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Localization of an *O*-glycosylation site in the α-subunit of the human platelet integrin GPIIb/IIIa involved in Bak^a (HPA-3a) alloantigen expression

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The human alloantigen system Bak$^{a/b}$ is associated with a Ile^{843} → Ser replacement on platelet glycoprotein IIb, the α-subunit of the integrin receptor for fibrinogen (GPIIb/IIIa). Recent immunological studies indicate that sialylated oligosaccharide chain(s) are also implicated in expression of the Bak^a determinant. Here we show that the GPIIb fragment 704–856 contains the whole Bak^a epitope, and that chemical cleavage of a single *O*-linked oligosaccharide chain within this GPIIb domain correlates with the loss of its anti-Bak^a antibodies binding ability. Ser^{847} was identified as the *O*-glycosylation site. Therefore, our results show that the Ser^{847} modification is responsible for the expression of the GPIIb-specific Bak^a alloantigen, and provide thus a link between the molecular biology and the immunologic observations.

Platelet GPIIb/IIIa complex; Alloantigen; Bak^a, *O*-Glycosylation

1. INTRODUCTION

Human glycoprotein (GP) IIb/IIIa complex, a Ca^{2+}-dependent heterodimer, is the major integrin receptor of the platelet membrane. It functions in adhesive interactions like platelet adhesion to the subendothelium and platelet aggregation, by serving on resting platelets as a low affinity adhesion receptor for surface-coated fibrinogen, and upon platelet activation as the inducible receptor for soluble fibrinogen and other adhesive plasma proteins [1–3]. Besides its central role in normal hemostasis, GPIIb/IIIa (integrin $a^{IIb}b_3$) bears a number of clinically important alloantigenic determinants that are implicated in the development of immunopathologic syndromes, such as posttransfusion purpura (PTP) and neonatal alloimmune thrombocytopenic purpura (NATP), due to platelet alloantigen incompatibility in recipients of blood transfusions (platelet transfusion refractoriness, PTR) and to maternal sensitization to paternal antigens on fetal platelets during pregnancy, respectively [4]. To date, six different alloantigen systems have been localized to the GPIIb/IIIa complex, and the molecular basis of these polymorphisms have been solved at the molecular genetic level. Thus, the amino acid substitutions within the mature GPIIIa polypeptide Leu^{33} → Pro [5], Arg^{143} → Gln [6], Pro^{407} → Ala [7], Arg^{489} → Gln [8], and Arg^{636} → Cys [9] are primarily responsible for the formation of the PLA$^{1/2}$ (= ZW$^{a/b}$, HPA-1), Pen$^{a/b}$ (= Yuk$^{b/a}$, HPA-4), Mo, CA/TU, and Sr(a) alloantigen systems, respectively. On the other hand, the Bak$^{a/b}$ (= Lek$^{a/b}$, HPA-3) system has been correlated with an Ile/Ser polymorphism at residue 843 of the heavy chain of glycoprotein IIb [10]. Serological data demonstrate that all GPIIb/IIIa alloantigens so far studied are diallelic systems showing a codominant Mendelian inheritance pattern, though the phenotype frequency of the different alloantigens on GPIIb/IIIa varies within the human gene pool [4,11].

Unlike other single amino acid replacements resulting in dysfunctional GPIIb/IIIa complexes and consequently in platelet Glanzmann thrombasthenic phenotypes (i.e. Asp^{119} → Tyr [12]; Arg^{214} → Gln [13] or Trp [14]; Ser^{752} → Pro [15] within GPIIIa, and Gly^{418} → Asp [16] and Ser^{573} → Arg [17] within GPIIb), there does not appear to be any notable functional difference related to GPIIb/IIIa alloantigen phenotype [4].

Interestingly, the PLA and Sr(a) alloantigen stability is maintained by the native disulphide bond pattern of GPIIIa [18,19]. Similarly, Pen epitopes are dependent upon proper folding of the mature GPIIIa polypeptide chain [6]. The Bak determinants, on the other hand, do not depend on the presence of intact protein structure [20] but require posttranslational processing of pro-GPIIb (both carbohydrate modification and cleavage into heavy and light chains), in addition to the amino acid difference Ile^{843} → Ser, for efficient epitope expression [21]. Moreover, both Bak^a (HPA-3a) and Bak^b (HPA-3b) are sensitive to removal of sialic acid [21,22]. Here, we report the localization of an *O*-glycosylated

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serine at the C-terminal end of the GPIIb heavy chain implicated in expression of the Bak alloantigen system.

2. MATERIALS AND METHODS

Anti-Bak^a antiserum was kindly donated by Dr. Nordhagen (National Institute of Public Health, Oslo, Norway) and was employed without further purification, at a 1:5 (v/v) dilution in 20 mM phosphate, 150 mM NaCl, pH 7.4 (PBS).

Glycoprotein IIbH (the heavy chain of GPIIb) was obtained as previously described [23] using purified GPIIb/IIIa [24] as the initial material.

Chemical degradation of GPIIbH with cyanogen bromide (CNBr) and size-exclusion and/or reverse-phase chromatographic isolation of the resulting fragments were done as previously described in detail [25].

SDS-gel electrophoresis was performed following [26], and electroblotting onto nitrocellulose sheets was done using the method of Towbin et al. [27]. As second antibody a goat anti-(total) human Ig, peroxidase-conjugated antiserum (Sigma) was employed at a 1 : 2000 (v/v) dilution in PBS. After washing with 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4 (TBS), the blots were developed with TBS containing 20% (v/v) methanol, 1 mg/ml 4-chloro-1-naphtol (BioRad) and 0.1 μl/ml H_2O_2.

Removal of *O*-linked carbohydrates from the 19 kDa C-terminal CNBr-fragment of GPIIbH was accomplished by alkaline β-elimination. To this end, the lyophilized polypeptide was resuspended at 1 mg/ml in 5 mM NaOH, and then incubated for 24 h at 37°C in a toluene atmosphere [28]. Thereafter, the peptide was extensively dyalized against deionized (Milli-Q) water, lyophilized, and stored at −20°C until use.

Amino acid and amino sugar analyses were done with a Biotronik LC5001 analyzer after hydrolysis at 110°C in 6 N HCl for 24 h and 4 N HCl for 4 h, respectively.

For proteolytic degradation isolated GPIIbH or its 19 kDa CNBr-derived fragment (5 mg/ml in 100 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.8) were incubated overnight at 37°C either with trypsin, chymotrypsin, endo Lys-C proteinase, or endo Glu-C proteinase (Boehringer Mannheim) at an enzyme:substrate ratio of 1:100 (w/w). After blocking the enzyme activity with a 25 molar excess of phenylmethylsulphonyl fluoride (PMSF) over enzyme concentration, the digestion mixtures were tested for their ability to bind anti-Bak^a antibodies by competitive enzyme-linked immunosorbent assay (ELISA) as previously described [29,30].

Tryptic peptides were purified by reverse-phase HPLC using a Lichrospher RP-100 (Merck) C18 column (25 × 0.4 cm, 5 μm particle size) eluted at 1 ml/min with a linear stepwise gradient of 0.1% (v/v) trifluoroacetic acid in (A) water and (B) acetonitrile (isocratically (10% B) for 5 min, followed by 10–40% B in 90 min). The isolated peptides were characterized by amino acid and amino sugar analysis and N-terminal sequencing (using either a Beckman 890C or an Applied Biosystems 473A sequencer following the manufacturers instructions).

3. RESULTS AND DISCUSSION

In order to investigate the structural requirements for expression of the Bak^a (HPA-3a) alloantigen on human platelet glycoprotein IIb, the isolated, reduced and carboxymethylated GPIIbH was degraded with cyanogen bromide, the fragments separated by SDS-gel electrophoresis, blotted onto nitrocellulose sheets and probed with and anti-Bak^a antiserum. Fig. 1 (lane b) shows that only a 19 kDa electrophoretic band was recognized by the antiserum. This band contained, however, two degradation products (GPIIbH 315–489 and 704–856) [25]

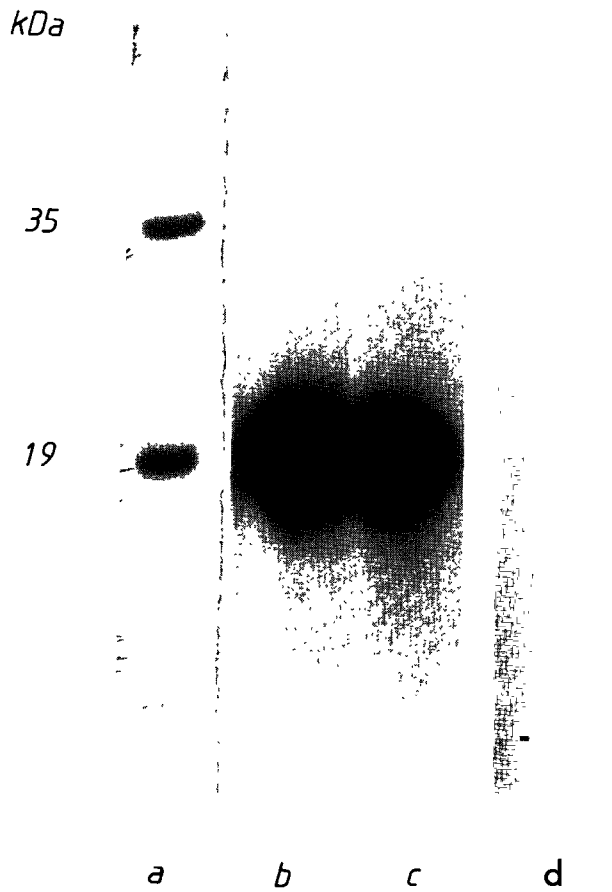

Fig. 1. Detection by immunoblotting analysis of anti-Bak^a binding activity. (Lane a) Electrophoretic control of the mixture of cyanogen bromide fragments obtained from reduced and carboxymethylated GPIIb. (Lane b) Binding of anti-Bak^a antibodies to the sample shown in (a). (Lane c) Binding of anti-Bak^a antiserum to HPLC isolated GPIIbH 704–856 fragment. (Lane d) No anti-Bak^a binding to isolated fragment 315–489 was observed. The numbers at the left side indicate the apparent molecular mass of the major fragments in kilodaltons (kDa).

but after separation by size-exclusion and reverse-phase chromatography [25,31] only the C-terminal fragment of GPIIbH retained the anti-Bak^a binding activity (Fig. 1 lane c). Fig. 1, lane d, shows that no anti-Bak^a binding to GPIIbH 315–489 could be detected. Moreover, the fragment GPIIbH 704–856, but not peptide 315–489, competed quantitatively with intact GPIIb for anti-Bak^a binding in an ELISA system (Fig. 2). In addition, enzymatic degradation of the 704–856 GPIIbH polypeptide with either trypsin, chymotrypsin, endoproteinase Lys-C or endoproteinase Glu-C did not affect antiserum recognition activity (Fig. 2), indicating that neither accessible basic amino acid side chains nor acidic or aromatic residues are involved in formation of the Bak^a epitope.

Amino sugar and amino acid analyses of the isolated 19 kDa GPIIbH 704–856 fragment revealed the presence of 0.92 mol galactosamine/mol peptide, suggesting

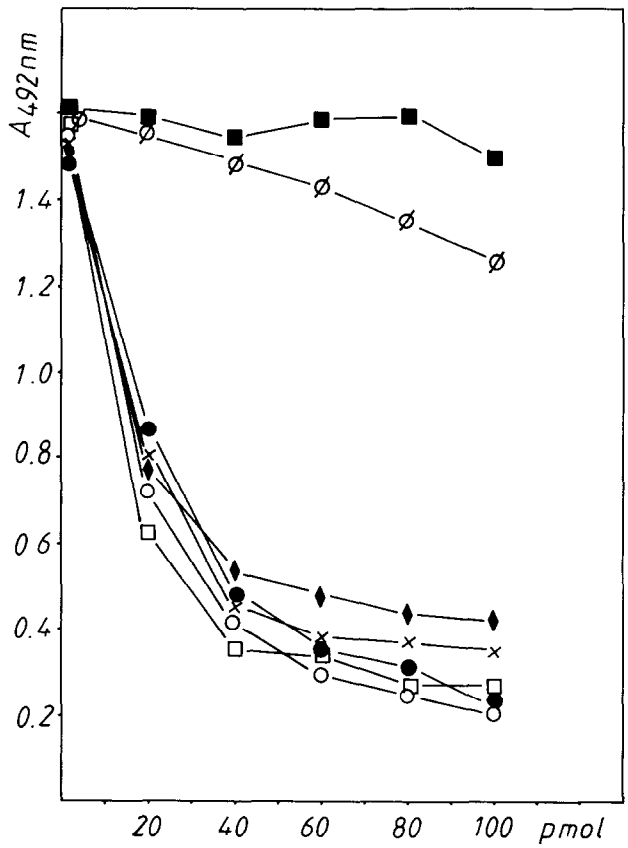

Fig 2. Detection by competitive enzyme-linked immunosorbent assay (ELISA) of anti-Bak[a] binding activity in reduced and carboxymethylated GPIIbH (○); HPLC-isolated 19 kDa cyanogen bromide fragment GPIIbH 704–856 (□), cyanogen bromide fragments of GPIIb 1–285; 286–314; 315–489, 490–549; 550–580;581 635, 636 660; 661–693; and 694–703 (■), isolated as described [25]. For simplicity only a curve is shown; mixture of tryptic or endoproteinase Lys-C peptides from GPIIbH 704–856 (●); mixture of endoproteinase Glu-C from GPIIbH 704–856 (×), mixture of chymotryptic peptides of GPIIbH 704–856 (♦); reverse-phase HPLC purified GPIIbH fragment 704–856 after deglycosylation by alkaline β-elimination (∅).

the existence of a single *O*-linked carbohydrate chain attached to any of the 15 serine or 2 theonine residues within the C-terminal part of GPIIbII. To test whether this oligosaccharide is actually involved in expression of the Bak[a] determinant, the GPIIbH 704–856 fragment was subjected to chemical deglycosylation by alkaline β-elimination. The degree of deglycosylation was apparently complete, as determined by amino acid and amino sugar analyses after purification of the NaOH-treated fragment by reverse-phase HPLC. When this peptide was tested, it did only show a very decreased anti-Bak[a] binding activity (Fig. 2; ∅). The epitopes recognized by monoclonal antibodies Mα[2] (GPIIbH$^{837\ 843}$) and M_3 (GPIIbH$^{849\ 857}$) [29] (data not shown) were not destroyed during the deglycosylation process, however. This strongly supports the hypothesis that the loss of the Bak[a] epitope was due to carbohydrate removal rather than to polypeptide damage. This conclusion agrees with recent reports showing that Bak[a] and Bak[b] epitope expression are sensitive to removal of sialic acid [21,22]. In addition, our study demonstrates that the carbohydrate implicated in Bak[a] determinant formation corresponds to an *O*-linked chain, and that this oligosaccharide is located within the C-terminal domain of GPIIbH.

To further localize the *O*-linked carbohydrate chain within the GPIIbH 704–856 stretch, the 19 kDa peptide was degraded with trypsin or chymotrypsin. Fig. 3 shows the HPLC isolation of the tryptic peptides purified by reverse-phase HPLC and characterized by N-terminal sequencing (Table I) and amino acid and amino sugar analyses. As a whole, the tryptic peptides recovered from the C18 column contained the complete amino acid sequence of the GPIIbH 704–856 fragment except the dipeptide Ser^{725}–Lys^{726}. Amino sugar analyses of this set of peptides revealed the presence of 0.8 mol/mol peptide in peptide R5 only. On the other hand, the missing tryptic dipeptide was found within the not glycosylated chymotryptic fragment 720–737 (Table I). This result corroborated our previous conclusion that the 19 kDa fragment may contain a single oligosaccharide chain and further localized its position within the very C-terminal portion of GPIIbH. Peptide R5 contains 2 serine residues and thus two potential glycosylation points. N-terminal sequencing analysis yielded the amino acid sequence: Val^{837}-Asp-Trp-Gly-Leu-Pro-Ile-Pro-Ser-Pro-Xaa-Pro-Ile-His-Pro-Ala ..., indicating therefore that Ser^{847} but not Ser^{845} is the modified residue. Since there are only three intervening residues between position 843, whose polymorphism Ile/Ser has been correlated with expression of the Bak$^{a/b}$ alloantigen system [10], and the glycosylated serine, the simplest explanation for their participation in Bak[a] epitope formation is that both residues will most probably occupy part of the ~750 Å^2 antigen surface area that usually an antibody recognizes.

Table I

Characterization by N-terminal sequence analysis of the tryptic peptides isolated in Fig. 3

Peptide	N-terminal sequence	Position
T1	A E A Q V E L R	744–751
T2	N S Q N P N S K	727–734
T3	E Q N S L D S W	769–776
T4	E Q N S L D S W G P K	769–779
T5	V D W G L P I P S P X P I H	837–856
T6	I V L L D V P V R	735–743
T7	G N S F P A S L V V A A	752–768
T8	L V S V G N L E E A G	704–724
T9	V E H T Y E L H N N	780 836
C	Q L Q I R S K N S Q N P N	720–737

Amino acid sequences are in the standard one-letter code. N-terminal sequence of a selected chymotryptic peptide (C) is also shown.

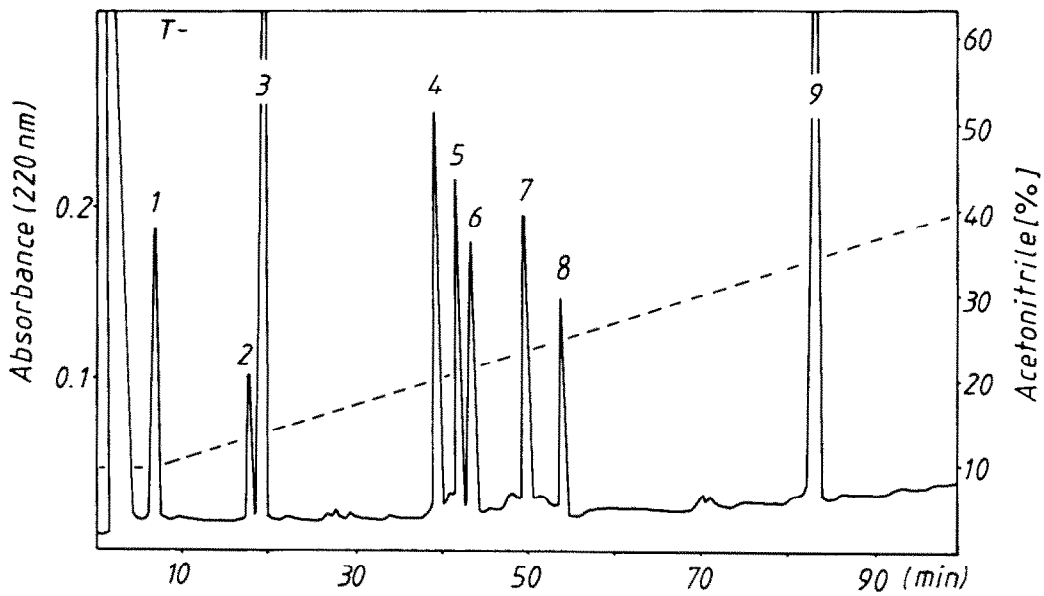

Fig. 3. Isolation by reverse-phase HPLC of tryptic peptides (T−) of reduced and carboxymethylated GPIIbH 704–856. For identification of each peak see Table I.

In a previous paper [25] we reported that anti-GPIIb monoclonal antibody M5 preferentially recognized Bak^a typified platelets. The M5 epitope has been located within the GPIIbH 550–558 polypeptide stretch, however [29]. Whether this region of GPIIb is spatially proximal to the Bak^a epitope within the quaternary structure of the GPIIb/IIIa complex needs further detailed investigation.

The structure of the oligosaccharide chain implicated in Bak^a formation remains to be established. It is also not clear whether the extra serine residue within the Bak^b determinant is glycosylated. This issues are currently being investigated in our laboratories.

The C-terminal domain of the heavy chain of GPIIb represents a very interesting region. It contains a neoepitope, GPIIbH$^{842-856}$, recognized by monoclonal antibody PMI-1, originally selected on the basis of its ability to block platelet adhesion to collagen [32]. This epitope is fully suppressed at physiological calcium ion concentration but results fully available upon receptor occupancy [33]. The ligand-induced binding site (LIBS) defined by the PMI-1 epitope is flanked by permanently surface-exposed regions recognized by monoclonal antibodies M3 (GPIIbH$^{849-856}$) and Mα^2 (GPIIbH$^{837-843}$) [29]. Remarkably, a single chymotryptic cleavage at GPIIbH Phe^{827} or Trp^{839} releases the C-terminal peptide of GPIIbH and correlates with acquisition of permanently available fibrinogen binding sites [34]. Our finding that Ser^{847} is glycosylated further indicates that this residue may be fully solvent accessible. Moreover, the region 842–851 is a proline-rich polypeptide for which a proline-based helix or a multiple turn conformation is predicted. If this were the case, then positions 843 and 847 would most probably reside on the surface-exposed face of the helix-like conformation, while the residues involved in expression of the PMI-1 determinant will occupy the hidden part of the structure. This suggestive hypothesis deserves further detailed investigation.

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