Comparative study of the glycosylation of platelet glycoprotein GPIIb/IIIa and the vitronectin receptor

Differential processing of their β -subunit

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The platelet glycoprotein GPIIb/IIIa and the vitronectin receptor (VNR) are $\alpha\beta$ -heterodimeric proteins and share the same β -subunit. By performing swainsonine treatment and digestion with endoglycosidase H (Endo H), we showed that the heavy chains of GPIIb and VNR α are glycosylated by complex-type oligosaccharide chains, and provided the first evidence for the presence of one complex carbohydrate residue on their light chains. The proteolytic cleavage of pro-GPIIb and the acquisition of Endo H-resistance are independent events occurring in the same Golgi compartment. We demonstrated the Endo H-sensitivity of GPIIIa and VNR β in all cellular systems tested. In addition, this β -subunit is differently glycosylated according to whether it is associated with GPIIb or VNR α , one carbohydrate chain being processed to the complex type on GPIIIa, but not on VNR β .

INTRODUCTION

Integrins are $\alpha\beta$ -heterodimers and encompass different groups of adhesion receptors, characterized by one β -subunit being associated with variable α -subunits [1,2]. The platelet glycoprotein IIB/IIIa and the vitronectin receptor (VNR) are members of one of these groups, the cytoadhesin family [3,4], and share the same β -subunit [5–8]. Platelet GPIIb/IIIa forms a Ca^{2+} -dependent heterodimer on the platelet surface [9], which serves, after platelet activation, as a receptor for fibrinogen, von Willebrand factor and fibronectin [10]. Endothelial cells synthesize a complex similar to GPIIb/IIIa [11–13] which interacts with vitronectin, fibrinogen and von Willebrand factor [14,15], and this complex has been shown to be identical with VNR [5]. The β -subunit of these two cytoadhesins (GPIIIa or VNR β) is a single-chain protein (100 kDa), whereas the two α -subunits, GPIIb and VNR α , are composed of a heavy (H) and a light (L) chain held together by disulphide bridges. Both α -subunits derive from a precursor form, the pro- α subunit, which contains the two polypeptidic chains [16–19]. In previous studies we demonstrated that an early assembly of pro- α -subunits with the β -subunits is critical for the intracellular processing of the heterodimer and its exposure on the cell surface [20]. Once the pro- $\alpha\beta$ heterodimer is formed, carbohydrate processing proceeds differently for the α - and the β -subunits in both megakaryocytes and endothelial cells. The oligosaccharide chains of the α -subunit are processed to the complex type in the Golgi system, whereas the β -subunit remains sensitive to endoglycosidase H (Endo H) in both cells [17–18].

Here we extended our studies on the biosynthetic pathways of cytoadhesins. Owing to controversial results already published [3,5,11,21], the molecular mass and the Endo H-sensitivity of the β -subunit were investigated in several cellular systems (human megakaryocytes, human endothelial cells, HEL and LAMA-84 cells, and surface-labelled platelets). The glycosylation of GPIIb/IIIa in megakaryocytes and of VNR in endothelial cells were further analysed and compared. The results indicate that: (1) the H- and L-chain of the α -subunits are both glycosylated and fully processed and (2) although the β -subunits remain not completely

processed in megakaryocytes and endothelial cells, a difference in glycosylation can be identified, suggesting that the β -subunit of the cytoadhesin family is heterogeneous.

MATERIALS AND METHODS

Materials

Carrier-free [^{35}S]methionine (1000 mCi/mmol), carrier-free ^{125}I , Amplifier and ^{14}C -labelled molecular-mass standards were from Amersham International (Amersham, Bucks., U.K.). Phorbol 12-myristate 13-acetate (PMA) was from Sigma (St. Louis, MO, U.S.A.). *Staphylococcus aureus* Cowan I Strain (Pansorbin) was from Calbiochem–Behring Corp. (La Jolla, CA, U.S.A.). Endo H, swainsonine and culture medium were from Boehringer (Meylan, France).

Methods

Cell culture. Human megakaryocytes were obtained from chronic-myelogenous-leukaemic (CML) patients as described in [22,17]. Human umbilical-vein endothelial (HUVE) cells were isolated by the method of Jaffe *et al.* [23] and cultured as described in [18]. HEL and LAMA-84 are two leukaemic cell lines that express GPIIb/IIIa [16,24,25]. Before metabolic labelling, HEL and LAMA-84 cells were induced for 3 days by 80 nM-PMA in RPMI 1640 containing 10% (v/v) fetal-calf serum.

Metabolic labelling and immunoprecipitation. Metabolic labelling, cell lysis and immunoprecipitation were performed as previously described, by using Pansorbin to collect antigen–antibody complexes [17,18,20]. When swainsonine was used, cells were preincubated overnight with swainsonine (2 $\mu\text{g}/\text{ml}$) and this concentration was kept constant during the metabolic labelling. Samples were analysed by SDS/PAGE on 7–12% linear-gradient gels [26].

Washing and surface iodination of human platelets. Platelets were isolated and surface-labelled in the presence of carrier-free ^{125}I using the lactoperoxidase method [17].

Abbreviations used: CML, chronic myelogenous leukaemia; Endo H, endoglycosidase H (EC 3.2.1.96); HUVE, human umbilical-vein endothelial; H, heavy; L, light; PMA, phorbol 12-myristate 13-acetate; VNR, vitronectin receptor.

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Fig. 1. Characterization of antibodies

Lysates (3×10^6 c.p.m.) from 8 h-metabolically-labelled human megakaryocytes (lanes 1–3) and HUVE cells (lane 4) were immunoprecipitated with the anti-GPIIIa antibody B2A (lanes 1 and 4), the anti-GPIIb antibody D12A (lane 2), or a mixture of antibodies B2A and D12A (lane 3). On lanes 1 and 2, dissociation of the GPIIb/IIIa complexes was performed before immunoprecipitation. Precipitates were subjected to SDS/7.5%–PAGE gels under reducing conditions and fluorography.

Specificity of the antibodies used. Immunoprecipitations were performed with two mouse monoclonal antibodies, B2A and D12A, prepared in our laboratory from a BALB/c mouse immunized with purified GPIIIa and GPIIb respectively as previously described [17,20]. In human megakaryocytes, when dissociation of the GPIIb/IIIa complexes was performed before immunoprecipitation by chelating Ca^{2+} with EDTA [27], the anti-GPIIIa antibody B2A immunoprecipitated GPIIIa (100 kDa), whereas antibody D12A immunoprecipitated pro-GPIIb (130 kDa) and GPIIb H-chain [GPIIb(H)] (116 kDa) (Fig. 1, lanes 1 and 2), demonstrating the specificity of these antibodies for GPIIIa and GPIIb epitopes respectively. When these antibodies were used in combination, they immunoprecipitated under non-dissociating conditions pro-GPIIb, GPIIb(H) and GPIIIa (Fig. 1, lane 3). Immunoprecipitations of HUVE-cell lysates were performed with the anti-GPIIIa antibody B2A, which immunoprecipitated VNR β (100 kDa) and co-immunoprecipitated pro-VNR α (128 kDa) and VNR α H-chain [VNR α (H)] (118 kDa) (Fig. 1, lane 4) [18].

Oligosaccharide digestion. ^{35}S -labelled proteins were isolated by immunoprecipitation and separated on reducing 7–12% linear-gradient gels [26]. Bands were excised from the gel, digested with Endo H (200 munits/ml; this concentration has proved optimal under our conditions) and analysed by SDS/PAGE on 7–12% linear-gradient gels as described in [17,18].

RESULTS AND DISCUSSION

Endo H-sensitivity of the different subunits of GPIIb/IIIa and VNR

The Endo H-sensitivity of the mature forms of GPIIIa, VNR β , GPIIb(H) and VNR α (H), was examined in cell lysates from 24 h-metabolically-labelled cells (human megakaryocytes, PMA-treated HEL and LAMA-84 cells, and HUVE cells) and surface-labelled platelets. Endo H specifically removes not-fully-processed oligosaccharide chains, but does not remove complex-type carbohydrates [28]. In all these cellular systems, the mature forms of GPIIIa and VNR β had a similar molecular mass of 100 kDa (Fig. 2, lanes 1–4 and 9), in agreement with their identical primary structures [6–8]. Consistent with our previous observations [17,18], GPIIIa and VNR β were sensitive to Endo H, since their molecular masses were decreased by approx. 10 kDa even after 24 h of metabolic labelling in megakaryocytes or endothelial cells (Fig. 2, lanes 5 and 8). The same results were

Fig. 2. Endo H-sensitivity of GPIIb/IIIa and VNR

Lysates from 24 h-metabolically-labelled human megakaryocytes (lanes 1 and 5), PMA-treated HEL (lanes 2 and 6) and LAMA-84 (lanes 3 and 7) cells, and HUVE cells (lanes 4 and 8), and surface-labelled human platelets (lanes 9 and 10), were immunoprecipitated with the anti-GPIIIa B2A (lane 4) or with a mixture of the anti-GPIIb D12A and the anti-GPIIIa B2A (lanes 1–3, and 9). After SDS/PAGE, bands from lanes 1–4 and 9 were excised from the gel, treated with Endo H (200 munits/ml), and analysed under reducing conditions by SDS/PAGE on a 7.5% gel (lanes 5–8, and 10).

observed with GPIIIa from HEL and LAMA-84 cells, and surface-labelled platelets (Fig. 2, lanes 6, 7 and 10). This indicates that the β -subunit of two members of the cytoadhesin family contains not-fully-processed carbohydrates. By contrast, in all cellular systems tested, the H-chains of GPIIb and VNR α were fully processed, since treatment with Endo H did not modify their respective molecular masses of 116 and 118 kDa (Fig. 2, lanes 5–8 and 10).

The Endo H-sensitivity of GPIIIa is in agreement (i) with its biochemical characterization [29,30], indicating that mannose residues represent 45% of the sugars of GPIIIa, and (ii) with the fact that GPIIIa binds preferentially to concanavalin A [31], suggesting the presence of *N*-linked oligosaccharide chains with branched mannose structure. Studies on the glycosylation of other integrins have already been published. Indeed, the β -subunits of the leukocyte adhesion molecules [32], the fibronectin receptor [33] and, surprisingly, the M21-melanoma-cell Arg-Gly-Asp receptor immunoprecipitated by a monoclonal anti-GPIIIa antibody [21], have been reported to be fully processed and Endo H-resistant. Thus the Endo H-sensitivity of the β -subunit is a characteristic of the cytoadhesin family.

Processing of GPIIb/IIIa and VNR α - and β -subunits

To characterize further the carbohydrate processing of the different subunits of GPIIb/IIIa and VNR, their sensitivity to Endo H was next examined in the presence of swainsonine. Swainsonine is an inhibitor of the Golgi mannosidase II, which blocks the formation of complex-type oligosaccharide chains and induces the formation of hybrid-type carbohydrates [34] that are sensitive to digestion by Endo H [35–37]. Swainsonine was used at $2 \mu\text{g/ml}$, a concentration which has proved optimal under our conditions with respect to the viability of the cell, the overall protein synthesis (as measured by trichloroacetic acid-precipitable radioactivity) and the maximal inhibition of the α -mannosidase II (results not shown). Processing of GPIIb/IIIa and VNR α - and β -subunits was analysed in lysates from 8 h-metabolically-labelled megakaryocytes or HUVE cells. In native megakaryocytes, we immunoprecipitated pro-GPIIb (130 kDa), GPIIb(H) (116 kDa), GPIIb L-chain [GPIIb(L)] (25 kDa) and GPIIIa (100 kDa) (Fig. 3, lane 1) as previously reported [17]. After treatment with swainsonine, the molecular masses of pro-GPIIb and GPIIIa were unmodified, whereas GPIIb(H) and GPIIb(L) migrated respectively at 114 and slightly below 25 kDa (Fig. 3, lane 2). In native HUVE cells, we immunoprecipitated pro-VNR α (128 kDa), VNR α (H) (118 kDa), VNR α L-chain [VNR α (L)] (25 kDa) and VNR β (100 kDa) (Fig. 4, lane 1) [18]. In swainsonine-treated cells, only the molecular masses of

Fig. 3. Endo H-sensitivity of GPIIb/IIIa α and β subunits after swainsonine treatment

Lysates from human megakaryocytes (lanes 1 and 2) metabolically labelled for 8 h in the absence (lane 1) or presence of 2 μ g of swainsonine (Sw)/ml (lane 2) were immunoprecipitated with a mixture of the anti-GPIIIa B2A and the anti-GPIIb D12A antibodies and analysed under reducing conditions by SDS/PAGE on a 7–12 % gradient gel (lanes 1 and 2). GPIIb and GPIIIa bands from lanes 1 and 2 were excised from the gel, treated by Endo H (200 munits/ml) and resubmitted to SDS/PAGE (lanes 3–6). Lanes 3 and 4, GPIIb(H) (top) and GPIIb(L) (bottom); lanes 5 and 6, GPIIIa. The lower part of the gel was autoradiographed for 7 days (lanes 1–4) instead of 16 h for the upper part.

Fig. 4. Endo H-sensitivity of VNR α - and β -subunits after swainsonine treatment

Lysates from 8 h-metabolically-labelled HUVE cells (lanes 1 and 2) in the absence (lane 1) or in the presence of 2 μ g of swainsonine (Sw)/ml (lane 2) were immunoprecipitated with the anti-GPIIIa B2A antibody and analysed under reducing conditions by SDS/PAGE on a 7–12 % gradient gel (lanes 1 and 2). VNR α and VNR β bands from lanes 1 and 2 were excised from the gel, treated with Endo H (200 munits/ml) and resubmitted to SDS/PAGE (lanes 3–6). Lanes 3 and 4, VNR α (H) (top) and VNR α (L) (bottom); lanes 5 and 6, VNR β . The lower part of the gel was autoradiographed for 21 days (lanes 1–4) instead of 24 h for the upper part.

VNR α (H) (116 kDa) and VNR α (L) (slightly below 25 kDa) were modified (Fig. 4, lane 2). After treatment with swainsonine, the change in molecular mass observed for GPIIb and VNR α H- and L-chains can be explained by the substitution of hybrid forms for complex ones [35–37]. In addition, the immunoprecipitation of GPIIb and VNR α H- and L-chains in swainsonine-treated cells indicated that, despite the inhibition of the carbohydrate processing by swainsonine, the proteolytic cleavage of pro-GPIIb and pro-VNR α still occurred. Thus complete processing of the carbohydrate moieties is not necessary for the proteolytic cleavage of the pro- α -subunits, and these two processing events would appear to occur independently. To investigate the Endo H-

sensitivity of the α - and β -subunits, bands were excised from the gel, treated with Endo H, and analysed by SDS/PAGE.

Processing of the α -subunits. As previously demonstrated, GPIIb(H) (116 kDa) (Fig. 3, lane 3) and VNR α (H) (118 kDa) (Fig. 4, lane 3) were resistant to digestion with Endo H. In addition, this experiment showed that the L-chains of these α -subunits, VNR α (L) and GPIIb(L) at 25 kDa, were not digested by Endo H (Fig. 3, lane 3 and Fig. 4, lane 3), suggesting either that they were not glycosylated or that they shared complex oligosaccharides. Treatment of cells with swainsonine resulted in the synthesis of Endo H-sensitive α -subunits: GPIIb(H) and

Table 1. Subunit molecular mass of, and oligosaccharide types in, GPIIb/IIIa and VNR

Molecular masses of the glycosylated and deglycosylated forms were deduced from SDS/PAGE analysis [26]. The putative *N*-glycosylation sites were obtained from cDNA sequence analysis [6–8,41–43] and biochemical characterization [40].

	Molecular mass (kDa)		Putative <i>N</i> -glycosylation sites	<i>N</i> -Linked oligosaccharide chains	
	Glycosylated forms	Deglycosylated forms		High-mannose or hybrid	Complex
GPIIIa	100	87	6	4 or 5	1
VNR β	100	87	6	5 or 6	None
GPIIb H	116	103	4	None	4
L	25	22	1	None	1
VNR α H	118	105	10	None	4
L	25	22	3	None	1

GPIIb(L) were decreased in molecular mass to respectively 103 and 22 kDa (Fig. 3, lane 4), and VNR α (H) and VNR α (L) shifted respectively to 105 and 22 kDa (Fig. 4, lane 4). Assuming an average molecular mass of 3 kDa for one complex oligosaccharide [38], the α -subunit H-chains, GPIIb(H) and VNR α (H), were predicted to share four oligosaccharide chains of the complex type and to have a deglycosylated mass of respectively 103 and 105 kDa (see Table 1). In addition, these experiments provided the first evidence that their L-chains, GPIIb(L) and VNR α (L), were glycosylated by one complex-type carbohydrate chain. Results concerning GPIIb are consistent with its previously reported biochemical characterization [39,40], which indicated that GPIIb H- and L-chains were rich in sialic acid and galactose, two sugar residues known to compose oligosaccharide chains of the complex type [38]. Our findings establish that the putative *N*-glycosylation sites in megakaryocytic GPIIb given by the nucleotide sequence analysis [41,42] and recent biochemical studies [40], are all glycosylated by complex oligosaccharides (Table 1). By contrast, the putative *N*-glycosylation points in VNR α [43] are not all glycosylated (Table 1). Indeed, it is now well established that the sequence Asn-Xaa-Ser(Thr) is a necessary, but not a sufficient, condition for glycosylation [38]. Although we cannot exclude the possibility of an incomplete digestion by Endo H for VNR α carbohydrate chains in swainsonine-treated cells, the glycosylation of the ten putative glycosylation sites in VNR α seems to be unlikely, as the molecular mass of its polypeptide backbone given by the cDNA sequence analysis [43] is 95 kDa.

In previous studies, we have shown that, in treated megakaryocytes and HUVE cells with monensin, an antibiotic blocking the transport of glycoproteins in the Golgi system [44], pro-GPIIb and pro-VNR α are not proteolytically cleaved into their respective H- and L-chains [17,18]. These data suggest that the proteolytic cleavage does not occur in the endoplasmic reticulum. In the present study we show that all GPIIb carbohydrate chains are Endo H-resistant (Table 1), in contrast with those of pro-GPIIb, which are all digested by Endo H [17]. This indicates that the acquisition of Endo H-resistance (which takes place after action of the *N*-acetylglucosamine transferase I and mannosidase II in the middle Golgi [45]) and the proteolytic cleavage of the pro-GPIIb probably occur in the same compartment of the Golgi system.

β -Subunits of GPIIb/IIIa and VNR are differently glycosylated in megakaryocytes and endothelial cells. VNR β from native and swainsonine-treated HUVE cells were decreased to 87 kDa after action of Endo H (Fig. 4, lanes 5 and 6), showing that VNR β contained not-fully-processed oligosaccharide chains only. By contrast, GPIIIa from swainsonine-treated megakaryocytes was more sensitive to digestion with Endo H than normal GPIIIa, as its molecular mass shifted to 87 kDa (Fig. 3, lane 6) instead of to 90 kDa as observed for normal GPIIIa (Fig. 3, lane 5), demonstrating the presence of one Endo H-resistant oligosaccharide chain on normal GPIIIa. The molecular mass of 87 kDa for the deglycosylated β -subunit is in agreement with our previous findings for the aglycosylated β -subunit obtained after tunicamycin treatment [17,18]. In addition, the complete digestion by Endo H of swainsonine-treated oligosaccharides confirms that Endo H was used under optimal conditions. These results for GPIIIa are supported by recent findings by Calvete *et al.* [46]. They obtained, by tryptic digestion of GPIIIa, two fragments of 23 and 80/70 kDa. The 23 kDa fragment, located in the *N*-terminal region, contained one putative *N*-glycosylation site [6–8] and was described as being enriched in sialic acid [46], suggesting the presence of a complex carbohydrate chain. The five other putative *N*-glycosylation sites were located on the

80/70 kDa fragment (four of them in the cysteine-rich region of GPIIIa), whose oligosaccharide chains contained 50% of mannose, and some sialic acid and galactose residues, suggesting the presence of high-mannose and/or hybrid-type oligosaccharide chains [38]. In addition, it has been suggested that processing is more extensive near the *N*-terminus of glycoproteins, where complex oligosaccharides are especially found, high-mannose types being located towards the *C*-terminus [47]. Taken together, these observations indicated that GPIIIa contains one complex-type oligosaccharide chain located in its *N*-terminal region and four or five chains of the high-mannose or hybrid type in the *C*-terminal region (assuming the molecular mass of each high mannose chain to be 2.5 kDa [38]). On VNR β , all oligosaccharide side chains remain incompletely processed (Table 1). Thus the same β -subunit is differently processed in megakaryocytes and in endothelial cells. Numerous studies have demonstrated that a single glycoprotein may be differently glycosylated in different cell types, owing to the individual glycosylation machinery of a particular cell, the protein structure and the relative accessibility of the *N*-linked oligosaccharides to the processing enzymes (for reviews, see [48,49]). In addition, it has already been reported for the leukocyte adhesion receptors LFA-1 and Mac-1 that the quaternary structure of a heterodimer could influence the glycosylation of a polypeptide [50]. Thus the differential subunit association of GPIIIa and VNR β with GPIIb and VNR α may explain the difference in glycosylation of the *N*-terminal glycosylation site.

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