possibility existed that it might function in the same manner that P_i would if mechanism I were operative. Again we found the substrate analogue unable to participate in the exchange reaction.

In conclusion, our studies show that *B. symbiosus* PPDK catalyzes two partial reactions: (1) ATP + P_i + E $\rightleftarrows$ AMP + PP_i + EP and (2) EP + pyruvate $\rightleftarrows$ E + PEP. The first partial reaction may proceed via a covalent pyrophosphoryl-enzyme intermediate as proposed earlier (Milner et al., 1978), or it may proceed via the intermediacy of ADP. At the present time studies that may allow us to distinguish between these possible chemical mechanisms are under way.

ACKNOWLEDGMENTS

We thank Drs. Nelson Phillips and Harland Wood for supplying us with their own *B. symbiosus* strain for comparison to the ATCC strain and for testing the ATP $\rightleftarrows$ AMP exchange reaction using PPDK prepared in our laboratory.

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Ristocetin-Dependent Reconstitution of Binding of von Willebrand Factor to Purified Human Platelet Membrane Glycoprotein Ib–IX Complex†

Michael C. Berndt,* Xiaoping Du, and William J. Booth
Research Centre for Thrombosis and Cardiovascular Disease, Department of Medicine, University of Sydney, Westmead Hospital, Westmead, NSW 2145, Australia

Received May 19, 1987; Revised Manuscript Received August 25, 1987

ABSTRACT: Whether the human platelet membrane glycoprotein (GP) Ib–IX complex is the receptor for ristocetin-dependent binding of von Willebrand factor (vWF) has been examined by reconstitution with the purified components using a solid-phase bead assay. Purified GP Ib-IX complex was bound and orientated on the beads via a monoclonal antibody, FMC 25, directed against the membrane-associated region of the complex. Specific binding of ^{125}I-labeled vWF to the GP Ib–IX complex coated beads was strictly ristocetin dependent with maximal binding occurring at ristocetin concentrations $\geq$1 mg/mL. Ristocetin-dependent specific binding of ^{125}I-labeled vWF was saturable. The observed binding was specific to the interaction between vWF and the GP Ib–IX complex since there was no ristocetin-dependent specific binding of vWF if the physicochemically related platelet membrane glycoprotein, GP IIb, was substituted for the GP Ib–IX complex in a corresponding bead assay. Further, neither bovine serum albumin nor other adhesive glycoproteins, such as fibrinogen or fibronectin, specifically bound to the GP Ib–IX complex in the presence of ristocetin. Ristocetin-dependent binding of vWF to platelets and to GP Ib–IX complex coated beads was inhibited by monoclonal antibodies against a 45 000 molecular weight N-terminal region of GP Ib but not by monoclonal antibodies directed against other regions of the GP Ib–IX complex. Similar correspondence between platelets and purified GP Ib–IX complex with respect to the ristocetin-dependent binding of vWF was obtained with anti-vWF monoclonal antibodies. The combined data indicate that the GP Ib–IX complex is the receptor involved in the ristocetin-dependent binding of vWF and that the specificity of this interaction is identical with that for the ristocetin-dependent binding of vWF to platelets.

The initial event in hemostasis in response to vascular injury involves the adhesion of platelets to the exposed subendothelial matrix. At high shear flow, the adhesion of platelets becomes von Willebrand factor (vWF)[1] dependent and involves a specific vWF receptor on the human platelet membrane (Chesterman & Berndt, 1986). In normal circulation, vWF does not bind to its platelet receptor. This interaction in vivo requires the prior binding of vWF to the subendothelial matrix

† This investigation was supported by Grant 6K14443 from the National Health and Medical Research Council of Australia. M.C.B. is the recipient of a Wellcome Australian Senior Research Fellowship.

[1] Abbreviations: EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; GP, glycoprotein; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane; vWF, von Willebrand factor.

0006-2960/88/0427-0633$01.50/0

(Sakariassen et al., 1979). Ristocetin, a glycopeptide antibiotic isolated from *Nocardia lurida*, mimics the active constituent(s) of the exposed vessel wall and causes the binding of human vWF to human platelets (Kao et al., 1979; Schneider-Trip et al., 1979) and platelet agglutination (Howard & Firkin, 1971).

There is now strong evidence that the human platelet membrane glycoprotein (GP) Ib–IX complex contains the binding domain for vWF involved in platelet adhesion and in the ristocetin-dependent agglutination of platelets (Berndt & Caen, 1984). Previous evidence from our laboratory indicates that GP Ib and GP IX exist as a heterodimer complex in the intact platelet membrane (Du et al., 1987). Both glycoproteins remain tightly associated after detergent solubilization since both GP Ib and GP IX copurify, coimmunoprecipitate with either anti-GP Ib or anti-GP IX monoclonal antibodies, and occur in the same immunoprecipitin arc in crossed immunoelectrophoresis (Berndt et al., 1985a,b). GP Ib has an apparent molecular weight on SDS–polyacrylamide gels of 170 000 and consists of two disulfide-linked subunits, GP Ib_α (mol wt 135 000) and GP Ib_β (mol wt 25 000). GP IX has an apparent molecular weight of 22 000 under both nonreducing and reducing conditions (Berndt et al., 1985b). The α-chain of GP Ib contains a central macroglycopeptide core (Wicki & Clemetson, 1985; Handa et al., 1986) with an apparent molecular weight of 118 000 (Okumura et al., 1976). At each end of the macroglycopeptide core is a proteolytically sensitive domain (Handa et al., 1986). Cleavage at both sites generates an approximately 45 000 molecular weight peptide tail region, macroglycopeptide, and an approximately 25 000 molecular weight fragment that remains membrane-associated, disulfide-linked to the β-subunit, and complexed with GP IX (Berndt et al., 1985a; Wicki & Clemetson, 1985; Handa et al., 1986). Polyclonal and monoclonal antibodies directed against the 45 000 molecular weight peptide tail region of the α-chain of GP Ib completely block the ristocetin-dependent interaction between vWF and platelets (Wicki & Clemetson, 1985; Handa et al., 1986).

In contrast, Stricker et al. (1985) have recently described a patient with an autoantibody against a 210 000 molecular weight platelet membrane protein. $(Fab)_2$ fragments of this antibody completely blocked the ristocetin-induced agglutination of human platelets in the presence of vWF. These data suggest that the 210 000 molecular weight protein may serve as a receptor for vWF. Alternatively, the antibody against the 210 000 molecular weight protein could interfere with binding of vWF to the GP Ib–IX complex. Conversely, the polyclonal and monoclonal antibodies directed against the peptide tail region of GP Ib could inhibit the ristocetin-dependent binding of vWF by sterically interfering with the 210 000 molecular weight protein. As precedent for this, we have described a monoclonal antibody, SZ 2, directed against the glycocalicin region of GP Ib (macroglycopeptide plus peptide tail) that inhibited both ristocetin- and collagen-induced platelet aggregation (Ruan et al., 1987). The inhibition of collagen-induced platelet aggregation by SZ 2 was shown to be due to steric interference by antibody with an adjacent collagen receptor.

Recently, we described a procedure for the purification of intact GP Ib–IX complex from human platelet membranes under mild conditions (Berndt et al., 1985b). We have now definitively examined whether the human platelet membrane GP Ib–IX complex is the receptor for the ristocetin-dependent binding of vWF by reconstitution with the purified components using a solid-phase bead assay. The combined data indicate that the GP Ib–IX complex is the receptor involved in the ristocetin-dependent binding of vWF and that the specificity of this interaction is identical with that for the ristocetin-dependent binding of vWF to platelets.

Materials and Methods

Bovine serum albumin (fraction V) was purchased from Sigma, St. Louis, MO; sodium periodate (Univar) from Ajax Chemicals, Sydney, Australia; Triton X-100 and Chloramine T from BDH, Poole, England; and lactoperoxidase from Boehringer-Mannheim, Sydney, Australia. Immunobead reagent, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDAC), and Affi-Gel 10 were purchased from Bio-Rad, Richmond, CA. Sodium [^{125}I]iodide was purchased from New England Nuclear, Boston, MA; sodium [^{3}H]borohydride from Amersham, Sydney, Australia; and protein A–Sepharose from Pharmacia, Uppsala, Sweden. Ristocetin sulfate was purchased from Lundbeck, Copenhagen, Denmark. Fibrinogen and fibronectin were purified according to the procedures of Palascak and Martinez (1977) and Coller (1980), respectively. Albumin, fibrinogen, and fibronectin were ^{125}I-labeled with Chloramine T.

Monoclonal Antibodies. All monoclonal antibodies were of the immunoglobulin G (IgG) class and either have been previously described or were prepared according to standard hybridoma technology (Ruan et al., 1987). Several of the monoclonal antibodies were generous gifts: AP 1 from Dr. T. Kunicki, Milwaukee, WI; SZ 1, SZ 21, and SZ 22 from Dr. C. Ruan, Suzhou, China; FMC 18 and FMC 25 from Dr. H. Zola, Adelaide, Australia; and HuPlm1 from Dr. P. Thurlow, Melbourne, Australia. The remaining antibodies were raised and characterized in our laboratory in conjunction with Dr. K. Bradstock and A. Kabral. AK 1, AK 2, AK 3, SZ 1, AP 1, WM 23, and FMC 25 are all directed against the human platelet GP Ib–IX complex. Epitope mapping was performed by immunoprecipitation (Berndt et al., 1983) with trypsin-treated, tritium-labeled GP Ib–IX complex essentially as described by Handa et al. (1986). Briefly, purified, tritium-labeled GP Ib–IX complex ($\simeq$100 μg/mL) in 0.01 M Tris buffer, 0.15 M sodium chloride, 0.001 M EDTA, 0.1% (v/v) Triton X-100, and 0.02% (w/v) sodium azide, pH 7.4, was incubated with 5 μg/mL of trypsin for 4 h at 25 °C. The reaction was terminated by adding an equal volume of the above buffer containing 100 μg/mL soybean trypsin inhibitor, 0.2 mM phenylmethanesulfonyl fluoride, and 200 μg/mL leupeptin.

WM 18, SZ 21, SZ 22, and HuPlm1 are directed against the human platelet GP IIb/IIIa complex. WM 18 is directed against a complex-specific epitope, while SZ 22 is directed against GP IIb and HuPlm1 and SZ 21 are directed against GP IIIa. 2C9 and 3F8 are previously described monoclonal antibodies that are directed against distinct epitopes on the vWF molecule (Booth et al., 1984b). FMC 18 is an irrelevant monoclonal antibody directed against *Toxoplasma gondii* and was employed where appropriate as a control antibody.

Monoclonal IgG was purified from mouse ascites by protein A–Sepharose chromatography as previously described (Ruan et al., 1987) except for FMC 25 IgG, which was purified from ascites by performing two 0–40% ammonium sulfate fractionations. Purified monoclonal antibodies were stored in 0.01 M Tris buffer and 0.15 M sodium chloride, pH 7.4.

Purification and Labeling of Human Platelet Membrane Glycoprotein Ib–IX Complex and Glycoprotein IIb. The GP Ib–IX complex was purified as previously described (Berndt et al., 1985b). Tritium label was incorporated into the GP Ib–IX complex by the periodate-labeling procedure again as previously described (Du et al., 1987). GP IIb was purified

by successive immunoaffinity chromatography using the anti-GP IIb/IIIa complex-specific monoclonal antibody, WM 18, and the anti-GP IIIa monoclonal antibody, HuPlm1. Human platelet membranes were prepared and detergent-extracted as previously described (Berndt et al., 1985b) except that the extraction buffers contained 1 mM calcium chloride instead of 1 mM EDTA. The extract was loaded onto a 10 × 1 cm column of WM 18 coupled Affi-Gel 10 (2 mg of WM 18 IgG/mL of agarose). After thorough washing with the extraction buffer, 0.01 M Tris buffer, 0.001 M calcium chloride, 0.1% (v/v) Triton X-100, and 0.02% (w/v) sodium azide, pH 7.4, dissociated GP IIb and GP IIIa were eluted with 0.038 M Tris, 0.1 M glycine, 0.01 M EDTA, and 0.1% (v/v) Triton X-100, pH 8.7 (Kunicki et al., 1981). The eluted fractions were immediately neutralized by the addition of 0.5 volume of 0.5 M Tris and 0.1% (v/v) Triton X-100, pH 6.8, and dialyzed exhaustively against buffer A. Buffer A contained 0.01 M Tris, 0.15 M sodium chloride, 0.001 M EDTA, 0.1% (v/v) Triton X-100, and 0.02% (w/v) sodium azide, pH 7.4. The dialyzate was loaded onto a 10 × 1 cm column of HuPlm1-coupled Affi-Gel 10 (2 mg of HuPlm1/mL of agarose) equilibrated with buffer A. The flow-through consisted of pure GP IIb and was devoid of GP IIIa as judged by SDS–polyacrylamide gel electrophoresis. ^{125}I-Labeled GP IIb was similarly prepared by radioiodination of washed platelets by the lactoperoxidase method (Booth et al., 1984a) prior to the preparation of the platelet membranes.

Purification and Labeling of von Willebrand Factor. Von Willebrand factor (vWF) was purified from fresh frozen human plasma as previously described (Booth et al., 1984b). Only the vWF that eluted in the void volume of the final Sepharose 2B column was used for these experiments. These fractions contained the high molecular weight multimers of vWF ($\simeq 1 \times 10^6$ to $>1 \times 10^7$) as judged by electrophoretic analysis (Ruggeri & Zimmerman, 1981). A weight-average molecular weight for the purified vWF of 4.3×10^6 was determined by laser densitometry and integration of the SDS–agarose electrophoretic pattern. vWF was ^{125}I-labeled with Chloramine T.

Preparation of FMC 25- and SZ 22-Coupled Immunobeads. Monoclonal IgG was coupled to the impermeable polyacrylamide immunobeads (average diameter = 10 μm) according to the manufacturer's instructions. Briefly, purified IgG was dialyzed against coupling buffer (0.003 M potassium phosphate, pH 6.3). The lyophilized immunobead reagent was reconstituted to 10 mg/mL with coupling buffer, centrifuged, and then resuspended to original volume with 0.5 mg/mL IgG in coupling buffer. Coupling was initiated by the addition of 2 mg of EDAC/mL of suspension and was allowed to proceed overnight at 4 °C. Coupled beads were washed with phosphate-buffered saline, adsorbed with 1% (w/v) bovine serum albumin, and stored at 4 °C as a 10 mg of beads/mL suspension in 0.01 M Tris and 0.15 M sodium chloride, pH 7.4. In both instances, the efficiency of coupling was ≃40%, i.e., ≃20 μg of IgG/mg of beads.

Reconstitution Assay. FMC 25 coupled beads were coated with purified membrane GP Ib–IX complex by incubating the beads at 22 °C with ≃250 μg/mL of the complex in buffer A for 2 h. The extent of complex binding was determined by the inclusion of a trace amount (10 μL/mL of suspension) of ^{3}H-labeled GP Ib–IX complex. Control experiments indicated that longer incubation times did not increase the amount of GP Ib–IX complex associated with the beads. The GP Ib–IX complex coated beads were washed once by centrifugation with buffer A and once with the final assay buffer (0.01 M Tris and 0.15 M sodium chloride, pH 7.4). Test assays performed in duplicate at 22 °C consisted of GP Ib–IX complex coated beads (4–5 mg/mL, final concentration), bovine serum albumin (0.1 mg/mL, final concentration), ^{125}I-labeled vWF (0–200 μg/mL), and ristocetin (0–2 mg/mL) in a final volume of 50 or 100 μL. In the studies on the effect of the monoclonal antibodies on the ristocetin-dependent binding of ^{125}I-labeled vWF to the GP Ib–IX complex coated beads, 50 μg/mL final concentration of monoclonal IgG was included in the assay in a total volume of 100 μL. The assay was terminated by sedimenting the beads in a Beckman microfuge at 8750*g* for 2 min. The supernatant was carefully removed by use of a Terumo syringe fitted with a fine bore (26 gauge) needle. ^{125}I-Labeled vWF associated with the pelleted beads was then measured in a γ counter. Nonspecific binding of ^{125}I-labeled vWF was determined both in the absence and in the presence of ristocetin either by the inclusion of a 50-fold excess of unlabeled vWF or from the binding of ^{125}I-labeled vWF to GP IIb coated, SZ 22 coupled immunobeads. GP IIb coated beads were prepared similarly to GP Ib–IX complex coated beads by incubating SZ 22 coupled beads (10 mg of beads/mL) with GP IIb at ≃300 μg/mL in buffer A for 2 h at 22 °C. The extent of coating was estimated by the inclusion of a trace amount of ^{125}I-labeled GP IIb. Related experiments to those described above were performed to address the binding of ^{125}I-labeled albumin, fibrinogen, or fibronectin (0–100 μg/mL) to GP Ib–IX complex coated or GP IIb coated beads in the absence or presence of 1 mg/mL ristocetin.

Ristocetin-Dependent Binding of ^{125}I-Labeled von Willebrand Factor to Platelets. The effect of the anti-GP Ib–IX complex and anti-vWF monoclonal antibodies on the ristocetin-dependent binding of ^{125}I-labeled vWF to platelets was determined by using a minor modification of the procedure of Michelson et al. (1986). The assay incorporated 1.25×10^7 washed platelets, 0.1 mg/mL bovine serum albumin, 50 μg/mL monoclonal IgG, 1 μg/mL ^{125}I-labeled vWF, and 1 mg/mL ristocetin in a final volume of 250 μL. Assay buffer was 0.01 M Tris and 0.15 M sodium chloride, pH 7.4. After a 50-min incubation at 22 °C, 200-μL aliquots were removed and loaded onto 500 μL of 17% (w/v) sucrose in assay buffer in microfuge tubes. Where appropriate, the sucrose cushion included ristocetin at a concentration of 1 mg/mL. Platelets were pelleted at 8750*g* for 5 min. After careful aspiration of the supernatants, ^{125}I-labeled vWF associated with the platelet pellets was measured in a γ counter.

Electrophoresis. Protein purity was assessed by electrophoretic analysis on SDS–7.5% linear polyacrylamide gels according to the method of Laemmli (1970). Immunoprecipitates were similarly electrophoresed on SDS–5–20% exponential gradient polyacrylamide gels. Protein staining, autoradiography, and fluorography were performed as previously described (Berndt et al., 1985a).

Results

Epitope Mapping of Anti-GP Ib–IX Complex Monoclonal Antibodies. The present study employed seven monoclonal antibodies, AK 1, AK 2, AK 3, SZ 1, AP 1, WM 23, and FMC 25, directed against the human platelet membrane GP Ib–IX complex. Three of these antibodies, AK 1, SZ 1, and FMC 25, have previously been described in detail and are directed against the membrane-associated region of the GP Ib–IX complex. AK 1 and SZ 1 are directed against complex-specific epitopes (Du et al., 1987), while FMC 25 is directed against GP IX (Berndt et al., 1985a,b). To epitope map the remaining four monoclonal antibodies, we performed immunoprecipitation with trypsin-treated, tritium-labeled GP

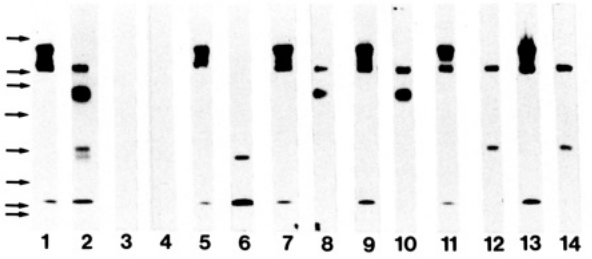

FIGURE 1: Fluorograph of a SDS–5–20% exponential gradient polyacrylamide gel run under nonreducing conditions of the immunoprecipitates (lanes 3–14) derived from tritium-labeled GP Ib–IX complex shown in lane 1 (odd-numbered lanes) or from trypsin-treated, tritium-labeled GP Ib–IX complex shown in lane 2 (even-numbered lanes). (Lanes 3 and 4) SZ 21 (negative control); (lanes 5 and 6) AK 1; (lanes 7 and 8) WM 23; (lanes 9 and 10) AK 3; (lanes 11 and 12) AK 2; (lanes 13 and 14) AP 1. Molecular weight markers in order of decreasing molecular weight are myosin (200 000), β-galactosidase (130 000), phosphorylase *b* (94 000), bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (29 000), soybean trypsin inhibitor (21 000), and lysozyme (14 000).

Ib–IX complex. Trypsin cleaves the GP Ib–IX complex into three distinct domains (Handa et al., 1986), a 45 000 molecular weight peptide tail region, an 85 000 molecular weight macroglycopeptide core, and an approximately 25 000 molecular weight fragment of the α-chain that is disulfide-linked to the β-subunit and complexed with GP IX (membrane-associated region). The immunoprecipitation results for WM 23, AK 3, AK 2, and AP 1 under nonreducing conditions are shown in Figure 1; AK 1, directed against an epitope on the membrane-associated region of the GP Ib–IX complex, has been included for comparison (lanes 5 and 6). All five monoclonal antibodies immunoprecipitated purified, tritium-labeled GP Ib–IX complex (lanes 5, 7, 9, 11, and 13). The purified, tritiated GP Ib–IX complex (lane 1) consisted of 90–95% intact complex (GP Ib, mol wt 170 000; GP IX, mol wt 22 000) (Berndt et al., 1985b). Two minor bands of lower molecular weight than that of GP Ib (Berndt et al., 1985a; Du et al., 1987) correspond to GP Ib–IX complex lacking the peptide tail region (upper band) and to glycocalicin (lower band), respectively.[2] Treatment of tritiated GP Ib–IX complex with trypsin (20:1 by weight, respectively) for 4 h yielded five peptides (lane 2): glycocalicin (mol wt 135 000), macroglycopeptide (mol wt 85 000), peptide tail (mol wt 45 000), remnant of α-chain disulfide-linked to the β-subunit (mol wt 41 000), and undigested GP IX (mol wt 22 000). Assignments are based on the data of Handa et al. (1986), the relative labeling intensity of each of the bands (Okumura et al., 1976), and the immunoprecipitation results under nonreducing (Figure 1) and reducing conditions (not shown). WM 23 and AK 3 immunoprecipitated residual glycocalicin and macroglycopeptide (lanes 8 and 10, respectively), indicating that these two monoclonal antibodies were directed against epitopes on the macroglycopeptide region of the GP Ib–IX complex. AK 2 and AP 1 immunoprecipitated residual glycocalicin and the 45 000 peptide tail (lanes 12 and 14, respectively), consistent with these two antibodies binding to epitopes in the peptide tail region of the GP Ib–IX complex.

Ristocetin-Dependent Binding of vWF to Purified GP Ib–IX Complex. Initial attempts to demonstrate the ristocetin-dependent binding of vWF to purified GP Ib–IX complex using sucrose gradient centrifugation analysis (Jennings & Phillips, 1982) were unsuccessful, presumably due to the dissociation

[2] J. E. B. Fox, L. P. Aggerbeck, and M. C. Berndt, unpublished observations.

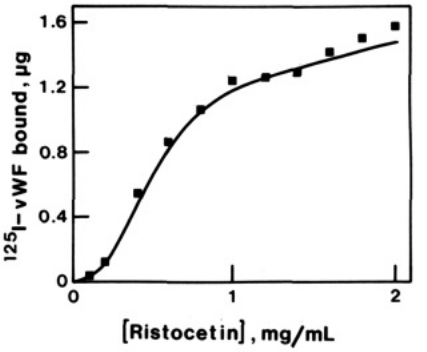

FIGURE 2: Ristocetin dependence of ^{125}I-labeled vWF (27 μg/mL) binding to GP Ib–IX complex coated beads (5 mg/mL) at 22 °C. Ordinate values were calculated from total bound ^{125}I-labeled vWF by subtracting the amount of nonspecifically bound ^{125}I-labeled vWF measured in the absence of ristocetin.

of the receptor–ligand complex during the time course of the centrifugation ($\simeq$6 h). We therefore chose to attempt the reconstitution of binding of vWF to GP Ib–IX complex using a solid-phase bead assay. Our approach was to indirectly bind and orientate the GP Ib–IX complex on the beads via a monoclonal antibody directed against the membrane-associated region of the complex. Immunobeads (Bio-Rad) were chosen as the insoluble matrix because the beads are uniform in size ($\simeq$10 μm in diameter), impermeable, specifically designed for the coupling of IgG, and because, like platelets (Coller, 1978), the beads have a net negative charge at neutral pH. Immunobeads were coupled with FMC 25, a monoclonal antibody directed against GP IX (Berndt et al., 1985a,b). FMC 25 maps into the membrane-associated region of the GP Ib–IX complex and has no effect on the ristocetin-mediated, vWF-dependent agglutination of platelets (Berndt et al., 1985a). Further, analysis of the structure of the GP Ib–IX complex by rotary shadowing electron microscopy indicates that the membrane-associated region of the complex is $\simeq$45 nm distant from the peptide tail region of the α-chain of GP Ib that probably contains the vWF binding domain.[2] Each milligram of coupled beads contained $\simeq$20 μg of monoclonal antibody and bound $\simeq$2 μg of GP Ib–IX complex at saturation. As a negative control for the assessment of vWF binding to the GP Ib–IX complex coated beads, we coupled the anti-GP IIb monoclonal antibody, SZ 22, to immunobeads and coated the beads with purified platelet membrane GP IIb. GP IIb was chosen as the negative control since it has a molecular weight [mol wt 136 000, consisting of two disulfide-linked subunits, GP IIb$_\alpha$ (mol wt 116 000) and GP IIb$_\beta$ (mol wt 23 000)] and an isoelectric point similar to those of the GP Ib–IX complex (Jennings & Phillips, 1982; Clemetson et al., 1982). Each milligram of SZ 22 coupled beads contained $\simeq$20 μg of monoclonal antibody and bound $\simeq$4.5 μg of GP IIb at saturation.

Preliminary time course experiments showed that binding of ^{125}I-labeled vWF to GP Ib–IX complex coated and GP IIb coated beads both in the absence and in the presence of ristocetin was complete within 10 min and that neither GP Ib–IX complex nor GP IIb dissociated from the monoclonal antibody coupled beads during the binding assay. In the absence of ristocetin, ^{125}I-labeled vWF bound nonspecifically ($\simeq$14% of total counts) to both the GP Ib–IX complex coated and GP IIb coated beads. That is, ^{125}I-labeled vWF bound linearly throughout the concentration range tested (0–200 μg/mL) without achieving saturation and without displacement by a 50-fold excess of unlabeled vWF (data not shown). With the GP Ib–IX complex coated beads the amount of ^{125}I-labeled vWF specifically associated with the beads increased with increasing concentrations of ristocetin and reached a plateau

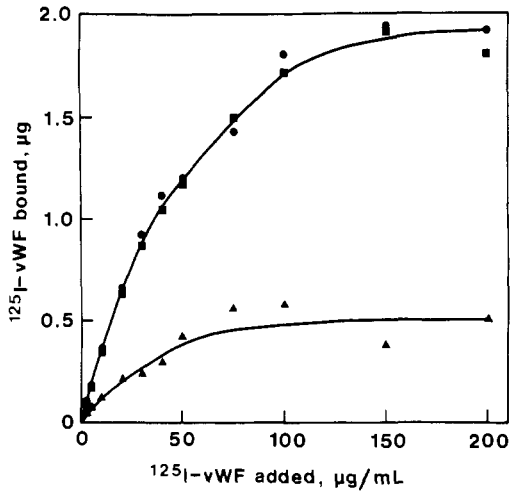

FIGURE 3: Ristocetin-dependent specific binding of ^{125}I-labeled vWF to GP Ib–IX complex coated beads (4 mg/mL) at 22 °C. Antibodies (50 μg/mL) were added to the beads 10 min before addition of ^{125}I-labeled vWF and ristocetin (final concentration, 1 mg/mL): (●) no antibody; (■) FMC 18; (▲) AK 2.

at ristocetin concentrations ≥1 mg/mL (Figure 2). The ristocetin dependence for the binding of vWF to the GP Ib–IX complex coated beads shown in Figure 2 was essentially identical with the ristocetin dependence for the maximal rate of agglutination of washed platelets (1×10^8/mL) in the presence of purified vWF (10 μg/mL) (data not shown) and with that previously reported for the ristocetin dependence of binding of vWF to platelets (Kao et al., 1979). In contrast, ristocetin concentrations up to 2 mg/mL did not augment the level of binding of ^{125}I-labeled vWF to the GP IIb coated beads.

In the presence of ristocetin (1 mg/mL), specific binding of ^{125}I-labeled vWF to the GP Ib–IX complex coated beads was saturable (Figure 3, circles). Under the assay conditions, ristocetin-dependent specific binding of vWF varied from ≃70% of the total added vWF at low vWF concentrations to ≃20% at 200 μg/mL of vWF. Since it was not technically feasible to use a 100-fold excess of cold vWF over the entire concentration range of ^{125}I-labeled vWF, specific binding was defined as the difference between vWF bound in the presence and absence of ristocetin. Experiments using excess unlabeled vWF at low concentrations of ^{125}I-labeled vWF indicated that this difference could be quantitatively accounted for solely in terms of specific binding. Further, ristocetin (1 mg/mL) did not increase the level of nonspecific binding of ^{125}I-labeled vWF with GP IIb coated beads under conditions identical with those described in the legend of Figure 3. Finally, ristocetin at 1 mg/mL did not increase the level of nonspecific binding (≃10%) of ^{125}I-labeled albumin or fibronectin to either GP Ib–IX complex coated or GP IIb coated beads. Ristocetin did increase the overall level of binding of ^{125}I-labeled fibrinogen (≃50%) to both GP Ib–IX complex coated and GP IIb coated beads, but this increased binding was nonspecific as it increased linearly with increasing fibrinogen concentration.

The monoclonal antibody AK 2 directed against the peptide tail region of the GP Ib–IX complex is a strong inhibitor of the ristocetin-mediated vWF-dependent agglutination of platelets. This antibody at 50 μg/mL also strongly inhibited the ristocetin-dependent binding of vWF to the GP Ib–IX complex-coated beads (Figure 3, triangles). In contrast, the irrelevant monoclonal antibody FMC 18, directed against an antigen on *Toxoplasma gondii* and of the same murine IgG subclass as AK 2, had no effect on the binding of vWF to the beads (Figure 3, squares), suggesting that AK 2 specifically

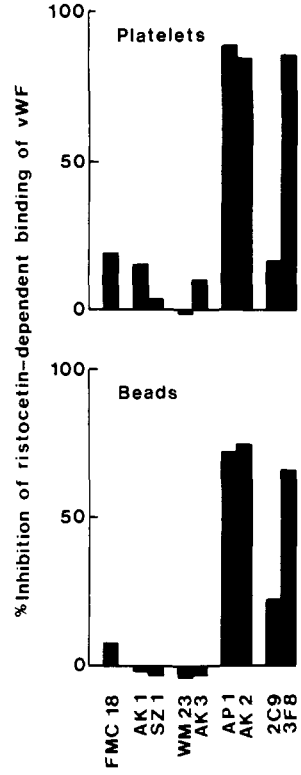

FIGURE 4: Inhibition of the ristocetin-dependent binding of ^{125}I-labeled vWF (1 μg/mL, platelets; 10 μg/mL, beads) to fresh washed platelets (upper panel; 5×10^8 platelets/mL) and to GP Ib–IX complex coated beads (lower panel; 4 mg/mL) by monoclonal antibodies at 22 °C. Platelets or beads were incubated with antibody (50 μg/mL) for 10 min before the addition of ^{125}I-labeled vWF and ristocetin (final concentration, 1 mg/mL).

inhibited the ristocetin-dependent association of vWF with the beads because it interferes with the vWF binding domain on GP Ib.

Effect of Anti-GP Ib–IX Complex and Anti-vWF Monoclonal Antibodies on the Ristocetin-Dependent Binding of vWF to Purified GP Ib–IX Complex. The analysis of the binding of vWF to the GP Ib–IX complex coated beads confirms the functional role of this complex as a major platelet vWF receptor. In similar manner to the ristocetin-dependent binding of vWF to platelets, specific binding of vWF to the GP Ib–IX complex coated beads was strictly ristocetin dependent (Kao et al., 1979; Schneider-Trip et al., 1979), had a similar dependence on ristocetin concentration (Kao et al., 1979), and was inhibited by a monoclonal antibody directed against the peptide tail region of GP Ib (Wicki & Clemetson, 1985; Handa et al., 1986). To further confirm that the ristocetin-dependent binding of vWF to the purified GP Ib–IX complex had a specificity identical with that of the platelet receptor, we examined the effect of a series of anti-GP Ib–IX complex and anti-vWF monoclonal antibodies on the ristocetin-dependent binding of vWF to platelets and to the GP Ib–IX complex coated beads (Figure 4). The irrelevant monoclonal antibody FMC 18 had little effect on the ristocetin-dependent binding of vWF to either platelets or the GP Ib–IX complex coated beads. Monoclonal antibodies (AP 1 and AK 2) against the 45 000 molecular weight peptide tail region of GP Ib that strongly inhibited the ristocetin-dependent binding of vWF to platelets also strongly inhibited the ristocetin-dependent binding of vWF to the GP Ib–IX complex coated beads. Control experiments indicated that the observed inhibition by AP 1 and AK 2 was not due to the dissociation of GP Ib–IX complex from the FMC 25 coupled immunobeads. Conversely, monoclonal antibodies against the membrane-associated region

of the GP Ib–IX complex (AK 1 and SZ 1) or the macroglycopeptide domain of the α-chain of GP Ib (WM 23 and AK 3) did not inhibit the ristocetin-dependent binding of vWF to either platelets or the GP Ib–IX complex coated beads. Similar functional correlations were obtained with anti-vWF monoclonal antibodies. 3F8, an anti-vWF monoclonal antibody that blocks the ristocetin-mediated, vWF-dependent agglutination of human platelets (Booth et al., 1984b), also inhibited the ristocetin-dependent binding of vWF both to platelets and to the GP Ib–IX complex-coated beads, while a separate anti-vWF monoclonal antibody, 2C9, had little effect on either binding interaction.

Discussion

In response to vascular injury, platelets rapidly adhere to the exposed vascular subendothelium. At high shear flow, this initial adhesive event is almost exclusively mediated by von Willebrand factor (vWF), either present in plasma or incorporated in the vessel wall subendothelial matrix (Stel et al., 1985; Turitto et al., 1985), and is dependent upon a specific platelet vWF receptor. Historically, this interaction between vWF and its platelet receptor has been more conveniently studied by using ristocetin, an antibiotic that mimics the subendothelium in causing vWF to bind to platelets (Berndt & Caen, 1984). The majority of the current evidence is consistent with the human platelet membrane glycoprotein (GP) Ib–IX complex being the receptor involved in the vWF-dependent adhesion of platelets to exposed vascular subendothelium and in the ristocetin-dependent binding of vWF to platelets. First, platelets from patients with the Bernard–Soulier syndrome, which genetically lack the GP Ib–IX complex (Berndt et al., 1983), adhere poorly to vascular subendothelium at high shear flow (Weiss et al., 1974) and fail to bind vWF in the presence of ristocetin (Moake et al., 1980a). However, besides lacking the GP Ib–IX complex, Bernard–Soulier syndrome platelets have been shown to be deficient in other membrane proteins, notably GP V and a protein of 210 000 molecular weight (Clemetson et al., 1982; Berndt et al., 1983; Stricker et al., 1985; Stricker & Shuman, 1986). Second, polyclonal and monoclonal antibodies against the 45 000 molecular weight tail region of the α-chain of GP Ib completely block the ristocetin-dependent interaction between vWF and platelets (Wicki & Clemetson, 1985; Handa et al., 1986). In contrast, Stricker et al. (1985) have described a patient with an autoantibody against the 210 000 molecular weight protein deficient in Bernard–Soulier syndrome platelets. $(Fab)_2$ fragments of this autoantibody also completely blocked the ristocetin-induced agglutination of human platelets. Finally, treatment of platelets with proteolytic enzymes that cleave off the peptide tail or glycocalicin (macroglycopeptide plus peptide tail) regions of the α-chain of GP Ib abolishes the ability of the platelets to agglutinate in the presence of vWF and ristocetin (Cooper et al., 1982; Yoshida et al., 1983; Brower et al., 1985).

Precise interpretation of these results is clouded by the occurrence of multiple membrane protein deletions in Bernard–Soulier syndrome platelets, by the possibility that specific antibodies sterically interfere with adjacent membrane proteins, and by the question of protease specificity in functional studies with whole cells. In this study, we have directly examined whether the human platelet membrane GP Ib–IX complex is the receptor for the ristocetin-dependent binding of vWF by reconstitution with the purified components using a solid-phase bead assay. The combined evidence from these studies strongly supports the functional role of the GP Ib–IX complex as the ristocetin-dependent platelet vWF receptor. *First*, specific binding of vWF to the GP Ib–IX complex coated beads was strictly ristocetin dependent with maximal binding of vWF at ristocetin concentrations ≥1 mg/mL. This ristocetin dependence for the binding of vWF was essentially identical with that for the ristocetin-dependent agglutination of washed platelets and for the ristocetin-dependent binding of vWF to platelets (Kao et al., 1979). *Second*, in the presence of ristocetin, vWF binding to the GP Ib–IX complex coated beads was saturable. Control experiments indicated that this binding was specific to the interaction between vWF and the GP Ib–IX complex. There was no ristocetin-dependent specific binding of vWF if the physicochemically related platelet membrane glycoprotein GP IIb was substituted for the GP Ib–IX complex in a corresponding bead assay. Further, neither bovine serum albumin nor other adhesive glycoproteins, such as fibrinogen or fibronectin, specifically bound to the GP Ib–IX complex coated beads in the presence of ristocetin. *Third*, inhibition studies with anti-GP Ib–IX complex and anti-vWF monoclonal antibodies indicated that the ristocetin-dependent binding of vWF to the GP Ib–IX complex coated beads had a specificity identical with that of the ristocetin-dependent interaction between vWF and human platelets. Monoclonal antibodies against the 45 000 molecular weight peptide tail region of GP Ib that strongly inhibited the ristocetin-dependent binding of vWF to platelets also strongly inhibited the ristocetin-dependent binding of vWF to the GP Ib–IX complex coated beads. In contrast, monoclonal antibodies against either macroglycopeptide or membrane-associated regions of the GP Ib–IX complex did not inhibit the ristocetin-dependent binding of vWF to platelets or to the GP Ib–IX complex coated beads. These results are in complete agreement with previous antibody studies which suggest that the 45 000 molecular weight peptide tail region of GP Ib contains the vWF binding domain (Wicki & Clemetson, 1985; Handa et al., 1986) and suggest that the inhibition of ristocetin-induced platelet agglutination by the autoantibody against the 210 000 molecular weight platelet membrane protein is steric (Stricker et al., 1985). Similar functional correlations between the ristocetin-dependent binding of vWF to platelets and to the GP Ib–IX complex coated beads were obtained with a more limited series of anti-vWF monoclonal antibodies.

In normal circulation, vWF does not bind to its platelet receptor unless vWF is bound to exposed subendothelial matrix (Sakariassen et al., 1979) or to fibrin (Loscalzo et al., 1986; Weiss et al., 1986). The molecular mechanism by which ristocetin allows the interaction between vWF and its normally cryptic platelet receptor is unclear. While there is conflicting evidence for ristocetin–vWF interaction (Floyd et al., 1977; Moake et al., 1980b), the available data are more consistent with a mechanism for ristocetin-mediated, vWF-dependent platelet agglutination that involves ristocetin binding to platelets (Coller & Gralnick, 1977; Coller, 1978; Jenkins et al., 1979; Moake et al., 1980b). Regardless of the precise mechanism, the successful ristocetin-dependent reconstitution of binding of vWF to purified human platelet GP Ib–IX complex in the present study indicates that ristocetin facilitates the binding of vWF to platelets by directly interacting with vWF or the GP Ib–IX complex or both. Purified GP Ib–IX complex (Berndt et al., 1985b) and its proteolytic derivative, glycocalicin (Okumara & Jamieson, 1976; Michelson et al., 1986), have previously been shown to inhibit ristocetin-dependent vWF–platelet interaction. These findings are supported by the reconstitution data which also suggest that this inhibition is directly due to the competition of soluble receptor with platelet receptor for the binding of vWF.

There is considerable discrepancy with respect to the characteristics for the ristocetin-dependent binding of vWF to platelets.[3] Kao et al. (1979), using Scatchard analysis, found a single class of binding sites (31 000 copies/platelet) with an estimated K_d of 0.45 nM. Morisato and Gralnick (1980) also used Scatchard analysis but found both high-affinity (K_d = 0.37 nM, 3500 copies/platelet) and low-affinity binding sites (K_d = 2.35 nM, 7500 copies/platelet). Girma et al. (1986) obtained a nonlinear Scatchard plot and estimated 15 500 binding sites/platelet. All these estimates for the number of ristocetin-dependent vWF binding sites on human platelets are in good agreement with the estimated number of copies of GP Ib–IX complex on intact platelets ($\simeq$25 000; Du et al., 1987). Our binding curve for the ristocetin-dependent binding of vWF to GP Ib–IX complex coated beads appears qualitatively similar to that obtained by Girma et al. (1986) for the corresponding ristocetin-dependent binding of vWF to platelets. The nonlinearity of Scatchard plots for binding of vWF to platelets in the presence of ristocetin has been attributed to the preferential binding of the higher molecular weight vWF multimers to platelets (Gralnick et al., 1981; Chopek et al., 1986). Chopek et al. (1986) have suggested that this increased affinity of the larger multimers of vWF for binding to platelets may be due to their interaction with more than one receptor, resulting in an increased apparent binding constant. Support for this conclusion can be derived from the work of Fox (1985a,b), who demonstrated with resting, discoid platelets that the GP Ib–IX complex spans the platelet plasma membrane lipid bilayer and is anchored to actin filaments via actin-binding protein. It is tempting to speculate that multimeric vWF consisting of linear flexible strands (Fowler et al., 1985) of repeating identical dimeric subunits (Fowler et al., 1985; Girma et al., 1986) can multiply interact with GP Ib–IX receptors evenly spaced along submembranous actin filaments. In the current study, if it is assumed that the stoichiometry of binding of vWF to the GP Ib–IX complex at saturation is 1:1, then a weight-average molecular weight for vWF of 1×10^6 can be calculated from the data in Figure 3 since each microgram of GP Ib–IX complex (mol wt $\simeq 2 \times 10^5$) bound $\simeq 5$ μg of vWF at saturation. However, the vWF employed in the current study consisted of multimers from 1×10^6 to $>10 \times 10^6$ in molecular weight (weight-average mol wt 4.3×10^6 by densitometry). This suggests, in agreement with the conclusions of Chopek et al. (1986), the presence of multiple GP Ib–IX complex binding sites within each individual vWF multimer. In this regard, the detailed analysis of binding of vWF dimer and vWF proteolytic fragments to purified GP Ib–IX complex and the examination of the topography of the GP Ib–IX complex in the intact membrane are under current investigation.

ACKNOWLEDGMENTS

We are indebted to Professor P. A. Castaldi and Dr. R. K. Andrews for helpful discussions. We thank Athena Webster for manuscript preparation and Peter Woods for his assistance in the preparation of the figures.

Registry No. vWF, 109319-16-6; ristocetin, 1404-55-3.

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[3] The K_d and number of binding sites have been calculated in individual papers using a weight-average molecular weight for vWF of $(1.1–2.15) \times 10^6$.

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Nanosecond Pulse Fluorometry of Conformational Change in Phenylalanine Hydroxylase Associated with Activation

Shinichi Koizumi,[‡] Fumio Tanaka,[§] Norio Kaneda,[∥] Koji Kano,[⊥] and Toshiharu Nagatsu*[,∥]

National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, Mie Nursing College, Tsu, Mie 514, Japan, Department of Biochemistry, Nagoya University School of Medicine, Nagoya 466, Japan, and Department of Applied Chemistry, Doshisha University, Kyoto 602, Japan

Received June 12, 1987; Revised Manuscript Received September 4, 1987

ABSTRACT: Conformational change in rat liver phenylalanine hydroxylase associated with activation by phenylalanine or *N*-(1-anilinonaphth-4-yl)maleimide was investigated by measuring fluorescence spectra and fluorescence lifetimes of tryptophanyl residues as well as the probe fluorophore conjugated with SH groups of the hydroxylase. The fluorescence spectrum of tryptophan exhibited its maximum at 342 nm. It shifted by 8 nm toward longer wavelength accompanied by an increase in its intensity, by preincubation with 1 mM phenylalanine. The fluorescence intensity of tryptophan increased by 36% upon the activation. On the other hand, the binding of (6*R*)-L-*erythro*-tetrahydrobiopterin, a natural cofactor of the enzyme, induced a decrease in the fluorescence intensity by 79% without a shift of the maximum wavelength. The fluorescence lifetime of tryptophan of phenylalanine hydroxylase exhibited two components with lifetimes of 1.7 and 4.1 ns. The values of the lifetimes changed to 1.4 and 5.6 ns, respectively, upon the activation. It is considered that the change in the longer lifetime is correlated with the shift of the emission peak upon the activation. The values of both the lifetimes decreased to 0.64 and 3.6 ns upon the binding of (6*R*)-L-*erythro*-tetrahydrobiopterin, which is coincident with the decrease in the fluorescence intensity. Conjugation of *N*-(1-anilinonaphth-4-yl)maleimide with SH of phenylalanine hydroxylase brought about a decrease in both the fluorescence intensity and the value of the shorter lifetime of the tryptophanyl residues, while the longer lifetime remained unchanged. These changes could be ascribed to excitation energy transfer from tryptophan with the shorter lifetime to the anilinonaphthyl group. When *N*-(1-anilinonaphth-4-yl)maleimide-conjugated phenylalanine hydroxylase was incubated with 1 mM phenylalanine, the energy-transfer efficiency decreased. The distances between both the tryptophan residues and the probe molecule are considered to be not very short compared to the critical transfer distance (1.7-1.8 nm). The fluorescence lifetime of *N*-(1-anilinonaphth-4-yl)maleimide exhibited a single component of 4.6 ns. This suggests that the surroundings of the anilinonaphthyl group are homogeneous in phenylalanine hydroxylase. It became heterogeneous upon activation by preincubation with 1 mM phenylalanine or binding of (6*R*)-L-*erythro*-tetrahydrobiopterin, so that a hydrophobic environment appeared around the fluorophore.

Rat liver phenylalanine hydroxylase [L-phenylalanine,tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1] catalyzes the conversion of phenylalanine to tyrosine, an obligatory step in the degrdn. of phenylalanine in mammals, with tetrahydropterin as a cofactor (Milstien & Kaufman, 1975). This monooxygenase is an allosteric enzyme (Shiman & Gray, 1980). Since an absence of this enzyme in humans causes phenylketonuria, the regulatory mechanism of its catalytic activity has been of interest both enzymatically and clinically. In the presence of a natural cofactor, (6*R*)-L-*erythro*-tetrahydrobiopterin (BH_4)[1] (Kaufman, 1963; Matsuura et al., 1980), the enzyme is in a low-activity state (Kaufman, 1970; Hasegawa & Kaufman, 1982). The rela-

* Author to whom correspondence should be addressed.
[‡] National Institutes of Health.
[§] Mie Nursing College.
[∥] Nagoya University School of Medicine.
[⊥] Doshisha University.

[1] Abbreviations: ANM, *N*-(1-anilinonaphth-4-yl)maleimide; BH_4, (6*R*)-L-*erythro*-tetrahydrobiopterin; $6MPH_4$, 6(*RS*)-methyl-5,6,7,8-tetrahydropterin; SDS, sodium dodecyl sulfate.

0006-2960/88/0427-0640$01.50/0