The von Willebrand Factor-binding Domain of Platelet Membrane Glycoprotein Ib

CHARACTERIZATION BY MONOCLONAL ANTIBODIES AND PARTIAL AMINO ACID SEQUENCE ANALYSIS OF PROTEOLYTIC FRAGMENTS*

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The glycoprotein Ib (GPIb), a two-chain integral platelet membrane protein, acts as a receptor for von Willebrand factor. In order to obtain information on the domain involved in this function, as well as on the structural organization of GPIb, the protein has been purified and submitted to limited proteolysis using three different enzymes. The resulting fragments were topographically oriented by means of partial NH₂-terminus sequence analysis and immunological identification using monoclonal antibodies. One of these antibodies (LJ-1b1) inhibited the von Willebrand factor-GPIb interaction completely, one (LJ-P3) partially, and one (LJ-1b10) had no inhibitory effect. Three distinct fragments, the 38-kDa fragment produced by Serratia marcescem protease as well as the 45- and 35-kDa fragments produced by trypsin, had the same NH₂ terminus as the intact GPIb a-chain (apparent molecular mass = 140 kDa). These fragments and the a-chain reacted with the inhibitory antibodies. On the other hand, three fragments produced by Staphylococcus aureus V8 protease, one of 92 kDa similar to the previously described "macroglycopeptide" and two others of 52 and 45 kDa, had NH₂-terminal sequences different from that of the GPIb a-chain and reacted only with the noninhibitory monoclonal antibody LJ-1b10. Thus, the binding domain for von Willebrand factor resides near the NH₂ terminus of the GPIb a-chain, whereas the carbohydrate-rich region is part of the innermost portion of GPIb and does not appear to be involved in the von Willebrand factor binding function.

The platelet membrane glycoprotein (GP) Ib plays an important role in primary hemostasis by serving as one of the surface receptors that anchor platelets to exposed subendothelium at sites of vascular injury (1–3). This process is mediated by the adhesive molecule von Willebrand factor, which binds to GPIb as well as to collagen and noncollagenous components in the subendothelial matrix (4–11).

A limited knowledge of the structural and functional characteristics of GPIb has been attained during the past years (12–16). This integral membrane protein is composed of two disulfide-linked chains and contains approximately 60% carbohydrate by weight. The structure of the carbohydrate moiety has been determined (17–20). An endogenous platelet calcium-dependent protease cleaves the a-chain, releasing a large soluble fragment, termed glyocalcin (21–24), which appears to contain the von Willebrand factor- and thrombin-binding domains (25–28). The molecular mass of glyocalcin corresponds to approximately 130 kDa.

In these studies, we have used three murine monoclonal antibodies raised against human GPIb to locate the domain involved in von Willebrand factor binding. For this purpose, several fragments generated by proteolytic cleavage of GPIb have been isolated and characterized in terms of their reactivity with the monoclonal antibodies. Furthermore, the NH₂-terminal sequences of these proteolytic fragments, as well as of the intact a-chain, have been determined. We found that the two monoclonal antibodies which blocked the von Willebrand factor-GPIb interaction reacted with fragments possessing the same NH₂ terminus as the intact a-chain. The smallest of these peptides had a molecular mass of 38 kDa. Moreover, a carbohydrate-rich fragment of GPIb, similar to the previously described "macroglycopeptide" (13), had an NH₂-terminal sequence distinct from that of the intact a-chain and reacted only with the antibody that failed to block the von Willebrand factor-GPIb interaction. These studies provide direct immunochemical evidence that the location of the von Willebrand factor-binding domain of GPIb is in proximity of the NH₂ terminus of the a-chain and that the carbohydrate-rich region is toward its middle portion.

EXPERIMENTAL PROCEDURES AND RESULTS*

Three enzymes were used to prepare proteolytic fragments of GPIb, namely, Serratia marcescem protease, trypsin, and...
Purified intact GPIb and LJ-Ibl were also analyzed by immunohlot analysis using the monoclonal antibody phase immunoisolation using monoclonal antibodies W-P3 (Fig. 1). The autoradiography of a reduced 5–15% gradient gel is shown. Purified intact GPIb (lane 4) and a 6-h digest (lane 5) were also analyzed by immunoblot analysis using the monoclonal antibody LJ-IblO. Electroeluted GPIb α-chain (lane 7) derived from purified GPIb (lane 6) and the electroeluted 38-kDa fragment (lane 9) obtained from the whole digest of purified GPIb (lane 8) are also shown after analysis by 5–15% gradient SDS-PAGE and staining with Coomassie Brilliant Blue R (CBB). The apparent molecular mass of the polypeptides is indicated. GPIb α-chain and glycocalcin, or the glycocalcin-like fragment generated by S. marcescens, are not well separated in this gel.

**Fig. 3.** Electroelution and immunoblotting of GPIb fragments generated by trypsin. The electroeluted 45-kDa fragment (lane 2) and the 35-kDa fragment (lane 3) isolated from whole trypsin digest of GPIb (lane 1) were analyzed by 5–15% gradient SDS-PAGE followed by staining with Coomassie Brilliant Blue R (CBB). The same samples, including the whole digest (lane 4), the 45-kDa fragment (lane 5), and the 35-kDa fragment (lane 6), were also analyzed by immunoblotting using monoclonal antibody LJ-IblO. This antibody did not react with the 35-kDa fragment. Some aggregates of the 45-kDa fragment are seen in lane 5.

**Fig. 4.** Analysis of immunosolated GPIb fragments generated by S. marcescens V8 protease. The whole digest of 125I-labeled GPIb (lane 1) and the fragments isolated with monoclonal antibodies LJ-P3 (lane 2) and LJ-IblO (lane 3) were analyzed by 5–15% gradient SDS-PAGE under reducing conditions, followed by autoradiography. Arrows indicate the position of isolated fragments with their apparent molecular masses. Note that LJ-P3 recognized only fragments of 70 and 40 in addition to the 127-kDa one, whereas LJ-IblO reacted with fragments of 92, 70, 60, 52, 45, and 40 kDa. The reactivity of LJ-IblO is shown better with the immunoblotting technique presented for Fig. 5.

The sequence of the first three amino acid residues was His-Pro-Ile and was identical to that of the intact α-chain. As with the α-chain, the yield of PTH-derivatives was 15–20% of the protein applied.

**Digestion with Trypsin**—Immunosolation of the tryptic fragments demonstrated that LJ-P3 and LJ-IblO (Fig. 2) as well as LJ-Ibl1 (not shown) reacted with the same species. Analysis of the isolated polypeptides under nonreducing conditions demonstrated fragments of 127 and 45 kDa, but not the 84-kDa one. Analysis after reduction of disulfide bonds demonstrated, in addition, fragments of 92 and 35 kDa (Fig. 2). Both the 45- and 35-kDa tryptic fragments were isolated by electroelution. When analyzed by immunoblotting, only the 45-kDa fragment reacted with antibody LJ-Ibl10 (Fig. 3). Both polypeptides were submitted to NH₂-terminal sequence analysis. The same sequence, His-Pro-Ile, was determined in both cases, identical to that of the intact α-chain and the 38-
kDa fragment generated by *S. marcescens* protease. The initial yield of PTH-derivatives was 15–20% of the applied protein in these cases also.

**Digestion with *S. aureus* V8 Protease—Immunosoliation of proteolytic fragments generated by *S. aureus* V8 protease demonstrated that LJ-P3 (Fig. 4) and LJ-Ibl1 (not shown) reacted only with polypeptides of 127, 70, and 40 kDa, whereas antibody LJ-Ibl0 reacted with all of the above as well as with fragments of 92, 60, 52, and 45 kDa (Figs. 4 and 5). The 92-, 52-, and 45-kDa polypeptides were isolated by electrophoresis (Fig. 5) and submitted to NH₂-terminal sequence analysis. The 92- and 45-kDa fragments gave the same sequence, and the first three amino acid residues were Asn-Ser-Leu. The first 2 residues of the 52-kDa peptide, on the other hand, were identified as Ser-Ile. The third cycle gave no PTH-derivative, and the fourth residue was phenylalanine. The NH₂-terminal sequences of these three peptides, therefore, were different from that of the intact α-chain. The yield of PTH-derivatives of amino acids was greater than 50% of applied protein in these cases.

**DISCUSSION**

These studies demonstrate that all GPIb fragments of molecular mass between 38 and 45 kDa that reacted with two monoclonal antibodies inhibiting von Willebrand factor binding to GPIb had the same NH₂-terminal sequence as the intact α-chain. This finding provides strong immunological evidence that the von Willebrand factor-binding domain is located in a region near the NH₂ terminus of the α-chain. This possible localization had been previously suggested (46), but alternative models had also been proposed whereby this functional domain was assigned to the "tail" region of the (γ-chain (3, 16, 47). The latter hypothesis is clearly in contrast with the present results. In studies not reported here, we also found that glycocalcin, a soluble proteolytic fragment released from platelets by an endogenous calcium-dependent protease, has the same NH₂-terminal sequence as the intact α-chain, thus demonstrating that this is the distal portion of

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**Fig. 5.** Immunoblotting and electroelution of GPIb fragments generated by *S. aureus* V8 protease (SV8) digestion. The 15-min (lane 1) and 6-h (lane 2) digests of GPIb were subjected to immunoblot analysis using monoclonal antibody LJ-Ibl10. The electroeluted 92 (lane 4), 52 (lane 5), and 45 (lane 6)-kDa fragments derived from a 15-min digest of GPIb (lane 3) were analyzed by 5–15% gradient SDS-PAGE, followed by staining with Coomassie Brilliant Blue R (CBB). Arrows indicate the apparent molecular mass of fragments. The electroeluted 92-kDa fragment stained poorly with Coomassie Brilliant Blue R because of its high carbohydrate content.

**Fig. 6.** Schematic representation of the structural organization of the GPIb α-chain. The orientation of the major proteolytic fragments generated by digestion of the GPIb α-chain with *S. marcescens* protease (SP), trypsin (T), and *S. aureus* V8 protease (SV8) is indicated by the corresponding NH₂-terminal sequence and reactivity with three distinct monoclonal antibodies. Localization of the epitopes corresponding to these antibodies (LJ-P3, LJ-Ibl1, LJ-Ibl10) is tentative, but within regions of the GPIb α-chain that can be defined with certainty. The localization of the NH₂ terminus of the fragments generated by *S. aureus* V8 protease digestion is indicated with broken lines because it cannot be established on the basis of these results. Since all these fragments react with LJ-Ibl10, however, their NH₂ termini must be distal to the LJ-Ibl10 epitope. The approximate molecular masses of the fragments are indicated. The von Willebrand factor (vWF)-binding domain lies within the NH₂-terminal region of the α-chain. The COOH terminus of the GPIb α-chain is indicated with a broken line because it is not yet known whether it has a transmembrane portion. One-letter abbreviation for amino acids indicates the NH₂-terminal sequence of fragments.
the molecule. On the other hand, all proteolytic fragments of GPIb reacting only with the noninhibitory antibody LJ-Ibl10 had an NH2-terminal sequence distinct from that of the intact α-chain. These included polypeptides that were rich in carbohydrate, thus demonstrating that the previously described macrocyclic peptide derived from GPIb does not extend into the NH2-terminal region of the α-chain, as suggested by some authors (16, 47).

The initial yield of PTH-derivatives was low for the intact α-chain and for the fragments that gave the same NH2-terminal sequence of His-Pro-Ile. Whereas we have no definitive explanation at present for this poor initial yield, it is noteworthy that its possible cause may be related to the presence of histidine and proline residues in the first two positions (48-50). The possibility of partial blockage at the NH2 terminus should also be considered, particularly in view of the fact that others have not been able to obtain an unambiguous NH2-terminal sequence for glycocalcin (46).

Our findings indicate the presence of at least one intrachain disulfide bond within the NH2-terminal region of the GPIb α-chain (Fig. 6) as also suggested by others.2 A 35-kDa trypsin-generated fragment that was detected only after reduction of the disulfide bonds possessed the same NH2-terminal sequence as a fragment of 45 kDa, seen both under nonreducing and reducing conditions, and as the intact α-chain. Therefore, the 35-kDa fragment derives from the 45-kDa one, and the cleavage that generates it must occur within a disulfide loop (Fig. 6). The two inhibitory antibodies LJ-P3 and LJ-Ibl1, unlike the noninhibitory antibody LJ-Ibl10, failed to react with GPIb or GPIb fragments maintained under reducing conditions. Therefore, intrachain disulfide bonds within the NH2-terminal region play an important role in providing the appropriate conformation of epitopes corresponding to antibodies that block the von Willebrand factor-GPIb interaction. It remains to be demonstrated whether the same applies to the function of the von Willebrand factor-binding domain itself.

It is also noteworthy that only the 45-kDa trypsin fragment, but not the 35-kDa one, reacted with the noninhibitory antibody LJ-Ibl10. Thus, the epitope recognized by LJ-Ibl10 can be localized with certainty in the region representing the portion of the 35-kDa S. marcescens protease-generated fragment extending beyond the 35-kDa trypsin-generated fragment (Fig. 6). In fact, these two fragments exhibited the same NH2 terminus, but only the 38-kDa polypeptide reacted with the antibody.

All three enzymes used in these studies generated, among others, fragments of approximately 127 and 92 kDa. The 127-kDa fragments, which may differ slightly from one another, are likely to derive from cleavages within a protease-sensitive region corresponding to the area where a cleavage by the platelet calcium-dependent protease results in the generation of glycocalcin (Fig. 6). All these "glycocalcin-like" molecules reacted with the three monoclonal antibodies used, including the two inhibitory ones. Thus, they are likely to have the same NH2 terminus as glycocalcin (the same as the intact α-chain) and comprise the von Willebrand factor-binding domain.

The fragments of approximately 92 kDa were found to be different from each other, but exhibited the common property of having the NH2-terminal sequence distinct from that of the α-chain. This was established for the S. marcescens protease-generated fragment because it did not react with antibody LJ-Ibl10 and therefore had no relationship with the 38-kDa fragment having the same NH2 terminus as the α-chain (Fig. 6). The 92-kDa fragment generated by trypsin, on

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Supplemental Material:

von Willebrand Factor Binding Domain of Platelet Glycoprotein Ib Glycoprotein Ib

Experimental Procedures

von Willebrand factor interaction was performed as previously described. 9 Aggregation was measured in the aggregometer (Chrono-log Corp., Haverton, PA). Aggregates were induced by the indicated agonists. (1984) Gene Anal. Technol. 1, 5–8

Antigenic analysis of purified von Willebrand factor was performed according to the method of Fakhouri et al. 31

Studies were performed by mixing washed platelets (1 x 10^9 cells/ml) with protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) or protein G-Sepharose (Sigma Chemical Co., St. Louis, MO) at 1 ml of Sepharose/ml of platelets. The mixture was incubated 30 minutes at 37°C and the platelets were recovered by centrifugation.

The binding of monoclonal IgG and of von Willebrand factor inactivator dependent binding to platelets has been previously described in detail. Two platelet fractions were washed with the antibody, density gradient techniques of Walsh et al. 33

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von Willebrand Factor-GPIIb Interaction

Immunoblotting. The procedure followed was that described by Twacht et al.3. "Rabbit" solution (see above) was used for washing the nitrocellulose membranes. The second antibody used (rabbit anti-mouse IgG) was labeled with 125I. Only L2-165 reacted with denatured GPIIb. The use of L2-F3 and L2-151 was attempted, but no protein band could be visualized.

Sequence analysis. The purified GPIIb, or proteinase digests, were run on 10% SDS-polyacrylamide gels, dried, and rehydrated over a 10% phosphoric acid (Sigma Chemical Co.) solution. After electrophoresis and staining, the bands were excised from the gel and digested with 25% acetic acid (Sigma Chemical Co.) and 3 M HCl for 30 min at 100°C. The digests were analyzed by amino acid analysis on a Beckman 120C amino acid analyzer.

RESULTS

Characterization of the purified GPIIb complex. Analysis by SDS-PAGE demonstrated that the purified GPIIb preparations consisted of a 170 kDa polypeptide, as determined under reducing conditions, that yielded a component of 140 kDa in non-reduced gels and one 128 kDa in non-reduced gels after reduction of disulfide bonds (Figure 7). Both components were stained with Coomassie blue and both were labeled with 125I. The 125I-labeled polypeptides were subjected to Edman degradation in a gas phase sequencer (Applied Biosystems, Foster City, CA, model 470A). The results were compared to Edman degradation of a GPIIb polypeptide in a GPIIb polypeptide standard (Peptron X-2 column, E.I. du Pont de Nemours) following the manufacturer's instructions.

![Image]

**Figure 7.** Analysis of purified GPIIb on 5-15% gradient SDS-PAGE. Sample containing approximately 100 micrograms of purified GPIIb were analyzed on unreduced (NR) or reduced (RD) gels stained with Coomassie brilliant blue R-250 or periodic acid-Schiff (PAS) reagent. The autoradiography of 125I-labeled GPIIb (inset), analyzed under reducing conditions, is also shown. Arrows indicate the location of major bands with the corresponding apparent molecular mass. GPIIb appears as a 170 kDa band on unreduced gels, which shifts to a lower molecular mass (140 kDa) when analyzed under reducing conditions. GPIIb is unreduced and reduced GPIIb show a identical migration pattern. The bands were excised from the gel and digested with 25% acetic acid (Sigma Chemical Co.) and 3 M HCl for 30 min at 100°C. The digest were analyzed by amino acid analysis on a Beckman 120C amino acid analyzer.

**Table I.** Amino acid composition of GPIIb. Values in parentheses are derived from reference 48 and 5 and are for comparison. The abbreviation n.d. indicates that a given residue could not be detected under the conditions used.

| Amino Acid | GPIIb α-chain | GPIIb β-chain |
|------------|---------------|---------------|
| Asp/Asn    | 7.8 (6.3)     | 7.9 (6.3)     |
| Thr        | 10.4 (8.6)    | 10.4 (8.6)    |
| Ser        | 7.5 (6.3)     | 7.5 (6.3)     |
| Gly/Ala    | 9.7 (8.5)     | 9.7 (8.5)     |
| Phe        | 7.0 (6.3)     | 7.0 (6.3)     |
| Val        | 6.9 (5.8)     | 6.9 (5.8)     |
| Leu        | 6.9 (5.8)     | 6.9 (5.8)     |
| Ala        | 9.4 (7.5)     | 9.4 (7.5)     |
| Ile        | 9.4 (7.5)     | 9.4 (7.5)     |
| Pro        | 1.0 (0.4)     | 1.0 (0.4)     |
| Tyr        | 3.4 (2.4)     | 3.4 (2.4)     |
| His        | 3.4 (2.4)     | 3.4 (2.4)     |
| Arg        | 3.4 (2.4)     | 3.4 (2.4)     |
| Lys        | n.d. (n.d.)   | n.d. (n.d.)   |

The α-chain and β-chain of GPIIb were isolated by electrophoresis from polyacrylamide gels. After electrophoresis under denaturing and reducing conditions. The amino acid composition was determined and is shown in Table 1. Our results are in excellent agreement with previously reported values.

Table II. Properties of the monoclonal antibodies directed against GPIIb. Subtypes of IgG were determined by precipitation using monospecific rabbit F(ab')2. Saturation constant (Ks) and number of binding sites were compared to those of standard type analysis, using the program Ligrand.20

| Antibody Subtype | Ks (nM) | Inhibition of binding to plates per platelet | VOH binding to platelets |
|------------------|---------|-----------------------------------------------|-------------------------|
| L2-F3            | 4.7     | 12.2                                         | Incomplete              |
| L2-JH1           | 9.0     | 6.7                                          | Complete                |
| L2-JH10          | 31.2    | 2.49                                         | Absent                  |

L2-F3, L2-JH10, and L2-JFH3, partially blocked ristocetin-induced platelet aggregation as well as von Willebrand factor (VWF)-mediated enhancement of platelet aggregation (Figure 8). The antibodies had the same effect on platelet aggregation induced by collagen or ADP, on fibrinogen binding induced by ADP.

![Image]

**Figure 8.** Effect of antibodies on von Willebrand platelet-platelet interaction. Upper panel. Washed platelets (1 x 10^8/ml) were mixed with monoclonal IgG (at the concentrations indicated) and ristocetin (1 mg/ml) for 1 min at 22-25°C. [125I]Fv' (10 ng/ml) was then added to the mixture and incubated for 5 min. The binding was measured as described. Lower panel Plasma- rich plasma (1 x 10^8/ml) was stirred at 1,000 rpm. In the aggregometer cuvette, 10% and 10% of the corresponding bands was the same under reducing and non-reducing conditions (Figure 9). Several other peptides with molecular mass < 25 kDa were also generated.

Characterization of proteolytic fragments generated by trypsin digestion. Treatment of radiolabeled GPIIb with trypsin resulted in the formation of a 65 kDa band. Upon analysis by SDS-PAGE under non-reducing conditions, demonstrated mobilities corresponding to 65 kDa and 85 kDa (Figure 10). These bands were excised from the gel and digested with 25% acetic acid (Sigma Chemical Co.) and 3 M HCl for 30 min at 100°C. The digest were analyzed by amino acid analysis on a Beckman 120C amino acid analyzer.

![Image]

**Figure 9.** Protonic clearance pattern of GPIIb treated with Bovine serum protease. [125I]GPIIb was incubated with the enzymes for the indicated periods of time. Aliquots of the digest (1 x 10^6 rpm) were then analyzed by 5-15% SDS-PAGE followed by autoradiography. The apparent molecular mass of the fragments is produced in the indicated. Intact GPIIb appears as high molecular weight aggregates which calculated under non-reducing conditions. GPIIb α-chain and glycoprotein are electrophoresed very close to the other in this gel system.

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The α-chain and β-chain of GPIIb were isolated by electrophoresis from polyacrylamide gels. After electrophoresis under denaturing and reducing conditions. The amino acid composition was determined and is shown in Table 1. Our results are in excellent agreement with previously reported values.
von Willebrand Factor-GPIb Interaction

Figure 10. Time course of GPIb digestion by trypsin. [125I]GPIb was digested with TPCK-trypsin for the indicated periods of time. Aliquots of digest (1.3 x 10^8 cpm) were then analyzed by 5-12% gradient SDS-PAGE under nonreducing (N-R) or reducing (R) conditions. The apparent molecular mass of relevant polypeptides is indicated. The 96 kDa and 84 kDa fragments are not well separated in this gel system, but can be differentiated by immunoblotting (see Fig. 2).

Characterization of GPIb fragments generated by Staphylococcus aureus V8 protease (SAP). For these studies, purified GPIb that had been labeled with either [125I] or [125I] was used. After digestion with SAP, the fragments were analyzed by SDS-PAGE followed by direct autoradiography or fluorography. The results of gels analyzed under reducing and nonreducing conditions were identical and only the former are presented (Figure 11). At short incubation times, polypeptides of apparent 127 kDa and 93 kDa were evident both in tritiated preparations and in trinitiated preparations. Incubation for longer periods resulted in the appearance of a 60 kDa species well within the trinitiated preparations (Figure 11). Concurrently, a 60 kDa species well within the trinitiated preparations increased in intensity with time. This band also exhibited a progressive slight increase in mobility (Figure 11). Additional polypeptides corresponding to 70 kDa, 52 kDa, 45 kDa and 40 kDa were also generated together with smaller bands whose mobility corresponded to between 14 kDa and 24 kDa (Figure 11).

Figure 11. Time course of GPIb digestion by Staphylococcus aureus V8 protease. Labeled GPIb was incubated with the enzyme for the indicated periods of time. Aliquots of [75S]GPIb (1.5 x 10^8 cpm) or [125I]GPIb (1 x 10^6 cpm) were analyzed by 5-12% gradient SDS-PAGE, under reducing conditions, followed by fluorography. Arrows indicate the apparent molecular mass of fragments. Note that the 127-, 96-, and 84-kDa fragments are rich in carbohydrate, as judged by the intense labeling with [75S].