical method for C-terminal sequencing was applied by us to known standard proteins, agreement with published results was obtained, e.g. for horse heart cytochrome *c*: glutamic acid and asparagine, for human serum albumin: leucine, glutamine and serine, and for whale sperm apomyoglobin: glycine and glutamic acid (data not shown). To confirm the result with GPIIbβ the C-terminal CNBr-fragment β117–137 was digested with trypsin, yielding two peptides, CR1 (β125–137: NRPPLEEDDEEG(E/Q)) and CR2 (β119–123), after reverse-phase HPLC separation (Fig. 2). The peptide CR1 (β125–137) was analyzed by mass spectrometry

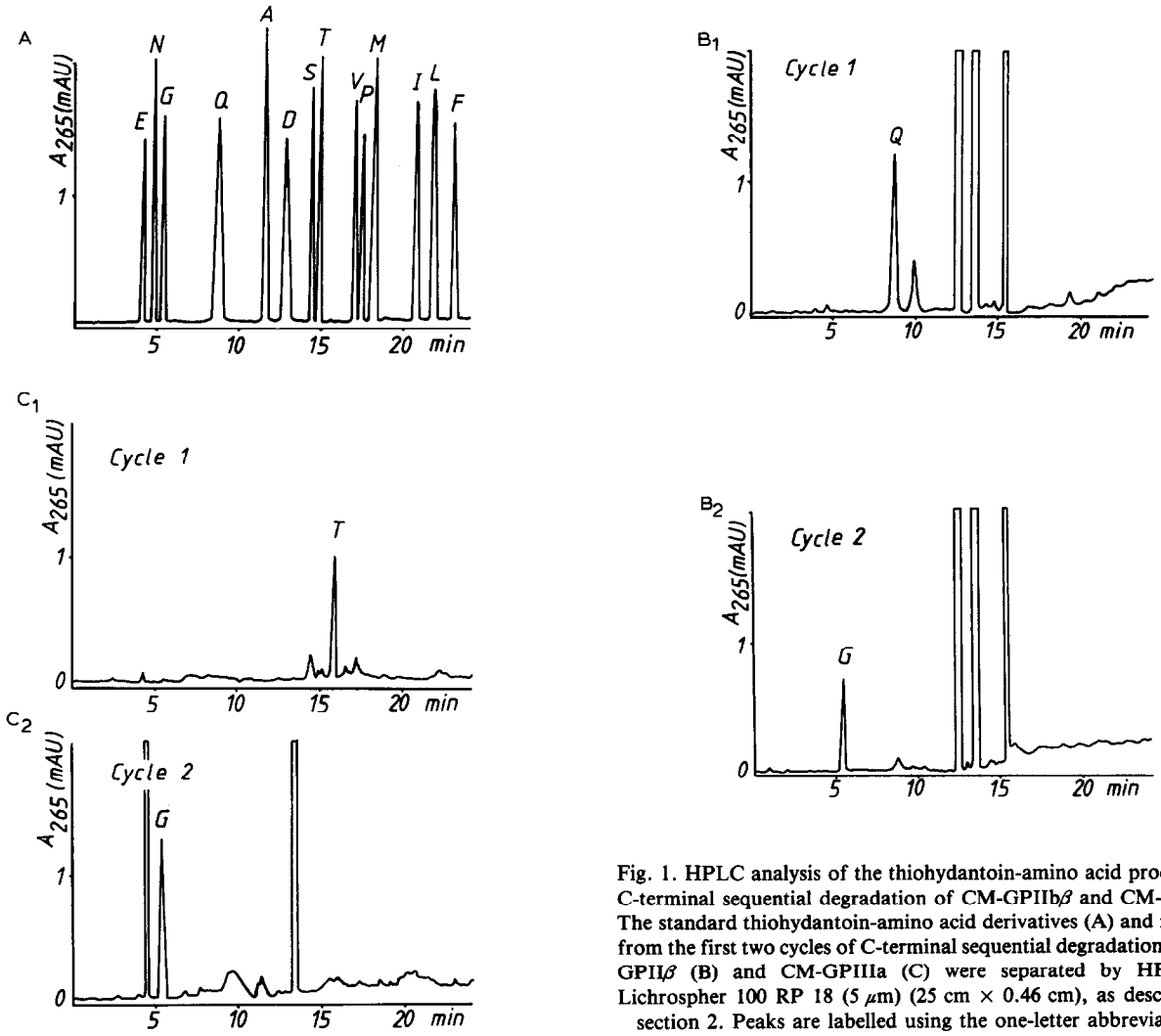

Fig. 1. HPLC analysis of the thiohydantoin-amino acid products of C-terminal sequential degradation of CM-GPIIbβ and CM-GPIIIa. The standard thiohydantoin-amino acid derivatives (A) and material from the first two cycles of C-terminal sequential degradation of CM-GPIIβ (B) and CM-GPIIIa (C) were separated by HPLC on Lichrospher 100 RP 18 (5 μm) (25 cm × 0.46 cm), as described in section 2. Peaks are labelled using the one-letter abbreviations.

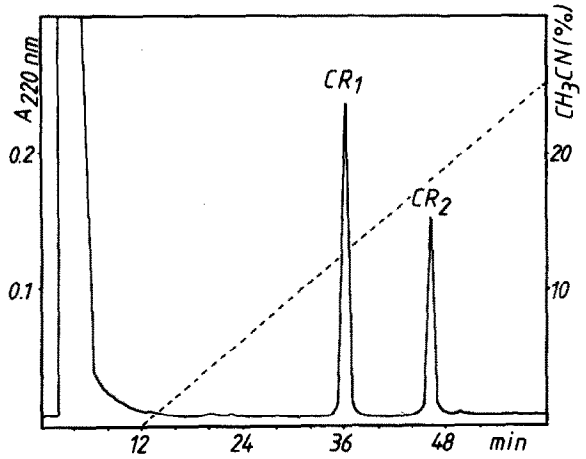

Fig. 2. Isolation of the tryptic digestion products of the CNBr-fragment CM-GPIIbβ117–137 by HPLC on Vydac C_4 (see section 2).

either underivatized or after esterification of the carboxyl group with isopropanol/HCl (see section 2). The molecular masses ($M+H^+$) obtained for the underivatized and the esterified β125–137 peptide (1528 and 1823, respectively) (Fig. 3) provided further evidence that the C-terminal amino acid residue of platelet GPIIbβ is glutamine. However, glutamic acid, and not glutamine, was predicted by cDNA sequencing for GPIIbβ in HEL and in megakaryocyte cells [7,12,22]. The most likely explanations for the discrepancy between the cDNA-predicted and the chemically established C-terminal residue for GPIIbβ are either that there are cell-type specific copies of the exon coding for the C-terminal, or that the C-terminus of the GPIIb precursor is posttranslationally modified, as already observed in other proteins [23]. Recently, Prandini et al. [24] have sequenced the 3′ end of the GPIIb gene from a genomic library, containing an exon encoding the 19 C-terminal amino acid residues of GPIIb and a 3′ untranslated region; the sequence showed glutamic acid as the C-terminal residue. Moreover, the same authors showed that there is only one copy of the GPIIb gene per human genome. This indicates that the first explanation can be ruled out. The role of glutamine, instead of glutamic acid, if any, in the interaction of the GPIIb/IIIa complex with

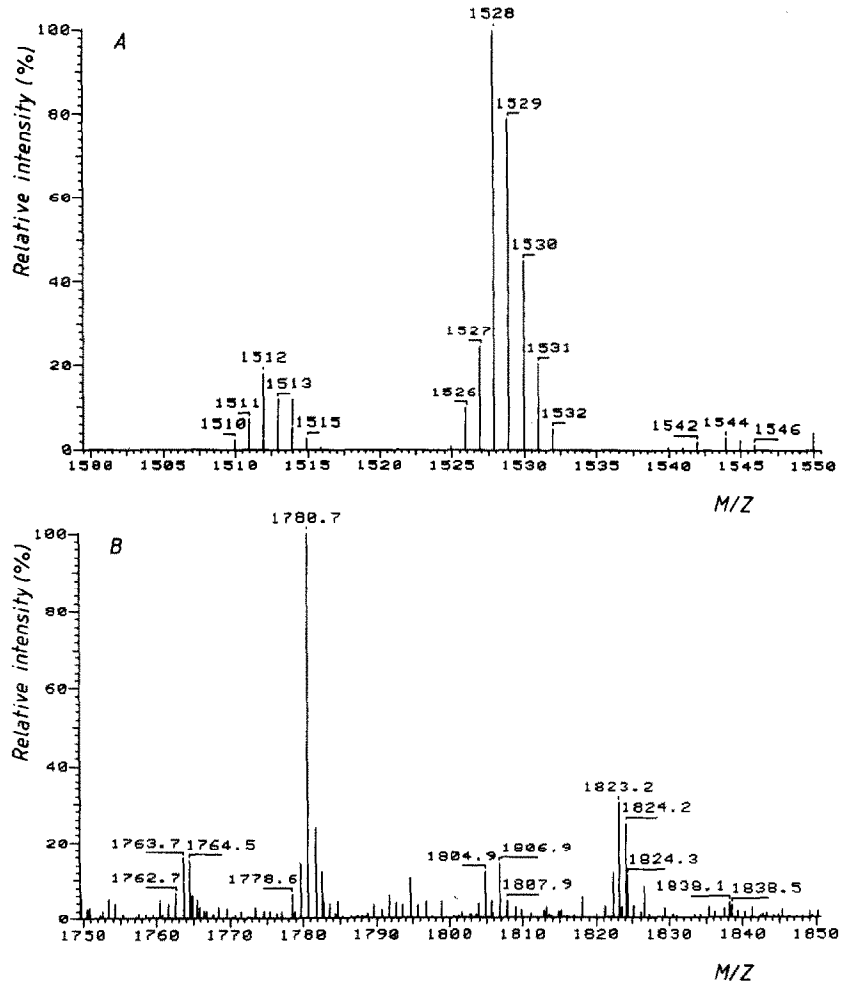

Fig. 3. Fast atom bombardment mass spectrometry of the tryptic peptide CR1 (β125–137) corresponding to the C-terminal end of GPIIbβ, both unmodified (A) and esterified (B) material being analysed.

elements of the activated platelet cytoskeleton requires further investigation.

Chemical C-terminal amino acid sequence analysis of fully reduced and carboxymethylated GPIIIa was also performed (see section 2). Two degradation cycles were successfully carried out, the amino acids obtained being threonine and glycine, respectively (Fig. 1C). This sequence is identical to that predicted by cDNA analysis for GPIIIa from HEL and endothelial cells [6,8,9].

During recent years our laboratories have been engaged in the protein-chemical characterization of GPIIb/IIIa. Thus, peptides which correspond to more than 70% of the whole GPIIIa structure have been isolated and analyzed for amino acid and amino sugar composition as well as N-terminal sequence. So far no differences from the cDNA sequences deduced for GPIIIa from HEL and endothelial cells [6,8,9] have been observed, with the exception of the sequence at positions 623–627 initially published as EPYMT [6,9] and subsequently corrected to be GALHD [8], where our data are in accordance with the latter report. All results together strengthen the conclusion that the three different cell types, HEL, endothelial cells and platelets express the identical glycoprotein IIIa.

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REFERENCES

[1] Phillips, D.R., Charo, I.F., Parise, L.V. and Fitzgerald, L.A. (1988) Blood 71, 831–843.
[2] Usobiaga, P., Calvete, J.J., Saiz, J.L., Eirin, M.T. and Gonzalez-Rodriguez, J. (1987) Eur. J. Biophys. 14, 211–218.
[3] Calvete, J.J. and Gonzalez-Rodriguez, J. (1986) Biochem. J. 240, 155–161.
[4] Calvete, J.J., Alvarez, M.V., Rivas, G., Hew, C.-L., Henschen, A. and Gonzalez-Rodriguez, J. (1989) Biochem. J. 261, 551–560.
[5] Eirin, M.T., Calvete, J.J. and Gonzalez-Rodriguez, J. (1986) Biochem. J. 240, 147–153.
[6] Fitzgerald, L.A., Steiner, B., Rall, S.C. jr, Lo, S.S. and Phillips, D.R. (1987) J. Biol. Chem. 262, 3936–3939.
[7] Poncz, M., Eisman, R., Heidenreich, R., Silver, S.M., Vilaire, G., Surrey, S., Schwartz, E. and Bennett, J.S. (1987) J. Biol. Chem. 262, 8476–8482.
[8] Rosa, J.-P., Bray, P.F., Gayet, O., Johnston, G.I., Cook, R.G., Jackson, K.W., Shuman, M.A. and McEver, R.P. (1988) Blood 72, 593–600.
[9] Zimrin, A.B., Eisman, R., Vilaire, G., Schwartz, E., Bennett, J.S. and Poncz, M. (1988) J. Clin. Invest. 81, 1470–1475.
[10] Shattil, S.J. and Brass, L.F. (1987) J. Biol. Chem. 262, 992–1000.
[11] Phillips, D.R., Jennings, L.K. and Edwards, H.H. (1980) J. Cell Biol. 86, 77–86.
[12] Uzan, G., Frachet, P., Lajmanovich, A., Prandini, M.H., Denarier, E., Duperray, A., Loftus, J., Ginsberg, M., Plow, E. and Marguerie, G. (1988) Eur. J. Biochem. 171, 87–93.
[13] Markwell, M.A.K., Haas, S.M., Bieber, L.L. and Tolbert, N.E. (1978) Anal. Biochem. 87, 206–210.
[14] Edman, P. and Henschen, A. (1975) in: Protein Sequence Determination (Needleman, S.B. ed.) pp.232–279, Springer, Berlin.
[15] Robinson, P.J., Dunnill, P. and Lilly, M.D. (1971) Biochim. Biophys. Acta 242, 659–661.
[16] Wachter, E., Machleidt, W., Hafner, H. and Otto, J. (1973) FEBS Lett. 35, 97–102.
[17] Hawke, D.H., Lahm, H.W., Shively, J.E. and Todd, R.W. (1987) Anal. Biochem. 166, 298–307.
[18] Stark, G.R. (1968) Biochemistry 7, 1796–1807.
[19] Stark, G.R. (1972) Methods Enzymol. 25, 369–384.
[20] Calvete, J.J., Henschen, A. and Gonzalez-Rodriguez, J. (1989) Biochem. J. 261, 551–560.
[21] Naylor, S., Finders, A.F., Gibson, B.M. and Williams, D.H. (1986) J. Am. Chem. Soc. 108, 6359–6363.
[22] Bray, P.F., Rosa, J.-P., Johnston, G.I., Shiu, D.T., Cook, R.G., Lau, C., Kan, Y.W., McEver, R.P. and Shuman, M.A. (1987) J. Clin. Invest. 80, 1812–1817.
[23] Wold, F. (1981) Annu. Rev. Biochem. 50, 783–814.
[24] Prandini, M.H., Denarier, E., Frachet, P., Uzan, G. and Marguerie, G. (1988) Biochem. Biophys. Res. Commun. 156, 595–601.