Tryptic digestion of human GPIIIa

Isolation and biochemical characterization of the 23 kDa *N*-terminal glycopeptide carrying the antigenic determinant for a monoclonal antibody (P37) which inhibits platelet aggregation

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Early digestion of pure human platelet glycoprotein IIIa (GPIIIa) leads to a single cleavage of the molecule at 23 kDa far from one of the terminal amino acids. Automated Edman degradation demonstrates that GPIIIa and the smaller (23 kDa) tryptic fragment share the same *N*-terminal amino acid sequence. A further cleavage occurs in the larger fragment (80 kDa), reducing its apparent molecular mass by 10 kDa. The 23 kDa fragment remains attached to the larger ones in unreduced samples. Stepwise reduction of early digested GPIIIa with dithioerythritol selectively reduces the single disulphide bond joining the smaller (23 kDa) to the larger (80/70 kDa) fragments. Two fractions were obtained by size-exclusion chromatography of early digested GPIIIa after partial or full reduction and alkylation. The larger-size fraction contains the 80/70 kDa fragments, while the 23 kDa fragment is isolated in the smaller. The amino acid compositions of these fractions do not differ very significantly from the composition of GPIIIa; however the 23 kDa fragment contains only 10.2% by weight of sugars and is richer in neuraminic acid. Disulphide bonds are distributed four in the 23 kDa glycopeptide and 20–21 in the 80/70 kDa glycopeptide. The epitope for P37, a monoclonal antibody which inhibits platelet aggregation [Melero & González-Rodríguez (1984) *Eur. J. Biochem.* **141**, 421–427] is situated within the first 17 kDa of the *N*-terminal region of GPIIIa, which gives a special functional interest to this extracellular region of GPIIIa. On the other hand, the epitopes for GPIIIa-specific monoclonal antibodies, P6, P35, P40 and P97, which do not interfere with platelet aggregation, are located within the larger tryptic fragment (80/70 kDa). Thus, the antigenic areas available in the extracellular surface of GPIIIa for these five monoclonal antibodies are now more precisely delineated.

INTRODUCTION

Glycoprotein IIIa (GPIIIa) is a 91.5 kDa major component of the platelet membrane (Clemetson, 1985; Usobiaga *et al.*, 1987) which together with GPIIb functions as a receptor for fibrinogen (Plow *et al.*, 1986) and other proteins, and plays a primary role in platelet aggregation (Nurden & Caen, 1974; Phillips *et al.*, 1975). GPIIb and GPIIIa have been isolated and characterized in several laboratories, both as individual glycoproteins or as a GPIIb–GPIIIa complex (Leung *et al.*, 1981; McEver *et al.*, 1982; Jennings & Phillips, 1982; Eirín *et al.*, 1986), and specific monoclonal antibodies have been raised to each of them as well as for the complex (McGregor, 1986). Previous studies had identified PL^{A1} alloantigen in GPIIIa (Kuniki & Aster, 1979), on a 66 kDa membrane-attached fragment of GPIIIa after chymotryptic digestion of whole platelets (Kornecki *et al.*, 1985), and on a 17 kDa polypeptide derived by tryptic digestion of a Triton X-114 extract of platelet membranes enriched in GPIIb and GPIIIa (Newman

et al., 1985). Recently, a family of glycoproteins immunologically and structurally related to GPIIb and GPIIIa were found in vertebrates (Charo *et al.*, 1986; Edelman, 1986; Hynes, 1987) and in invertebrates (Leptin *et al.*, 1987).

In order to characterize the regions of GPIIIa exposed to the extracellular surface, we combined partial tryptic digestion of isolated GPIIIa with localization of epitopes for monoclonal antibodies directed to the extracellular surface of GPIIIa. Because GPIIIa is highly cross-linked by intramolecular disulphide bonds (Eirín *et al.*, 1986), selective cleavage by reduction was used to isolate the two earlier tryptic products. We report here that the epitope for a GPIIIa-specific monoclonal antibody (P37) which inhibits platelet aggregation (Melero & González-Rodríguez, 1984) is located on the *N*-terminal region of GPIIIa (17 kDa), whereas the epitopes for four monoclonal antibodies which do not inhibit aggregation are located on the *C*-terminal fragment (80/70 kDa). This appears to confer a special functional interest to the *N*-terminal region of GPIIIa. Furthermore, the amino acid

Abbreviations used: GPIIb, glycoprotein IIb; GPIIIa, glycoprotein IIIa.

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and sugar compositions and the disulphide contents of the two earlier tryptic glycopeptides are given.

MATERIALS AND METHODS

Materials

Chymotrypsin-free trypsin was from Worthington Co. (Freehold, NJ, U.S.A.). The rest of the chemicals and biochemicals, chromatographic columns and buffers, as well as the preparation of human platelets, platelet membranes and isolation of GPIIb and GPIIIa were as described before (Eirín *et al.*, 1986).

Analytical methods

Assays of proteins (Lowry *et al.*, 1951; Markwell *et al.*, 1978), sialic acid (Hammond & Papermaster, 1976), neutral sugars (Alpenfels, 1981), hexosamines (Hjerpe *et al.*, 1980), amino acids, free thiol groups (Ellman, 1959) and disulphide bonds (Zahler & Cleland, 1968), bound eosin, and SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970), were done as described previously (Eirín *et al.*, 1986; Calvete & González-Rodríguez, 1986).

Immunoelectroblotting

After gel electrophoresis the glycoprotein bands were transferred to nitrocellulose by a standard procedure (Towbin *et al.*, 1979) at 250 mA for 3–5 h. The first antibodies used, all in the form of 50% $(\text{NH}_4)_2\text{SO}_4$ precipitate fraction of ascitic fluids or antisera, were mouse anti-GPIIIa monoclonal antibodies (P6, P35, P37, P40, P97) described previously (Melero & González-Rodríguez, 1984) and rabbit anti-GPIIIa sera (M. Maruri & J. González-Rodríguez, unpublished work). The second antibodies were anti-(mouse IgG)-peroxidase and anti-(rabbit IgG)-peroxidase conjugates (Sigma). The substrate was 4-chloro-1-naphthol.

Trypsin digestion of pure GPIIIa

Pure GPIIIa (2 mg/ml) was treated with TPK-trypsin at different glycoprotein/enzyme weight ratios, either in 50 mM- NH_4HCO_3 /1% (v/v) *N*-ethylmorpholine, pH 8.0 (ammonium bicarbonate buffer) or in 10 mM-Tris/HCl/0.15 M-NaCl/1 mM-EDTA/1% Triton X-100, pH 7.4 (Tris buffer), always at 37 °C. Proteolysis was stopped with a 25-fold molar excess of phenylmethanesulphonyl fluoride over trypsin, and the digestion products were used directly or kept frozen in solution until use.

Cleavage of early-digested GPIIIa by reduction and isolation of the 23 kDa and 80/70 kDa fragments with different degrees of reduction and carboxymethylation

Samples of GPIIIa, previously digested with trypsin at a 250:1 protein/enzyme ratio for 15 min in ammonium bicarbonate buffer (early or restricted digestion), were lyophilized, resuspended in 50 mM-Tris/HCl/1 mM-EDTA containing 0.1 or 2% (w/v) SDS, pH 8.0, and stepwise reduced with increasing concentrations of dithioerythritol for 1 h at room temperature. The thiol groups formed were titrated with 5,5'-dithiobis(2-nitrobenzoic acid) (Zahler & Cleland, 1968). Before electrophoretic analysis, samples were carboxymethylated with a 2-fold molar excess of iodoacetate

over the reducing agent used in each sample, and dialysed against electrophoretic sample buffer.

Fully reduced and carboxymethylated tryptic fragments (23 kDa and 80/70 kDa) were prepared by reduction of 5–10 mg of early-digested GPIIIa with a 150-fold molar excess of dithioerythritol over theoretical half-cystine residues in the glycoprotein, for 1 h at room temperature, and further carboxymethylation with a 1.5-fold molar excess of iodoacetate over reducing agent, all in the same buffer as above (2% SDS). Partially reduced (an average of 1 ± 0.05 half-cystine residues reduced per molecule of 23 kDa fragment) and carboxymethylated tryptic fragments were prepared as above by reduction of early-digested GPIIIa but using a 0.6-fold molar excess of dithioerythritol over theoretical half-cystine content in GPIIIa (Eirín *et al.*, 1986), followed by carboxymethylation. The partially or fully carboxymethylated fragments were isolated by size-exclusion chromatography on a Sephacryl S-200 column (140 cm $\times$ 1 cm) using 0.1 M-sodium phosphate/1 mM-EDTA (pH 6.8) containing 0.1% SDS and 0.025% NaN_3 as elution buffer.

Eosin labelling of partially reduced 23 kDa and 80/70 kDa tryptic fragments of GPIIIa

Early digested GPIIIa (5–10 mg) was lyophilized, resuspended in 1 ml of 50 mM-Tris/HCl/1 mM-EDTA/0.1% SDS, pH 8.0, and reduced using a 5-fold molar excess of dithioerythritol with respect to the theoretical half-cystine content of GPIIIa, for 1 h at room temperature. The reduced sample was labelled with eosin-maleimide (1.5-fold molar excess over reducing agent), diluted with 1 ml of 50 mM-Tris/HCl/1 mM-EDTA, pH 8.0, to bring the SDS concentration below its critical micellar concentration (Usobiaga *et al.*, 1987), and the unbound dye was separated by passage through a Sephadex G-50 column (26 cm $\times$ 1.6 cm) equilibrated with 20 mM-phosphate/0.025% SDS, pH 6.8. The eosin-labelled peptides were isolated by chromatography on Sephacryl S-200 as described above.

Automated Edman degradation

For automated Edman degradation the peptide (2–10 nmol) was loaded in a Beckman 890 C protein sequencer in the presence of Polybrene (Pierce Chemicals). Amino acid sequence determination was carried out using the 0.1 M-Quadrol program. After conversion in the presence of 25% aqueous trifluoroacetic acid for 30 min at 70 °C, the phenylthiohydantoin derivatives of the amino acids were analysed by reverse-phase high performance liquid chromatography using a Beckman Ultrasphere PTH column (4.6 mm $\times$ 250 mm) and a precolumn at 48 °C, as previously described (Hew *et al.*, 1986). The repetitive yield of the Edman cycle was higher than 93%.

RESULTS

Electrophoretic and kinetic patterns of tryptic digestion of pure GPIIIa

The electrophoretic patterns of reduced and unreduced products of limited proteolysis of GPIIIa with trypsin in ammonium bicarbonate buffer and in Tris buffer at increasing digestion times are shown in Fig. 1. The appearance after reduction of two distinct products of 23

Fig. 1. Time-dependence of the reduced and unreduced electrophoretic patterns of digestion of pure GPIIIa with trypsin in two different buffers

Gels of 12.5% polyacrylamide were used and stained with Coomassie Blue. Reduced samples were incubated with 1% 2-mercaptoethanol at 100 °C for 2 min before electrophoresis. Digestion times are at the bottom. Apparent molecular masses in kDa are at the side. Abbreviations: M, platelet membrane; Myo, myosin; (GPIIIa)₂, dimer of GPIIIa; GPIIb α , α subunit of GPIIb; Act, actin. Protein loadings: platelet membrane, 100 μ g; GPIIIa control, 10 μ g; digestion samples, about 50 μ g each. (a) Reduced pattern of digestion in Tris buffer at a GPIIIa/trypsin ratio of 100:1 (see the Materials and methods section). (b) The same as (a) but unreduced. (c) Reduced pattern of digestion in ammonium bicarbonate buffer at a GPIIIa/trypsin ratio of 250:1 (see the Materials and methods section). (d) Pattern of digestion in the same conditions as (c) after full reduction and alkylation (see the Materials and methods section); control samples were unreduced.

and 80 kDa apparent molecular mass is observed from the beginning. These two tryptic fragments are very slightly cleaved further within the next 3 h when the digestion is carried out in Tris buffer (Figs. 1a and 1b). However, in ammonium bicarbonate buffer, the 80 kDa fragment is successively degraded to 70 and 52 kDa

peptides within the next 24 h, when trypsin is replenished every 6 h (Fig. 1c), and the 23 kDa fragment is more readily degraded to products of 21, 19 and 17 kDa, finally disappearing in the form of very small peptides.

The unreduced tryptic products, at the stage of undegraded 80/70 kDa and 23 kDa, appear as a main

band and two minor bands of lower electrophoretic mobility than unreduced GPIIIa (Fig. 1b), meaning that the 23 kDa fragment is joined to both the 70 and 80 kDa products by disulphide bonds. Total reduction and carboxymethylation of these products increase their apparent molecular masses to 28, 105 and 120 kDa, respectively (Fig. 1d). These three fragments are glycopeptides, as revealed by sugar-specific staining of the electrophoretic gels (not shown).

From now on, whenever we refer to a tryptic peptide we will use the apparent molecular mass of the reduced form found by SDS/polyacrylamide-gel electrophoresis, independently of the state of reduction and carboxymethylation.

Isolation of the fully reduced and carboxymethylated 23 kDa and 80/70 kDa tryptic fragments

After early digestion of GPIIIa with trypsin in ammonium bicarbonate buffer, the reaction products were separated by size-exclusion chromatography after full reduction and carboxymethylation (Fig. 2). The large-size fraction contains a mixture of peptides of apparent molecular mass 80 and 70 kDa, the relative contribution of each being dependent on the GPIIIa/trypsin ratio, pH, temperature and reaction time used. The smaller-size fraction contains the pure 23 kDa fragment. About 80 % of the initial glycoprotein mass is recovered between the two fractions, 60 % in the larger and 20 % in the smaller.

Amino acid and sugar composition of the 80/70 kDa and 23 kDa tryptic fragments

From Table 1 one can observe the similarities between the amino acid compositions of the larger and the smaller fractions and that of GPIIIa. The amino acid composition of early digested GPIIIa calculated from the composition of the individual fragments and their contribution to the total mass of GPIIIa (75 % and 25 % for the larger and the smaller fraction, respectively) is in good agreement with the known composition of GPIIIa (Eirín *et al.*, 1986), except for histidine, aspartic acid and glycine for which the calculated values are 44, 18 and 18 % higher, respectively, and tyrosine for which the calculated value is 20 % lower.

The 80/70 kDa fraction contains 14.5 % by weight of sugars, whereas the 23 kDa glycopeptide contains only 10.2 %. Sugars are not uniformly distributed. Whereas the molar percentages of mannose and galactosamine in the 23 kDa fragment are nearly half of those in the 80/70 kDa fraction, sialic acid is enriched in the small fragment.

From the amino acid and sugar compositions we calculate (Hoy *et al.*, 1974) an average molecular mass of 71 kDa for the 80/70 glycopeptide mixture in the larger fraction, and 23 kDa for the smaller glycopeptide, in evident disagreement with the molecular masses obtained by gel electrophoresis of the fully reduced and carboxymethylated forms; similar observations have been reported for GPIIIa (Eirín *et al.*, 1986). The agreement between the sum of the molecular masses of the two fractions with the known molecular mass of GPIIIa (91.5 kDa) confirms the electrophoretic observations that these are the only major tryptic products of early digestion of GPIIIa and that the 80 kDa (apparent molecular mass) glycopeptide is the main contributor to the larger fraction in our conditions of digestion.

Fig. 2. Electrophoretic analysis of the fully reduced and carboxymethylated tryptic fragments of early digestion of GPIIIa isolated by size-exclusion chromatography

Electrophoresis was done in a 10 % polyacrylamide gel and all samples were reduced with 1 % 2-mercaptoethanol as stated in Fig. 1. Lane a, early digested GPIIIa in ammonium bicarbonate buffer, at a 250:1 GPIIIa/trypsin weight ratio for 15 min (50 µg); lane b, the same sample as in lane a after full reduction and carboxymethylation; lane c, larger molecular size fraction (80/70 kDa) obtained by chromatography on a Sephacryl S-200 column of sample as in lane b (25 µg); lane d, smaller molecular size fraction (23 kDa) obtained in the same separation as sample in lane c (15 µg). Numbers at the side are as in Fig. 1.

Automated N-terminal sequence analysis

Automated Edman degradation of pure GPIIIa and of the 23 kDa fragment shows that both contain an N-terminal amino acid sequence identical to that determined by Charo *et al.* (1986) for human platelet membrane glycoprotein IIIa: Gly-Pro-Asn-Ile-Cys-Thr. Furthermore, when the 17 kDa fragment, derived by further tryptic digestion of the 23 kDa glycopeptide and isolated by gel electrophoresis, was subjected to automated Edman degradation, the sequence found was: Gly-Xaa-Asn-Ile-Cys.

Selective cleavage of the disulphide bonds joining the 23 kDa fragment to the 80/70 kDa fragments in tryptically digested GPIIIa

As we have shown above, when GPIIIa is digested with trypsin, the 23 and the 80/70 kDa fragments formed are still joined by disulphide bonds. Stepwise reduction

Table 1. Amino acid and sugar compositions of the 23 kDa and 80/70 kDa tryptic fragments of GPIIIa

The experimental results are an average of two determinations with two different preparations. Abbreviation: n.d., not determined.

Residue	Composition					
	(mol/100 mol of total amino acids)			(residues/molecule)*		
	23 kDa	80/70 kDa	GPIIIa†	23 kDa	80/70 kDa	GPIIIa†
Asp	12.4	13.5	11.44	23	74	82
Thr	4.8	5.4	5.10	9	30	37
Ser	8.2	8.3	8.03	15	46	58
Glu	11.2	11.2	11.06	21	61	80
Pro	5.0	4.2	4.56	10	23	33
Gly	6.5	7.7	7.32	20	43	53
Ala	5.7	4.7	5.33	10	26	33
Cys	5.3	6.3	5.97	9	35	43
Val	6.2	5.9	5.63	11	32	41
Met	3.8	4.1	4.06	7	23	29
Ile	4.4	4.1	4.63	8	23	33
Leu	8.8	7.8	8.91	16	43	64
Tyr	2.8	2.2	2.93	5	12	21
Phe	2.9	2.9	3.25	5	16	23
His	1.5	1.9	1.25	3	10	9
Lys	5.6	5.8	5.58	10	32	40
Arg	4.5	3.9	4.23	8	22	30
Trp	n.d.	n.d.	0.72	—	—	5
Gal	1.5	1.7	1.31	3	9	9
Man	2.6	5.2	4.37	5	29	31
GlcNAc	1.4	2.9	2.20	3	16	17
NeuAc	0.9	0.8	0.85	2	4	6

* Assuming M_r 23 000 for the 23 kDa fragment and M_r 71 000 for the 80/70 kDa fragment.

† From Eirin *et al.* (1986).

with dithioerythritol of early digested GPIIIa in 50 mM-Tris/HCl/1 mM-EDTA, pH 8.0, and 0.1% or 2% SDS, cleaves selectively those linkages as monitored by SDS/polyacrylamide-gel electrophoresis (Fig. 3). In 2% SDS a 0.6-fold molar excess of dithioerythritol with respect to the half-cystine content in GPIIIa is required to cleave the 23 kDa glycopeptide from the larger fragments, exposing 3.2 ± 0.5 thiol groups per cleaved molecule of digested GPIIIa to titration with 5,5'-dithiobis-(2-nitrobenzoic acid). However, when the reduction is done in 0.1% SDS, a 5-fold molar excess of dithioerythritol with respect to the half-cystine content is required to selectively cleave the single interfragment disulphide bond.

Eosin-maleimide labelling and isolation of the labelled fragments: quantification of the thiol and intrafragment disulphide bonds in the 23 kDa and 80/70 kDa glycopeptides

After selective cleavage by reduction of the single interfragment disulphide bond with a 5-fold molar excess of reducing agent in 0.1% SDS, the newly formed thiol groups were blocked either with eosin-maleimide or with iodoacetate, and the fragments were separated by size-exclusion chromatography as above, for quantification of the thiol groups exposed in each fragment and determination of the intrafragment disulphide bonds. From the eosin content we found that 1 ± 0.06 and 2.3 ± 0.4 thiol groups were formed per molecule of 23 kDa and 80/70 kDa polypeptides, respectively. This is in good agreement with the number of thiol groups per molecule of early digested GPIIIa determined, after

selective cleavage by reduction and before size-exclusion chromatography, either from the eosin content (3.4 ± 0.6) or, as we have shown above, by titration with 5,5'-dithiobis-(2-nitrobenzoic acid) (3.2 ± 0.5). A maximum of 7.9 ± 0.9 thiol groups per molecule of 23 kDa glycopeptide were titratable with 5,5'-dithiobis-(5-nitrobenzoic acid) after reduction of the isolated glycopeptide with a 150-fold molar excess of dithioerythritol, in good agreement with the half-cystine residues determined by amino acid analysis (Table 1) and the thiol groups determined by eosin labelling after selective cleavage of the interfragment disulphide. A maximum of 37.7 ± 1.8 thiol groups per molecule of 80/70 kDa glycopeptide were titratable with 5,5'-dithiobis-(5-nitrobenzoic acid) after reduction of the isolated glycopeptide with a 150-fold molar excess of dithioerythritol, which (together with the thiol groups determined by eosin labelling) represent 114% of the half-cystine residues determined by amino acid analysis for this glycopeptide (Table 1). If we add up the total thiol groups determined in the 23 and 80/70 kDa glycopeptides, we end up with 49, six more than those determined previously in intact GPIIIa (Eirin *et al.*, 1986).

Localization of the epitope for monoclonal antibody P37 in the 23 kDa fragment

The distribution of the epitopes for five murine GPIIIa-specific monoclonal antibodies (P6, P35, P37, P40 and P97) between the tryptic products of early digestion of pure GPIIIa was studied by immunoelectroblotting (Fig. 4). All these antibodies recognize

Fig. 3. Electrophoretic analysis of the cleavage in early digested GPIIIa of the interfragment disulphide bond joining the 23 kDa fragment to the 80/70 kDa fragment

Early digested GPIIIa in ammonium bicarbonate buffer was lyophilized, and resuspended in 50 mM-Tris/HCl/1 mM-EDTA containing 0.1% SDS, pH 8.0. Stepwise reduction of early digested GPIIIa was done at increasing molar excess of dithioerythritol with respect to the half-cystine content of GPIIIa as determined by amino acid analysis. Samples were alkylated with iodoacetate, and electrophoresis was carried out under non-reducing conditions. Lane a, GPIIIa (10 μ g); lane b, early digested GPIIIa (35 μ g); lanes c-k, early digested GPIIIa (35 μ g each) reduced with 0.5, 1, 2, 4, 6, 10, 15, 20 and 30-fold molar excesses of dithioerythritol over the half-cystine content of GPIIIa respectively. Note the gradual appearance of the 23 kDa fragment and the change in electrophoretic mobility of both the 80/70 kDa and the 23 kDa fragments on increasing reduction and alkylation. Numbers at the side are as in Fig. 1.

unreduced digested GPIIIa, but only P37, an antibody which inhibits ADP-induced platelet aggregation and binds to fully reduced and carboxymethylated GPIIIa, recognizes the 23 kDa fragment (Fig. 4a) both in its partially and fully reduced and carboxymethylated forms. The rest of the monoclonal antibodies recognize the reduced form of the 80/70 kDa fragment (Fig. 4b) but not the partially or fully alkylated forms, except for P6 which recognizes both the 80 and the 70 kDa fragments, in their partially reduced and carboxymethylated form (Fig. 4c).

Digestion products of the 23 kDa fragment are recognized by P37 down to the 17 kDa product. P6 is the only monoclonal which recognizes further digestion products of the 70 kDa fragment down to the 52 kDa product.

DISCUSSION

The first tryptic cleavage of GPIIIa occurs preferentially at 23 kDa from one of the end terminal amino acids of the glycoprotein. This end terminal was identified as the *N*-terminal, after finding that the *N*-terminal amino

acid sequences of intact GPIIIa and of the 23 kDa fragment were identical and coincided with the sequence determined by Charo *et al.* (1986) for human platelet GPIIIa: Gly-Pro-Asn-Ile-Cys-Thr. This first cleavage gives rise to two main glycopeptides joined by a single disulphide, which is the first disulphide to be reduced by dithioerythritol in early digested GPIIIa. About this interfragment disulphide we can say that the region of the smaller fragment (23 kDa) joined by this disulphide must be somewhere within the first 17 kDa from the *N*-terminal of GPIIIa, because a 17 kDa fragment (the smallest tryptic product still recognized by P37 and still carrying the GPIIIa *N*-terminal sequence) is liberated from the 70 kDa fragment only after reduction (see Fig. 1c). As far as the total thiol group content in the 23 and 80/70 kDa glycopeptides is concerned, we determined now 49 ± 3.3 after dithioerythritol reduction, six more than those determined previously in intact GPIIIa (Eirin *et al.*, 1986); this may be due to an easier access of dithioerythritol to all the disulphide bonds in digested GPIIIa.

The amino acid and sugar compositions of the 23 kDa and 80/70 kDa tryptic fragments of early digested GPIIIa are very close to those of GPIIIa. None of the tryptic fragments therefore can be considered as low polarity membrane peptides, as it happens with GPIIIa (Eirin *et al.*, 1986). On the other hand, the uneven distribution of sugars between the two fragments (Table 1) is most probably due to heterogeneity among the oligosaccharide chains of GPIIIa. The molecular masses obtained from the chemical composition of these fragments, 23 kDa and 71 kDa, are in good agreement with the known molecular mass of GPIIIa (91.5 kDa) determined from its chemical composition (Eirin *et al.*, 1986) and by physical measurements (Jennings & Phillips, 1982; Usobiaga *et al.*, 1987).

The electrophoretic pattern of unreduced early digested GPIIIa (Fig. 1b) shows a major component of apparent molecular mass about 120 kDa, and two minor bands of electrophoretic mobilities slightly lower than those of GPIIIa dimer and myosin. The lower mobility of the major component is most probably due to a conformational change induced by proteolysis, while the high molecular mass minor bands are due to associations of the major component of the same nature as those responsible for dimerization of GPIIIa (Calvete *et al.*, 1987).

If we compare our results with those of Newman *et al.* (1985) and Kornecki *et al.* (1985), we conclude that the PL^{A1} alloantigen cannot be on the 23 kDa *N*-terminal region of GPIIIa. On the one hand, the 73, 68 and 17 kDa tryptic products found by Newman *et al.* (1985) appear as a 15–19 kDa broad band, when analysed under reducing conditions. At this advanced stage of digestion we do not expect to find our 17 kDa *N*-terminal tryptic product, because it would be completely digested (Fig. 1c). Therefore the 17 kDa fragment on which Newman *et al.* (1985) locate the PL^{A1} alloantigen has to come from the more resistant 70 kDa fragment formed, in our experimental conditions, after the second tryptic cleavage of pure GPIIIa. On the other hand, the PL^{A1} alloantigen was identified on a 66 kDa membrane-attached fragment of GPIIIa after chymotryptic digestion of whole platelets (Kornecki *et al.*, 1985). This fragment was recognized by monoclonal antibodies P6, P35, P40 and P97, specific for our 80/70 kDa tryptic fragment, but

Fig. 4. Localization by immunoelectroblotting of the epitopes for GPIIIa-specific monoclonal antibodies among the products of early tryptic digestion of pure GPIIIa

Immunoelectroblotting was carried out after electrophoresis in 12% polyacrylamide gels. Sample reduction was as stated in Fig. 1. (a) Analysis with monoclonal antibody P37 (at 1:1000 dilution) of the same samples as in Fig. 1(a) after reduction. Lanes a–d are the tryptic products of GPIIIa after 0, 5, 15 and 300 min of digestion, respectively. (b) Analysis with monoclonal antibody P6 at (1:2000 dilution) of the same samples as in Fig. 1(c) after reduction. Lanes a–e are the tryptic products of GPIIIa after 0, 5, 15, 30 and 60 min of digestion, respectively. (c) Analysis with monoclonal antibodies P6 and P35 (both at 1:1000 dilution). Lanes a and b are reduced GPIIIa and partially reduced and carboxymethylated early digested GPIIIa, respectively, exposed to P6 after blotting; lanes c and d are control GPIIIa and early digested GPIIIa, respectively, reduced before electrophoresis and exposed to P35 after blotting.

not by P37, specific for our 23 kDa *N*-terminal fragment of GPIIIa (J. J. Calvete & J. González-Rodríguez, unpublished work). Therefore, we conclude that the particulate 66 kDa fragment obtained by Kornecki *et al.* (1985) lacks the *N*-terminal region of GPIIIa, and that our 23 kDa tryptic fragment of GPIIIa does not contain the PL^{A1} alloantigen.

It is remarkable that whereas monoclonal antibodies P6, P35, P40 and P97 recognize the 80/70 kDa fragment of early digested GPIIIa, in both unreduced and reduced forms, only P6 recognizes the partially reduced and alkylated form of the 80/70 kDa fragment, and its further digestion products down to 52 kDa. That means that it is not the reduction of the interfragment disulphide bond that destroys the epitopes for P35, P40 and P97, but the alkylation of the thiols formed afterward. The conformational change in this glycopeptide determined by alkylation must be very large, because not only does it affect the electrophoretic mobility (Fig. 4c) but also destroys the epitopes for P35 and P40 (as could be expected given the cross-competitive binding for GPIIIa between themselves and with P37) and even the epitope for P97, which does not seem to be closely located to any epitope of the other monoclonal antibodies studied (Melero & González-Rodríguez, 1984).

We had delineated previously five different antigenic areas in the extracellular surface of GPIIIa (Melero & González-Rodríguez, 1984), some of which can now be more specifically located within the glycoprotein. Thus, while the epitopes for P6, P35, P40 and P97, which do not interfere with platelet aggregation, are located within

the larger tryptic fragment (80/70 kDa), the epitope for P37, the monoclonal antibody which inhibits platelet aggregation, is situated within the first 17 kDa of the *N*-terminal region of GPIIIa. This finding gives a special functional interest to this region of GPIIIa, and reveals a characteristic in common with other cell adhesion molecules such as neural cell adhesion molecule (N-CAM) whose *N*-terminal domain is known to mediate homophilic binding (Edelman, 1986). Whether GPIIIa shares other features with N-CAM such as a C-terminal domain, part of which spans the plasma membrane, or whether the *N*-terminal region of GPIIIa forms the binding site for fibrinogen, remains to be demonstrated. Finally, while the discontinuous determinants for P35 and P40 must be close to this functional region, the determinants for P97 and P6 must be further away, because these monoclonal antibodies do not compete with P37, and the binding site for P6 is somewhere within the first 52 kDa from the C-terminal amino acid of GPIIIa.

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