Identification of a Site in the α Chain of Platelet Glycoprotein Ib That Participates in von Willebrand Factor Binding*

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The binding of von Willebrand factor (vWF) to the platelet receptor glycoprotein (GP) Ib-IX complex is a key event in hemostasis and may participate in the development of thrombotic vascular occlusion. We present here evidence that residues Ser231-Tyr279 in the GP Ib α-chain participate in this function. Initial studies suggested that the modality of vWF interaction with GP Ib depended on the conditions used for induction of binding, either in the presence of ristocetin, or botrocetin, or with asialo-vWF. In fact, only the 45-kDa amino-terminal fragment of GP Ibα inhibited the vWF-GP Ib interaction under all conditions tested, while the 84-kDa macroglycopeptide was significantly effective only in the presence of ristocetin. Moreover, the 45-kDa fragment with reduced disulfide bonds still inhibited ristocetin-induced binding but had no effect, at the concentrations tested, on botrocetin-mediated or direct asialo-vWF binding. In order to localize in more detail the functional site, the entire sequence of the 45-kDa fragment was reproduced in 27 overlapping synthetic peptides that were then used in inhibition of binding assays. This led to the identification of a linear GP Ibα sequence (residues Ser231-Tyr279) that effectively inhibited platelet interaction with vWF mediated by ristocetin and, at higher concentration, also by botrocetin. A shorter peptide overlapping with the longer one (residues Gly271-Glu285) was the second most active inhibitory species. This region of the molecule contains several residues with a high surface probability index, as expected for a site involved in ligand binding. Thus, while native conformation of GP Ibα appears to be important for optimal interaction with vWF, the results obtained with short synthetic peptides may help in defining the amino acid residues participating in this essential function.

The membrane glycoprotein (GP) Ib-IX complex, constituted by the equimolar noncovalent association of GP Ib and GP IX (1), is a major receptor of platelets which, by interacting with the adhesive protein von Willebrand factor (vWF) (2-4), mediates platelet adhesion at sites of vascular injury (5). This event is crucial in the process of normal hemostasis, particularly under flow conditions characterized by high wall shear rate (6), and may be an important factor in the pathogenesis of thrombotic vascular occlusion (7, 8). Understanding the chemical nature of this interaction is, therefore, of theoretical as well as practical importance, and may lead to the formulation of new anti-thrombotic strategies. To this end, considerable efforts have been devoted to elucidating the structure and structure-function relationships of the GP Ib-IX complex. Among the results already attained are the definition of the primary sequences of the α (9, 10) and β (11) chains of GP Ib, and the demonstration that the amino-terminal domain of the α-chain, the 45-kDa fragment comprising residues 1-293 (10), contains a vWF-binding site that functions independently of other structures in the complex (12).

The molecular mechanisms responsible for vWF binding to GP Ib in vivo remain to be elucidated. In fact, while vWF and platelets coexist in the circulation without any interaction normally occurring, GP Ib acts as a competent receptor for vWF exposed onto the subendothelium (13-15). This may be the result of specific functional conformations acquired by vWF associated with components of the vessel wall. Alternatively, or in addition, it may be the consequence of changes in the GP Ib receptor itself induced by unidentified agonist substances and/or rheological conditions, as demonstrated following exposure of platelets to high shear stress (16). Experimentally, vWF-GP Ib interaction can be demonstrated in the presence of certain molecules, like the glycopeptide ristocetin (17, 18) or the protein botrocetin (19), or following chemical manipulation of the vWF molecule itself by removal of terminal sialic acid from carbohydrate side chains (20). The pathophysiological counterparts of these experimental conditions, if any, remain unknown, and only a detailed definition of the mechanisms involved may eventually permit to relate in vitro findings to pertinent in vivo functions. Until then, it seems appropriate to assume that the different experimental conditions tested may reflect distinct pathophysiological pathways leading to vWF-GP Ib interaction. Thus, it is important to know whether vWF binding to GP Ib in the different assay systems mentioned above involves similar or distinct functional domains in the receptor molecule.

With these considerations in mind, we have undertaken the studies reported here aimed at identifying functionally relevant sequences in the 45-kDa domain of GP Ibα, the region of the GP Ib-IX complex containing a major vWF binding site (12). The assays employed, based on inhibition of vWF
binding to platelets measured in the presence of ristocetin, botrocetin, or with asialo-vWF, indicate that the sequence comprised between residues Ser251-Tyr270 includes a site that is negatively charged, recognized by synthetic peptides containing the sequence Ser251-Cys252-Lys253-Asp254. The inhibitory activity of synthetic peptides was evaluated in the same manner, with peptides diluted in the assay mixture to give the desired final concentration.

**EXPERIMENTAL PROCEDURES**

**Purification of Glycocalcin and Preparation of Its Two Major Tryptic Fragments**—Glycocalcin was purified from outdated platelet concentrates by affinity chromatography on wheat germ agglutinin and a specific anti-GP Iba monoclonal antibody, as previously reported in detail (12). The 45-kDa amino-terminal fragment, representing the outermost extracytoplasmic portion of GP Iba, and the carboxyl-terminal carbohydrate-rich half of the molecule, also known as macroglycopeptide, were isolated by high-performance liquid chromatography (HPLC) following tryptic digestion of purified glycocalcin, as previously described (12). No cross-contamination of the two fragments could be demonstrated using a sensitive radioimmunoassay (12). Reduction of disulfide bonds with dithiothreitol and iodoacetamide were achieved under conditions previously described (19, 91). Purified proteins were stored in a buffer composed of 20 mM HEPES and 150 mM NaCl, pH 7.4 (Hepes buffer), at -70°C until used.

**Preparation and Purification of Synthetic Peptides**—All peptides used in these experiments were synthesized using the method of simultaneous multiple peptide synthesis which has been described in detail elsewhere (22). After synthesis, they were analyzed by reversed-phase HPLC (Perkin-Elmer) using a 1 × 25-cm Vydac C18 column (TP Silica; pore diameter 300 Å) with a 0-60% acetonitrile linear gradient in 0.1% trifluoroacetic acid. The peptides were then purified using the best conditions suggested by the analytical chromatography. Amino acid compositions were determined with an automatic amino acid analyzer (LKB) after a 24-h hydrolysis in 6 M HCl at 110°C. Stock solutions of each peptide were prepared at a concentration of 2.5 mM in distilled water. The required amount of peptide was weighed before use, one-tenth volume of 10-fold concentrated HEPES buffer was added to each stock solution of peptide. The pH of the solution was checked and, if necessary, adjusted to 7.4 with titrated amounts of 1 or 0.1 N NaOH or HCl. In some cases, peptides were dissolved directly in HEPES buffer. The solutions were centrifuged at 12,000 × g for 10 min. The concentration of peptides that appeared to be not completely soluble, the concentration in the supernatant was tested with a colorimetric assay (Fierce Chemical Co.). A few of the peptides prepared for these studies could not be tested in the assays described below because they were poorly soluble in aqueous buffers.

**Measurement of vWF Binding to GP Ib-IX Complex**—In previous publications we have described in detail the methods used for the purification of vWF (4), the preparation of its desialylated derivative (asialo-vWF) (20, 23), and the radioiodination of these molecules (4, 23). We have also described previously the assays employed for measuring the binding of vWF to washed platelets in the presence of ristocetin (4) or botrocetin (24), and the direct (without addition of a crosslinking agent) binding of asialo-vWF to platelets in platelet-rich plasma (25). In these studies, vWF binding to platelets was measured by means of a competitive binding assay performed at 4°C in distilled water. The required amount of peptide was weighed and dissolved in aqueous buffers.

**Inhibition of vWF Binding to Platelets by Glycocalcin**—Inhibitory activity of synthetic peptides was evaluated according to a procedure utilized and described in a previous publication (12). The inhibitory effect of synthetic peptides was evaluated in the same manner, with peptides diluted in the assay mixture to give the desired final concentration.

**RESULTS**

The ristocetin-mediated platelet binding of vWF was inhibited by glycocalcin as well as its two tryptic fragments, the 45-kDa amino-terminal domain and the carbohydrate-rich macroglycopeptide representing the carboxyl-terminal half of the extracytoplasmic domain of GP Iba. The concentration of these different molecules necessary to inhibit 50% of binding (IC50) was between 1 and 8 μM and it was lower for intact glycocalcin than for the two fragments (Fig. 1). These IC50 values were higher than the dissociation constant (Kd) measured for ristocetin-dependent vWF binding to intact platelets (1.7–8.7 × 10-9 M, range of five determinations; molar concentrations calculated assuming a molecular mass of 275 kDa for the vWF subunit). The fact that glycocalcin had greater inhibitory activity than its two fragments supports the concept that elements in both the amino-terminal domain and the macroglycopeptide region may be involved in ristocetin-dependent vWF-GP Ib interaction.

In contrast, vWF binding to platelets mediated by botrocetin was inhibited equally well by glycocalcin and the 45-kDa fragment. The calculated IC50 was in the order of 10 nM, namely 100-fold lower than for ristocetin-mediated binding (Fig. 2). Yet, the Kd value estimated for botrocetin-dependent vWF binding to intact platelets was 2.21–10 × 10-9 M (range of five determinations), thus of the same order of magnitude as measured for ristocetin-mediated binding. Although the macroglycopeptide fragment of glycocalcin was also apparently effective in inhibiting botrocetin-mediated binding, its
FIG. 2. Effect of glycocalicin and its two tryptic fragments on botrocetin-dependent vWF binding to platelets. These experiments were conducted essentially as described in the legend to Fig. 1, with the only exception that purified botrocetin (5 μg/ml) was used instead of ristocetin. Note that the concentrations of glycocalicin and 45-kDa fragment used to obtain a dose-response curve are lower than those shown in Fig. 1.

FIG. 3. Effect of glycocalicin and its two tryptic fragments on direct platelet binding of asialo-vWF. In this experiment, conducted following the principles described in the legend to Fig. 1, 125I-labeled asialo-vWF, with or without various competing fragments, was added at a final concentration of 20 μg/ml to a suspension of platelet-rich plasma containing trisodium citrate (11 mM). Final platelet count was 2 × 10⁷/ml. EDTA was then added to a final concentration of 0.8 mM, and the incubation continued for 30 min at 37 °C before separation of platelet-bound from free ligand. Note that, unlike in the case of the ristocetin-dependent binding shown in Fig. 1, glycocalicin and its 45-kDa amino-terminal fragment have similar inhibitory activity, while the 84-kDa fragment is without inhibitory effect. The apparent increase in platelet-associated asialo-vWF in the presence of higher concentrations of 84-kDa fragment (Fig. 3) may have been caused by aggregate formation with asialo-vWF.

Reducation of disulfide bonds followed by S-carboxymethylation with iodoacetamide had no effect on the ability of the 45-kDa fragment of glycocalicin (the only domain of the molecule containing cysteine residues) to inhibit ristocetin-mediated vWF binding to platelets (compare Fig. 4 with Fig. 1). In contrast, the reduced and alkylated fragment had minimal inhibitory effect on binding mediated by botrocetin or on the direct binding of asialo-vWF, even when tested at concentrations corresponding to those of nonreduced fragment resulting in nearly complete inhibition (compare Fig. 4 with Figs. 2 and 3).

The results of the experiments described to this point indicated that the amino-terminal 45-kDa domain of glycocalicin may contain a functional site participating in vWF-GP Ib interaction. In order to identify with more precision this functional domain in the α-chain, overlapping synthetic peptides encompassing the entire amino acid sequence of the 45-kDa fragment were tested for their ability to inhibit vWF binding to platelets (Fig. 5). Because of the significant differ-
and 25) inhibited the ristocetin-dependent vWF binding to platelets by more than 50%, but only one of these (peptide a preliminary screening assay at the final concentration of by more than 30% under the conditions of the screening assay tides, mostly 15 amino acid residues in length, were tested in dependent vWF binding assays. Twenty-six different pep-ences described above, evaluation of the inhibitory activity was performed both in ristocetin-dependent and botrocetin-dependent vWF binding assays. Twenty-six different pep-ptides, mostly 15 amino acid residues in length, were tested in a preliminary screening assay at the final concentration of 500 μmol/liter. Three distinct peptides (identified as 9, 14, and 25) inhibited the ristocetin-dependent vWF binding to platelets by more than 50%, but only one of these (peptide 25) was also capable of inhibiting botrocetin-mediated binding by more than 30% under the conditions of the screening assay (Fig. 5).

Peptide 25, which was more effective than any of the others in two different experimental systems employed to measure vWF binding to GP Ib, was used for additional studies performed to characterize in more detail the mechanisms of its inhibitory activity. The average IC<sub>50</sub> for inhibition of ristocetin-related to the concentration of vWF in the assay, as shown both for ristocetin-dependent (Fig. 7) and botrocetin-dependent binding (Fig. 8). In the presence of a constant peptide concentration (250 μmol/liter), ristocetin-induced binding could be completely inhibited at the lower concentrations of vWF tested (Fig. 7), while botrocetin mediated vWF binding appeared to be effectively but only partially inhibited (Fig. 8).

FIG. 5. Effect of synthetic peptides on vWF binding to platelets. The top part of the figure shows the amino acid sequence of the amino-terminal region of GP Ibα, in single letter notation. T indicates the sites of tryptic cleavage that give origin to the 45-kDa domain (10, 33). Numerals above the sequence line indicate the first residue in a synthetic peptide sequence, and the same number below the sequence line indicates the last residue in that peptide. The heavy bar underlines the sequence of the longer peptide (29 residues) used in subsequent studies. The lower part of the figure displays in a bar graph the inhibitory effect of all the peptides tested on ristocetin-dependent (black bars) and botrocetin-dependent (hatched bars) vWF binding to platelet GP Ib-IX. Each peptide, used at a final concentration of 500 μmol/liter with a <sup>125</sup>I-vWF concentration of 2 μg/ml, is identified by the same numeral used in the top part of the figure. Note that ristocetin-dependent binding was inhibited by five groups of peptides (mainly those identified by numbers 3-4, 8-9, 14, 21, and 23-25), while botrocetin-dependent binding was significantly inhibited only by peptides 8 and 22-25.

These results suggest that ristocetin and botrocetin mediate vWF-GP Ib interaction through distinct mechanisms. A survey of the results presented in Fig. 5 revealed that the greatest inhibitory activity on vWF binding to GP Ib was associated with peptides partially overlapping with peptide 25 on its amino-terminal side. Moreover, the peptide with sequence Pro-Glu-Glu-Asp-Thr-Glu, corresponding to the last 5 carboxyl-terminal residues in peptide 25, had no inhibitory activity (not shown here), in agreement with the fact that peptide 26, overlapping in those 5 residues with peptide 25, was also inactive (Fig. 5). Consequently, we hypothesized that a longer peptide containing the first 10 residues of peptide 25 and an additional sequence on its amino-terminal side might prove a more effective inhibitor of the VWF-GP Ib interaction. In order to test this hypothesis, a longer peptide composed of 29 amino acids and corresponding to the sequence Ser<sup>251</sup>-Tyr<sup>279</sup> of the α-chain of GP Ib (Fig. 5) was synthesized and tested. This molecule inhibited ristocetin-mediated vWF binding to platelets with an IC<sub>50</sub> of 170 μmol/liter, less than half the corresponding value for peptide 25 (compare Figs. 6 and 9). Its effect on botrocetin-mediated binding, however, was not remarkably greater than that of peptide 25, with inhibition averaging 44% at a concentration of 500 μmol/liter (compare Figs. 6 and 9). Moreover, the direct binding of asialo-vWF was inhibited only by 23% (average of three
Fig. 7. Inhibitory effect of the synthetic peptide Gly\textsuperscript{271}-Glu\textsuperscript{280} on ristocetin-mediated binding as a function of vWF concentration. These experiments were performed with constant peptide concentration of 250 \textmu molar/liter and varying \textsuperscript{125}I-vWF concentrations; ristocetin was at 1 mg/ml. The top panel shows the values of residual binding calculated as described in the legend to Fig. 6; the lower panel shows the actual counts/min bound in the presence of HEPES buffer (columns with horizontal bars), anti-GP Ib antibody L3 Ib1 (black columns), or synthetic peptide (columns with diagonal bars). As expected, binding was a function of the indicated \textsuperscript{125}I-vWF concentration. The inhibitory effect of the peptide was more pronounced at lower vWF concentrations.

In experiments (when the peptide was at a concentration of 500 \textmu M, and no inhibitory activity was detected when lower peptide concentrations were tested. Since the “long peptide” used contains 1 cysteine residue (see Fig. 5), with the potential for dimerization, studies were conducted to characterize the nature of the predominant molecular species in solution. Analysis by reversed-phase high performance liquid chromatography demonstrated that dimers represented the prevalent form after incubation at the temperature, pH, and for the time used in the inhibition of vWF binding assay.

DISCUSSION

The present studies identify a discrete sequence in the amino-terminal domain of the GP Ib\textalpha chain that appears to be part of a vWF-binding site, as judged by its ability to inhibit the vWF-GP Ib interaction. The residues involved in this function are located between amino acids Ser\textsuperscript{261}-Tyr\textsuperscript{279} in the region of the molecule near the carboxyl-terminal end of the 45-kDa extracytoplasmic domain and proximal to the carbohydrate-rich domain (12, 26). This segment of sequence is located on the carboxyl-terminal side of the region corresponding to the leucine-rich repeats (residues 29–193 in GP Ib\textalpha) that are homologous to those found in leucine-rich glycoprotein and GP Ib\textbeta (9–11). It appears, therefore, that the function of binding vWF involves structural elements unique to GP Ib\textalpha and not present in other homologous proteins. Twelve of the residues between positions 251 and 279, corresponding to the “long” peptide, and 13 of the 15 residues in peptide 25 have a surface probability index greater than four, as calculated according to Emini et al. (27), suggesting that in the folded polypeptide they may indeed have a favorable orientation to participate in a ligand-binding site. Moreover, the localization of this functional domain in the 45-kDa amino-terminal domain of GP Ib\textalpha is supported by the observation that only this fragment exhibited inhibitory activity in three independent experimental assays used to probe the vWF-GP Ib interaction. Such conclusion does not exclude the possibility that the macroglycopeptide region of the molecule, which was effective in inhibiting only ristocetin-induced vWF
vWF Binding Site in GP Ibα

binding, may play an accessory role under certain functional circumstances.

The fact that a limited region of GP Ibα is capable of inhibiting platelet binding of vWF in the presence of either ristocetin or botrocetin suggests that both substances induce vWF-GP Ib interaction through at least partially common sites in the receptor and ligand molecules. This concept is supported by the finding that the same anti-GP Ibα monoclonal antibody inhibited binding induced both by ristocetin and botrocetin (compare Figs. 7 and 8). In spite of these analogies, however, it is apparent that differences must exist in the molecular mechanisms involved in the binding-promoting activity of ristocetin and botrocetin. This is convincingly demonstrated by the following observations. The isolated 45-kDa amino terminal of GP Ibα with intact disulfide bonds exhibited a much lower IC50 when tested in botrocetin-dependent rather than ristocetin-dependent binding. Moreover, alteration of the native conformation of the 45-kDa fragment following reduction of intrachain disulfide bonds resulted in significant loss of inhibitory activity on vWF binding mediated by botrocetin, as well as on the direct binding of asialo-vWF, but had less effect on inhibition of ristocetin-induced binding. These variations in the extent of inhibition by distinct GP Ibα fragments cannot be explained by differences in the KD of the vWF-platelet of GP Ib interaction under the various experimental conditions tested, since a constant amount of vWF was used in each assay when comparing different fragments, and this was of the same order of magnitude as the experimentally determined KD value for any specific assay. Finally, while a synthetic peptide with linear sequence of GP Ibα could completely block, when a 4-fold lower concentration of vWF than used in ristocetin- or botrocetin-dependent binding. This may explain the relatively low inhibitory activity of the longer peptide tested, particularly of its disulfide-linked dimeric form, might result from the presence of additional relevant residues, possibly with more favorable spatial orientation in adjacent chains. As mentioned above, variations in the extent of inhibition observed with various peptides in ristocetin-dependent as compared to botrocetin-dependent binding may be due to specific mechanisms operating in each of these two distinct experimental conditions, which appear to have different susceptibility to loss of native conformation.

Binding mediated by ristocetin, unlike that mediated by botrocetin or the direct binding of asialo-vWF, may involve multiple sites located both in the 45-kDa amino-terminal half of GP Ibα and in the carboxyl-terminal carbohydrate-rich domain. Nevertheless, since the mechanisms by which these different sites may participate in the receptor function of GP Ibα remain obscure at the moment, it is not possible to exclude that the inhibitory effect of some of the synthetic peptides, or even of the macroglycopeptide fragment of glycocalcin, may pertain not only to vWF binding in a proper sense, but also to the likely, although not established, interactions between ristocetin and platelets and/or vWF (18, 31, 32). In some cases, for instance, inhibition may have been the consequence of forming a complex with ristocetin, thereby affecting its ability to mediate vWF binding to platelets, rather than signify involvement of a certain GP Ibα sequence in interacting with vWF. This possibility, however, does not seem relevant for those peptides, including the sequence Ser251-Tyr279, whose inhibitory effect on vWF binding to platelets was not limited to ristocetin-dependent assays. In fact, our decision to synthesize peptides representing the sequence of the 45-kDa fragment, and not of the 84-kDa fragment, was dictated by the observation that only the former had inhibitory effect independent of the nature of the substance used to induce platelet binding of native vWF, suggesting that it might contain the biologically relevant functional site.

In conclusion, the present studies provide evidence that amino acid residues participating in the vWF binding activity of the GP Ib-IX complex are contained in a limited sequence of the α-chain, between residues Ser251-Tyr279. Considerable additional work is now required to define the overall structure of this functional site.

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