Characterization of a Fragment of Bovine von Willebrand Factor That Binds to Platelets†

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ABSTRACT: Bovine von Willebrand factor was digested with human plasmin in order to isolate and characterize a fragment that can bind to human platelets. A terminal plasmin digest of bovine von Willebrand factor is composed of five fragments, ranging in relative molecular weight (M_r) from 250 000 to 35 000. The major fragment has a M_r of 250 000 and consists of four disulfide-linked polypeptide chains with M_r from 69 000 to 35 000. The M_r 69 000 and 49 000 polypeptides possess carbohydrate moieties, as indicated by their reaction with periodate–Schiff reagent. Gel filtration studies suggest that, at physiological ionic strength, four of the M_r 250 000 fragments associate into a limited noncovalent oligomer. Monoclonal antibodies were prepared against native von Willebrand factor and used to characterize the distribution of epitopes on native vWF and the M_r 250 000 major fragment. Two of the monoclonal antibodies that recognize the major fragment (2 and H-9) inhibit platelet agglutination. The M_r 250 000 fragment binds to human platelets, and the binding is inhibited by monoclonal antibodies 2 and H-9. The M_r 250 000 fragment does not agglutinate platelets, consistent with a requirement for high molecular weight oligomers of von Willebrand factor for platelet agglutination. The M_r 250 000 fragment can compete with intact, bovine von Willebrand factor for binding to human platelets. However, its affinity is one-tenth that of intact von Willebrand factor.

von Willebrand factor (vWF)[1] is a high molecular weight, oligomeric, plasma glycoprotein that is required for platelets to recognize and adhere to damaged endothelial surfaces at high shear rates (Baumgartner et al., 1980). As such it can be considered an adhesive protein. It circulates in blood in a noncovalent complex with factor VIII:C procoagulant protein (Owen & Wagner, 1972). It is synthesized in endothelial cells (Jaffe et al., 1973) and megakaryocytes (Nachman et al., 1977) and is stored in the α granules of platelets (Nachman et al., 1975).

vWF is a complex of apparently identical subunits that have an apparent M_r of 230 000 (Counts et al., 1978). The monomeric subunits are covalently linked by disulfide bonds to form heterogeneous oligomers, ranging in M_r from 1×10^6 to 20×10^6 (Ruggeri & Zimmerman, 1980a). It has been proposed that organization of newly synthesized subunits into multimers involves the formation of dimers (Counts et al., 1978) followed by a secondary polymerization to higher order sets of multimers. The majority of this protein in plasma, though, exists as high molecular weight oligomers (Ruggeri & Zimmerman, 1980b). Recognition of damaged endothelial surfaces for platelet adhesion requires vWF in high molecular weight oligomers (Zimmerman & Ruggeri, 1982).

The platelet receptor for vWF is composed, at least in part, of a protein termed glycoprotein Ib (Coller et al., 1983). In vitro, the function of human vWF is assayed by its capacity to agglutinate platelets in the presence of the nonphysiological agonist ristocetin (Howard & Firkin, 1971). Bovine vWF agglutinates human platelets directly (Forbes & Prentice, 1973), without the need for ristocetin. Several lines of evidence suggest that the mechanism of platelet agglutination by bovine and human vWF is similar. For instance, agglutination of human platelets by bovine and human vWF is inhibited by antibodies to platelet glycoprotein Ib (Tobelem et al., 1976). Platelets from individuals with Bernard–Soulier syndrome are deficient in glycoprotein Ib and are not agglutinated by either bovine or human vWF (Caen et al., 1976). Also, bovine and human vWF bind to human platelets in a reversible manner and compete for binding to platelets (Suzuki et al., 1980).

Much of what is known regarding vWF has been derived with the aid of immunological and proteolytic analysis. Antibodies to vWF demonstrated that vWF and factor VIII:C are distinct from one another (Zimmerman & Edgington, 1973). Affinity chromatography on insolubilized antibodies to vWF has been used to purify factor VIII:C by virtue of its noncovalent association with vWF (Fulcher & Zimmerman, 1982). Monoclonal antibodies have been prepared against both human (Meyer et al., 1984b; Sola et al., 1982; Schullek et al., 1984; Stel et al., 1984) and porcine vWF (Katzmann et al., 1981), and initial studies have described the distribution of epitopes on human vWF and the identification of regions responsible for its varied functions (Meyer et al., 1984a; Sixma et al., 1984).

Several authors have attempted to study the structure of vWF by cleaving the molecule with proteases and analyzing

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[1] Abbreviations: vWF, von Willebrand factor; PS, phosphate saline buffer; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; IgA, immunoglobulin A; IgM, immunoglobulin M; IgG, immunoglobulin G; PEG, poly(ethylene glycol); EDTA, ethylenediaminetetraacetate; STI, soybean trypsin inhibitor; NMS, normal mouse serum; cpm, counts per minute; SPIRA, solid-phase immunoradiometric assay; M_r, relative molecular weight; Tris, tris(hydroxymethyl)aminomethane; DMEM, Dulbecco's minimal essential medium; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; DEAE, diethylaminoethyl.

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the fragments for platelet binding ability (Kirby et al., 1974; Atichartakarn et al., 1978; Hamilton et al., 1985; Fujimura et al., 1986). Martin et al. (1980) have isolated a tryptic digest fragment of human vWF (M_r 116 000) that agglutinates human platelets in the presence of ristocetin. A similar tryptic fragment is recognized by a monoclonal antibody that inhibits vWF-mediated platelet agglutination (Sixma et al., 1984). However, the affinity of this fragment of human vWF for platelets has not been established. This study reports the isolation and partial characterization of a fragment of bovine vWF that binds to platelets. The size of the fragment, and its epitope composition, suggests that it retains most of the polypeptide content of vWF. The fragment's affinity is compared to that of native vWF.

Materials and Methods

Reagents. Materials were nitrocellulose filter sheets, 0.45 μm (Millipore Corp., Bedford, MA); Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril) (Pierce Chemical Co., Rockford, IL); Sepharose CL-4B and Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ); normal goat serum, bovine IgM, and immunopurified goat-anti-mouse IgG (Pel-Freez Biologicals, Rogers, AR); bentonite (Prolabo, Paris, France); human fibrinogen (Kabi Diagnostics, Helena Laboratories, Beaumont, TX); acrylamide, *N,N'*-methylenebis-(acrylamide), and Bio-Gel A-1.5m (100–200 mesh) (Bio-Rad, Richmond, CA); and X-ray film type XAR-5 (Eastman Kodak, Rochester, NY). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA) and Sigma Chemical Co. (St. Louis, MO). Human plasmin was a generous gift from Dr. Andrei Budzynski, Thrombosis Research Center, Temple University.

Buffers. Phosphate saline buffer (PS) contained 0.01 M phosphate, 0.15 M NaCl, and 3.1 mM NaN_3, pH 6.5. SPIRA buffer was 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 147 mM NaCl, and 3.1 mM NaN_3, pH 7.2, containing 0.1% bovine serum albumin (BSA), 0.1% normal goat serum, and 0.005% Tween 20. Tris/NH_4Cl buffer contained 0.017 M Tris and 0.144 M NH_4Cl, pH 7.2.

Cells were grown in medium containing hypoxanthine and thymidine (HT medium) prepared as described by Kennet (1978). DMEM–Hepes medium, used for washing and suspending cells, was serum-free Dulbecco's minimal essential medium, high glucose (Gibco, Los Angeles, CA), containing 10 mM Hepes, pH 7.3.

Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gels (7.5%) were prepared, and electrophoresis was performed according to the procedures of Laemmli (1970) and Weber and Osborn (1969). Gels were stained with Coomassie Brilliant Blue R-250 or with periodate–Schiff stain according to the procedure of Fairbanks et al. (1971). Discontinuous $NaDodSO_4$–agarose gels (2% with 0.8% stacking gels) were prepared as described previously (Lynch et al., 1983), with the following modifications: A 15 × 13.3 cm horizontal electrophoresis apparatus (Aquebogue Machine and Repair Shop, Aquebogue, NY) was used. A 2.5 mm thick, 2% running gel, pH 8.8, was poured (50 mL) and allowed to cool for 30 min. Two centimeters were cut from the end of the running gel. A 0.8% stacking gel was then poured with a sample comb in place. Twenty microliters of sample was diluted with 10 μL of diluent buffer, and the samples were boiled for 3 min and loaded onto the gel. Both chambers were filled with running buffer to the level of the sides of the gel (but not immersing the gel). Samples were electrophoresed into the stacking gel at 30 mA. After all the sample had entered the gel, the gel was immersed in running gel buffer (approximately a 1-cm depth) and electrophoresed at 25 mA for 16 h at room temperature. Agarose gels were stained with Coomassie Blue.

Protein Determinations. Protein concentrations were determined by the procedure of Lowry et al. (1951) or from the absorbance of the protein at 280 nm, using a value of 12 for $E^{1\%}$. The A_{280} was corrected for light scattering by subtracting 1.7 times the absorbance at 320 nm.

Purification of vWF and Other Proteins. Bovine vWF was purified as described (Kirby, 1982), with the following modifications: A volume of 9 L of barium sulfate treated plasma was allowed to thaw partially overnight. Bovine vWF was precipitated from plasma by slow addition of 20% poly(ethylene glycol) (PEG 8000) in PS to a final concentration of 2.5%. The temperature was kept near 0 °C. The precipitate was collected by centrifugation at 0 °C (7000*g* for 15 min). The precipitate was dissolved in one-tenth the original plasma volume of 0.05 M sodium citrate and 0.04 M ε-aminocaproic acid buffer, pH 6.8, at room temperature. Phenylmethanesulfonyl fluoride and soybean trypsin inhibitor (STI) were added to a final concentration of 100 μg/mL, and diisopropyl fluorophosphate was added to a final concentration of 1 mM. Fibrinogen was partially removed by adding 1 L of a 10% suspension of bentonite in 0.15 M NaCl, stirring for 10 min at room temperature, and centrifuging at 7000*g* for 15 min. The supernatant was diluted with an equal volume of 0.5 M succinate buffer [which had been titrated to pH 7.0 with tris(hydroxymethyl)aminomethane]. The vWF was precipitated by slow addition of finely powdered glycine (125 g/L of solution). The glycine precipitate was collected by centrifugation at 7000*g* for 15 min. The precipitate was redissolved in a minimum volume of PS containing 100 μg/mL phenylmethanesulfonyl fluoride and soybean trypsin inhibitor and applied to a 5.2 × 150 cm Sepharose CL-4B column equilibrated in PS containing 1 mM EDTA. vWF activity was eluted at the void volume. Active fractions were pooled and concentrated to one-fourth volume by dialysis against solid PEG 20 000. Minor traces of fibrinogen were removed from the final product by adsorption with zinc–Sepharose (Scully & Kakkar, 1982) or heparin–agarose when necessary. $NaDodSO_4$–polyacrylamide gel electrophoresis of the purified vWF showed a single band with an apparent M_r of 230 000.

Human vWF, purified by the method of DeMarco et al. (1981), was a gift from Drs. L. DeMarco and S. Shapiro, Cardeza Foundation of Thomas Jefferson University, Philadelphia, PA.

Bovine fibronectin was obtained from the supernatant of the glycine precipitation step in the vWF purification and was further purified by adsorption to gelatin–Sepharose (Engvall & Ruoslahti, 1977) and elution with 4 M urea–10 mM *N*-ethylmaleimide in PS. The fibronectin was dialyzed against PS and stored at −90 °C. Bovine fibrinogen was obtained during the purification of bovine vWF by taking a fraction that eluted late from the Sepharose 4B column and did not contain vWF.

The goat antibody to mouse IgG used for initial screening of hybridoma cultures was prepared by ammonium sulfate precipitation of goat antiserum against mouse IgG. Immunoblotting assays used goat-anti-mouse IgG that had been immunopurified.

Iodination of Proteins. vWF and goat-anti-mouse IgG were labeled with ^{125}I by the Iodogen method (Markwell et al., 1978). vWF (0.8–1 mg) was dialyzed against 0.1 M phosphate, pH 7.0. NaI was added to the sample to a final concentration of 8 μM followed by 400 μCi of $Na^{125}I$ (New England Nuclear, Boston, MA; sp act. = 17 Ci/mg). The

solution was transferred to a 5-mL capped septum vial that had been coated with 50 μg of Iodogen. The vial was swirled gently for 10 min at room temperature. Labeling was stopped by transferring the sample to a vial containing 0.1 mL of a 0.1 M NaI solution in water. The sample was passed over a column (1 × 10 cm) of Sephadex G-25 equilibrated in PS. Void volume fractions with the highest specific activity were pooled. Incorporation of ^{125}I was 50–60%, and >95% of the radioactivity in the labeled protein was trichloroacetic acid precipitable. ^{125}I-vWF had 0.5–1 atom incorporated/subunit with a specific activity of 200–400 μCi/mg vWF. Previous studies (Kirby, 1982) have shown that up to two atoms of iodine could be incorporated per vWF subunit without significant loss of either platelet binding affinity or platelet agglutinating activity. The IgG fraction of goat-anti-mouse IgG was labeled similarly with 500 μCi of $Na^{125}I$. Free ^{125}I was removed by a microcolumn technique (Tuszynski et al., 1980).

Preparation of Hybridoma Cell Lines. Balb/c mice were immunized with 20 μg each of highly purified bovine vWF in Freund's complete adjuvant, injected intraperitoneally. One month later immunization was repeated, with vWF in incomplete adjuvant, and 2 months later with vWF suspended in aluminum hydroxide gel. Serum was obtained from each mouse and titered by solid-phase immunoradiometric assay (see below). Only mice having antisera that were positive at greater than 10^6 dilution were used for fusions.

Mice were boosted intraperitoneally with 20–50 μg of vWF in saline, 72 and 48 h before removal of spleens for fusions. Spleen cells were suspended in DMEM–Hepes buffer and incubated for 1 h at 37 °C in polystyrene T-75 flasks, and adherent cells were discarded. Red cells were lysed by incubation with Tris–NH_4Cl buffer for 10 min at 0 °C. The cell suspension was centrifugated at 250*g* for 10 min and resuspended in DMEM–Hepes. After a second centrifugation the spleen cells were resuspended in DMEM–Hepes to approximately 10^7 cells/mL.

Fusions were performed with P3 Ag8 × 63.653 myeloma cells, an immunoglobulin nonsecreting mutant of P3 Ag8 × 63 (Kearney et al., 1979). Log-phase myeloma cells were suspended in DMEM–Hepes, and the spleen cells and myeloma cells were mixed in a ratio of 5:1. The mixture was pelleted at 250*g* for 5 min, and the cell pellet was gently suspended in 30% PEG 1000 (w/v) in DMEM–Hepes and incubated for 4 min at room temperature. The suspension was centrifuged at 100*g* for 3 min and then DMEM–Hepes buffer added slowly to dilute the PEG. After 5 min, the cells were again centrifuged and resuspended in HT medium. After incubation for 24 h at 37 °C in HT medium, methotrexate (10^{-6} M) was added to select hybrid cells and the incubation continued at 37 °C for a total of 72 h.

Viable cells were obtained by centrifugation at 6.4% Ficoll–10% Hypaque and collected from the interface. Cells were washed by centrifugation and diluted in HT medium containing methotrexate to approximately 4 × 10^5 cells/mL. Aliquots of 0.1 mL were plated in 96-well flat-bottom microtiter plates and grown at 37 °C. As soon as definite colonies were visible, supernatants were assayed by a solid-phase immunoradiometric assay. Wells that were positive for antibodies to vWF were retained, and 2–3 weeks after fusion, cells were cloned at limiting dilution in HT medium. One to six clones from each line producing antibody were selected.

Sixteen independent cloned hybridoma lines were prepared. Seven of these lines were further recloned. Cells were subsequently injected intraperitoneally into Balb/c mice that had been primed by intraperitoneal injection of 0.5 mL of pristane 1 week earlier. Ascites fluid was collected 10–20 days after the cells were injected.

Solid-Phase Immunoradiometric Assays. Poly(vinyl chloride) 96-well microtiter plates (Dynatech, Alexandria, VA) were incubated with 100 μL/well purified bovine vWF (1 μg/mL) in PS for approximately 16 h at 4 °C. The solution was removed, and the plates were washed with SPIRA buffer. Excess protein-binding sites were blocked by incubation of the plates for at least 2 h at room temperature with SPIRA buffer containing an additional 3% BSA and 3% normal goat serum. The plates were subsequently washed with SPIRA buffer and stored at 4 °C in a humid chamber.

For assay of culture supernatants, ascites fluids, or mouse sera, the antibody-containing samples were diluted in SPIRA buffer and 100-μL aliquots were placed in the wells of the vWF-coated plates. After incubation for at least 16 h at 4 °C, the samples were aspirated and the plates washed 5 times with SPIRA buffer (200 μL/well). ^{125}I-Labeled goat-anti-mouse IgG (immunochemically purified) was added to each well and incubated for at least 2 h at 37 °C, and then the plates were washed 5 times with SPIRA buffer. After being dried at 37 °C for 30 min, the plates were sliced with a hot wire and each well was counted in a 16-well Nuclear Enterprises γ counter. Assays were performed in a similar manner with microtiter plates coated with human vWF or with bovine fibrinogen, fibronectin, or serum albumin to determine the specificty of binding.

Antibody Subtyping. Supernatants from cultures of each of the recloned cell lines were precipitated at 4 °C by addition of an equal volume of saturated ammonium sulfate. The IgG-containing precipitates were dissolved in Tris–saline buffer to 20% of the initial volume and dialyzed against this buffer overnight.

Subtyping was performed by double immunodiffusion on microscope slides coated with 1% agar in borate buffer, pH 8.6. Each sample was tested against selected antisera directed against IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM and κ and λ chains (Litton Bionetics, Inc., Charleston, SC). Normal mouse serum was used as a reference, as were murine myelomas of each subclass to validate the antiserum specificity. After diffusion for 48 h at room temperature, the slides were washed and stained with Amido Black.

Subtyping of antibody bound to antigen was confirmed with an ELISA using polystyrene microtiter plates coated with either bovine vWF (5 μg/mL) or goat-anti-mouse immunoglobulins with reagents supplied by Boehringer-Mannheim (Indianapolis, IN).

Purification of IgG from Ascites Fluids. Ascites fluids from mice injected with each of the recloned cell lines were centrifuged to remove cells and debris and then precipitated by adding an equal volume of saturated ammonium sulfate at 4 °C. The IgG-containing precipitates were redissolved in 0.02 M Tris, pH 7.2, to 20% of the initial volume and dialyzed against this buffer. Approximately 2 mL of the dissolved precipitates were applied to 7-mL columns of DEAE-Affi-Gel Blue, equilibrated with 0.02 M Tris, pH 7.2, as described by Bruck et al. (1982). The columns were eluted with gradients of 100 mL of 0.02 M Tris, pH 7.2, and 100 mL of 0.02 M Tris and 0.1 M NaCl, pH 7.2. The IgG eluted near the middle of each gradient. Other proteins were subsequently eluted by washing the columns with 0.02 M Tris and 1.0 M NaCl, pH 7.2.

Recognition of Denatured vWF by Monoclonal Antibodies. Bovine vWF was denatured by incubation with 1% $NaDodSO_4$ in the absence or presence of 10 mM dithiothreitol. Nitro-

cellulose sheets were then incubated in these two solutions, washed, and blocked by incubation in SPIRA buffer containing 3% BSA and 1% normal goat serum. Dilutions of the purified monoclonal antibodies were spotted on these sheets and incubated, and then the sheets were washed extensively. Bound antibody was detected by incubation with ^{125}I-labeled goat-anti-mouse IgG and subsequent autoradiography.

Digestion of vWF with Plasmin. Human plasmin was added to bovine vWF to a concentration of 0.5 CTA unit/mg of vWF[2] and incubated at 37 °C in PS. Digestion was stopped by the addition of soybean trypsin inhibitor to a final concentration of 0.25 mg/mL.

Preparation of Formalin-Fixed Platelets. Platelet concentrates that were less than 24 h old were provided by the Penn-Jersey Regional Red Cross Blood Program. The platelet concentrates were formalin-fixed as described (Kirby, 1982). The formalin-fixed platelets were stored at −90 °C in PS containing 25 mg/mL BSA.

Binding Studies. Frozen formalin-fixed platelets were thawed, kept at room temperature for the 1–2 h needed for most experiments, and then prewarmed to 37 °C 10 min prior to doing binding studies. All binding analyses were performed in 1.5-mL Eppendorf tubes in the presence of BSA (10 mg/mL), to decrease nonspecific binding. The proteins were diluted in PS containing 1% BSA, added to the tubes, and adjusted to 300 μL with the same buffer. A suspension of formalin-fixed platelets (700 μL) was added to give a final concentration of 2.2×10^8 platelets/mL. Tubes were inverted to mix the contents and then incubated at 37 °C for 6 min without stirring. The platelets were pelleted by centrifugation for 2 min at 12000*g* (Eppendorf Model 3200 centrifuge equipped with a swinging-bucket rotor). Supernatants were removed with siliconized Pasteur pipets that had been drawn out to fine capillaries. The tip of the centrifuge tube was cut off with a razor blade, and supernatants and pellets were counted for ^{125}I γ emission (Nuclear Enterprises Model 1600 γ counter).

Agglutination Studies. Platelet agglutination studies were performed on a Payton dual-channel aggregometer as previously described (Kirby, 1982).

Immunoblotting Procedure. Proteins were electrophoresed on 7.5% $NaDodSO_4$–polyacrylamide gels. The proteins were transferred to nitrocellulose (Towbin et al., 1979) at 100 mA (30 V) for 24 h at room temperature. Nonreacted sites of the nitrocellulose were blocked by incubation in PS containing 3% BSA for 1 h at room temperature with gentle shaking. The nitrocellulose sheets were incubated with each monoclonal antibody in SPIRA buffer for 24 h at 4 °C with mixing and then with ^{125}I-labeled goat-anti-mouse IgG. The blots were washed between antibody incubations with three $^1/_2$-h washings with SPIRA buffer.

Gel Chromatography. Gel filtration columns (Sepharose CL-6B) were standardized with bovine IgM (M_r 1×10^6), bovine thyroglobulin (M_r 670000), bovine ferritin (M_r 450000), and human IgG (M_r 150000).

Results

Digestion of vWF by Plasmin. When the time course of plasmin digestion of ^{125}I-labeled vWF was analyzed, a stepwise decrease in higher molecular weight multimers was observed by $NaDodSO_4$–agarose gel analysis of the unreduced protein (Figure 1). At 24 h, a predominant fragment was present whose relative mobility was slightly less than that of the intact vWF subunit. An autoradiogram of the nonreduced samples analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis is shown in Figure 2. Native vWF did not enter the stacking gel, due to its high molecular weight. However, as time of proteolysis increased, fragments of vWF small enough to enter the gel were observed. After 24 h of proteolysis, plasmin cleaved vWF into several fragments ranging in M_r from 250000 to 35000. The same fragments, and no others, were also visualized by Coomassie staining (data not shown). The major fragment had a M_r of 250000 and accounted for more then 60% of the total protein. Four smaller fragments could be seen on autoradiograms or stained gels. The pattern of fragments seen in a 24-h digest was unaltered after 48 h of digestion or when additional plasmin was added (data not shown). Therefore, the five fragments of the 24-h digest shown in Figure 2 were terminal degradation products.

Aliquots of each time point were tested for platelet agglutinating activity (Figure 3). Platelet agglutinating activity was unaltered at the early stages of digestion (up to 60 min). $NaDodSO_4$–acrylamide and agarose gels showed that, even

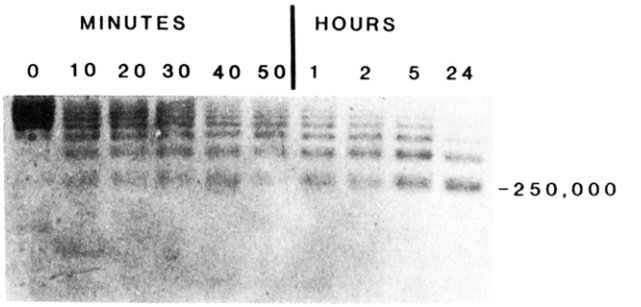

FIGURE 1: Time course of plasmin digestion, analyzed by $NaDodSO_4$–agarose gel electrophoresis. Bovine vWF (0.75 mg/mL) was incubated with plasmin. At the indicated times, aliquots were removed and electrophoresed on a 2% $NaDodSO_4$–agarose gel. The gel was stained with Coomassie Blue.

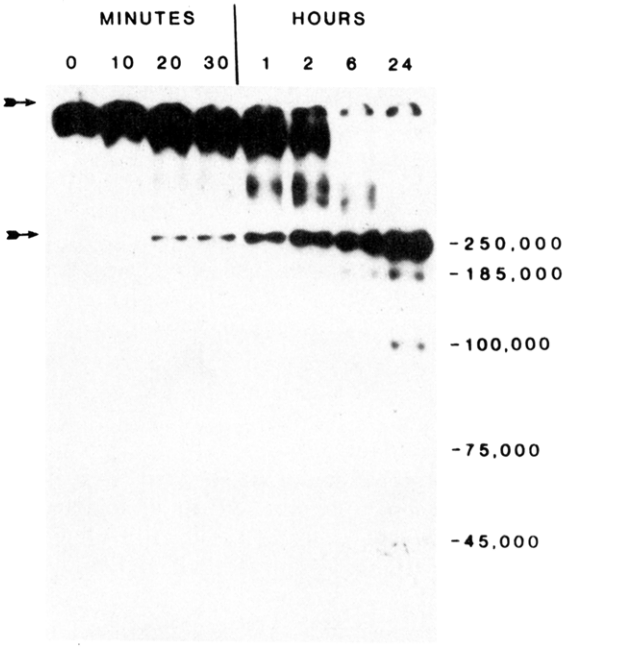

FIGURE 2: Time course of plasmin digestion, analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. ^{125}I-Labeled bovine vWF (0.45 mg/mL) was incubated with plasmin at 37 °C. At the indicated times, 20-μL samples were electrophoresed. An autoradiogram of the gel is shown. Arrows at the left indicate the top of the stacking gel and the top of the running gel.

[2] One CTA unit (Committee on Thrombolytic Agents unit) is equal to 1.1 Remmert and Cohen units, which is equivalent to the liberation of 450 μg of tyrosine equivalents/h from casein at 37 °C.

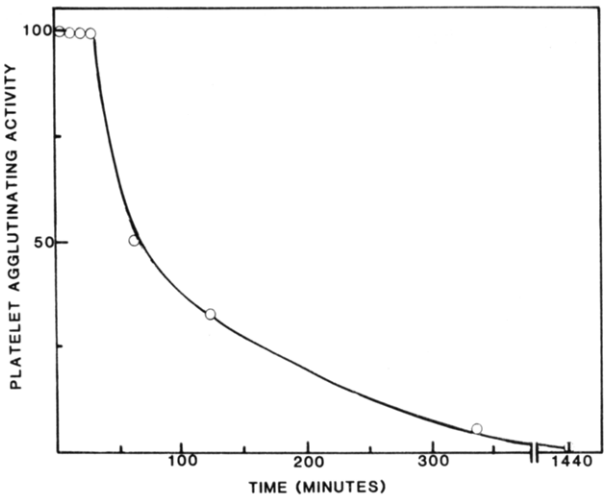

FIGURE 3: Time course of plasmin digestion, analyzed by platelet agglutination. ^{125}I-vWF (0.1 mg/mL, 1×10^6 cpm/mL) was digested with plasmin. Samples (2.2 μg) were taken at intervals and assayed for their ability to agglutinate formalin-fixed platelets (2.2×10^8 platelets/mL).

after 2 h of digestion, a significant portion of vWF still existed as high molecular weight oligomers, consistent with the hypothesis that the oligomeric forms of vWF are required for platelet agglutination. After 24 h of digestion, the vWF did not agglutinate platelets.

Isolation of the Major Plasmic Fragment of vWF. In order to separate the fragments produced by plasmin, a 24-h plasmin digest was chromatographed on a Bio-Gel A-1.5 m column (Figure 4). Five peaks eluted from the column. Fractions of each peak were pooled and analyzed on nonreduced Na-DodSO_4–polyacrylamide gels. An autoradiogram of the gel showed that the protein in peak 1 did not enter the stacking gel, suggesting that the protein in peak 1 could be vWF that had been only partially digested. Peak 2 contained predominantly the M_r 250 000 fragment. Peak 3 contained the M_r 185 000, 100 000, and 45 000 fragments. Peak 4 contained the M_r 75 000 fragment. Peaks 1–4 contained 9%, 68%, 18%, and 5%, respectively, of the total protein-bound radioactivity eluted from the column. Peak 5 contained soybean trypsin inhibitor that had been added to arrest proteolysis. No low molecular weight radioactive fragments were observed.

Characterization of the M_r 250 000 Fragment. Analysis of the reduced M_r 250 000 fragment on Na-DodSO_4–polyacrylamide gels demonstrated five polypeptide chains, ranging in M_r from 120 000 to 35 000 (Figure 5). The M_r 120 000 chain seen in the preparation used in Figure 5 was an intermediate product, since with further digestion (not shown), it disappeared. The M_r 120 000, 69 000, and 49 000 polypeptide chains each stained with periodate–Schiff reagent, indicating the presence of carbohydrate.

The major plasmin fragment (M_r of 250 000 by Na-DodSO_4–polyacrylamide gel electrophoresis) eluted from the gel filtration column at a M_r of 1×10^6 in PS (Figure 6A). In higher ionic strength (0.25 M Na_2SO_4), the fragment eluted from the column with an apparent M_r of 250 000 (Figure 6B). The M_r selectivity of the column was not altered by the high salt; human IgG eluted at the same volume in 0.15 M NaCl or 0.25 M Na_2SO_4. Peak fractions of the column in Figure 6B were pooled and dialyzed against PS and reapplied to the same column equilibrated in PS. The fragment then eluted at an apparent M_r of 1×10^6. This reassociation of the fragments occurred at relatively low protein concentration (7 μg/mL), indicating a high affinity for association between fragments.

Measurement of Binding of the M_r 250 000 Fragment to Platelets. In order to determine whether the platelet binding site was present on the M_r 250 000 fragment, binding studies were performed (Figure 7). The data indicate that the M_r 250 000 fragment binds well to platelets. Addition of native vWF inhibited approximately 90% of the binding of the

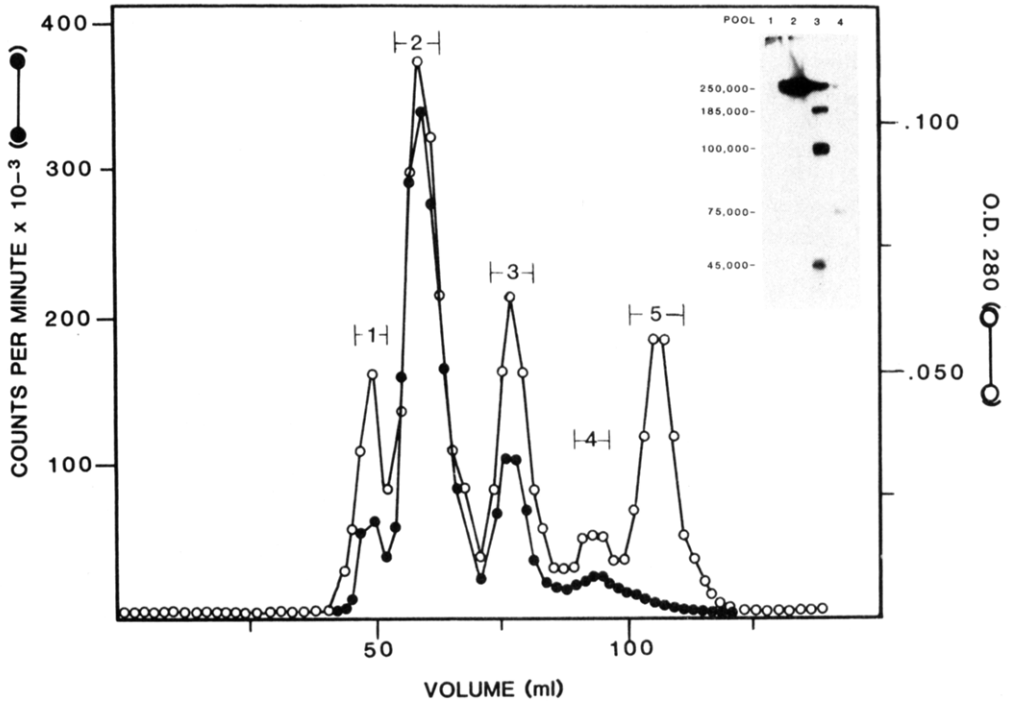

FIGURE 4: Analysis of a 24-h plasmin digest of vWF by molecular exclusion chromatography on A-1.5m. ^{125}I-vWF (0.8 mg/mL, 1×10^6 cpm/mL) was digested with plasmin for 24 h. The digest was dialyzed against 0.05 M phosphate, 0.5 M NaCl, and 0.02% NaN_3, pH 7.0, and applied to a 1.5 × 60 cm Bio-Gel A-1.5 m column. Fractions of each peak were pooled as indicated by the bars. Aliquots of each pool were analyzed by electrophoresis on nonreduced Na-DodSO_4–polyacrylamide gels and autoradiography (inset). The presence of the band at 250 000 in lane 3 was primarily due to contamination by the sample in lane 2.

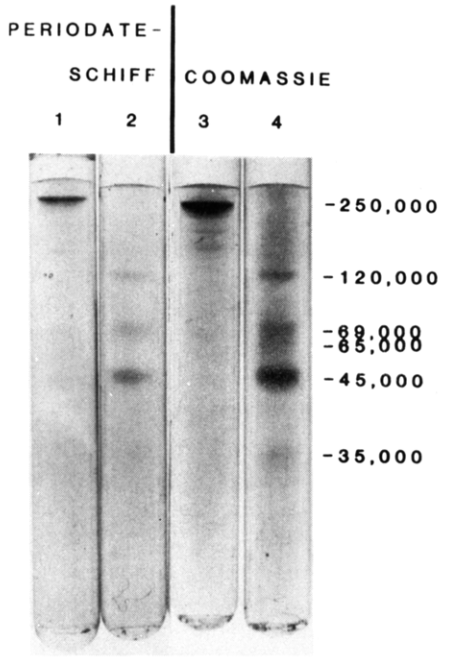

FIGURE 5: Analysis of the M_r 250 000 fragment on nonreduced and reduced $NaDodSO_4$–polyacrylamide gels. Isolated M_r 250 000 fragment (20 μL, 9 μg) was electrophoresed nonreduced on gels 1 and 3 and reduced on gels 2 and 4 with 5% $NaDodSO_4$–polyacrylamide gels. The gels were stained with either Coomassie Blue (gels 1 and 2) or periodate–Schiff stain (gels 3 and 4).

fragment. In contrast, BSA had no effect on the fragment's ability to bind to platelets. In order to compare the binding of the M_r 250 000 fragment to that of native vWF, the M_r 250 000 fragment, native vWF, and human fibrinogen were analyzed for their ability to complete with labeled, native vWF for binding to platelets (Figure 8). It required approximately 2.2 μg of native vWF for 50% inhibition of binding of ^{125}I-vWF to platelets. In contrast, it required approximately 22 μg of the M_r 250 000 fragment to inhibit binding of the ^{125}I-vWF to platelets by 50%. Fibrinogen did not inhibit binding of vWF to platelets. These results indicate that the apparent affinity of the M_r 250 000 fragment for binding to platelets is one-tenth that of native vWF and that, at suitable molar ratios of the M_r 250 000 fragment to platelet binding sites, saturable binding can be observed.

Immunological Characterization of vWF and the M_r 250 000 Fragment. Sixteen independent primary hybridomas were selected from four different fusions of spleen cells from mice immunized with bovine vWF. These were selected and recloned on the basis of binding of secreted antibody to bovine vWF in the solid-phase immunoassay. No specific binding of any of the antibodies was observed to wells coated with bovine fibrinogen or bovine fibrinonectin.

The selection from different fusions and initial limiting dilutions ensured that each hybridoma was derived from a distinct fusion event and that the hybridoma lines were distinct from one another. The fact that several similar antibodies were derived from the same fusion procedure (see Table I) suggests that their similarity could have been derived from clonally related spleen cells of an individual mouse. However, differences between the antibodies in each class were apparent (see below).

The characteristics described in Table I and subsequent competitive binding experiments allowed the preliminary grouping of the 16 hybridoma cell lines into five general specificity classes (A–E). Representatives of each of these classes (cell lines numbered 1, 6, 10, 16, 2, 7, and 9) were

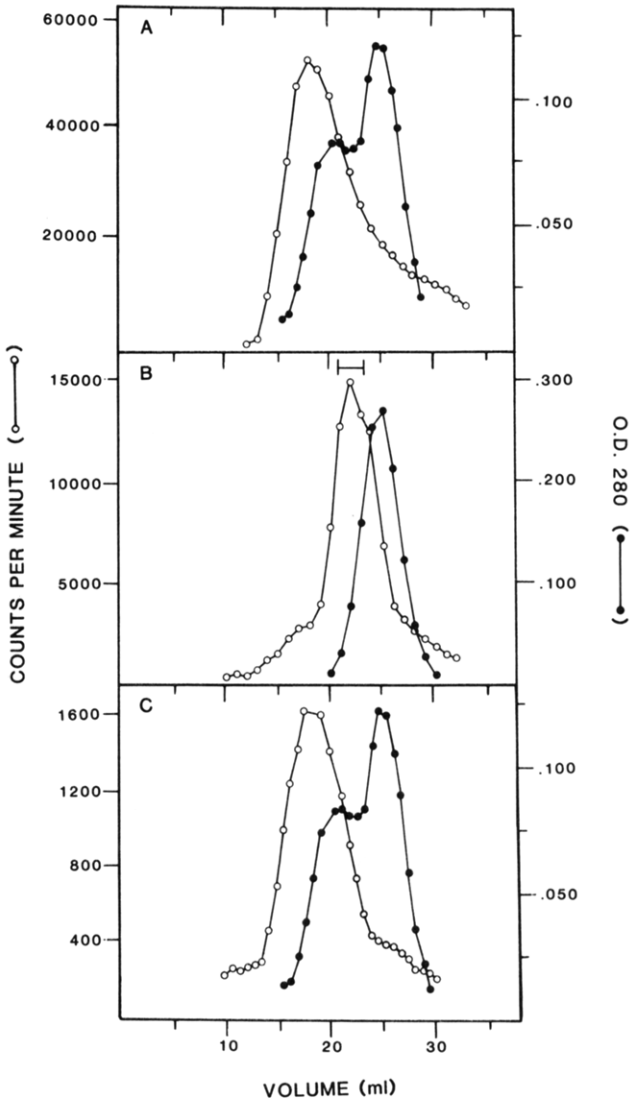

FIGURE 6: Elution profile of M_r 250 000 fragment under different ionic conditions. (A) The ^{125}I-labeled M_r 250 000 fragment (109 μg, 700 000 cpm) in PS was mixed with molecular weight markers of bovine thyroglobulin (400 μg) and human IgG (800 μg). This sample was applied to a 0.9 × 50 cm Sepharose CL-6B column equilibrated in PS. The presence of the standard proteins did not alter the elution properties of the vWF fragment. (B) The ^{125}I-labeled M_r 250 000 fragment (40 μg, 250 000 cpm) was mixed with human IgG (1.2 mg) and dialyzed against 0.1 M phosphate and 0.25 M Na_2SO_4, pH 7.0. The proteins were applied to a 0.9 × 50 cm Sepharose CL-6B column equilibrated with the same buffer. Fractions were pooled as indicated by the bars. (C) Peak fractions (7 μg, 40 000 cpm) from the column shown in Figure 6B were pooled and dialyzed against PS. They were then mixed with bovine thyroglobulin (400 μg) and human IgG (800 μg) and applied to a 0.9 × 50 cm Sepharose CL-6B column equilibrated in PS.

recloned and characterized further.

All of the antibodies had κ light chains. More than one heavy chain subclass was present in the class A antibodies. This was found consistently through repeated subcloning. The myeloma P3 × 63Ag8.653 demonstrated reversion to secretor status when reexamined. The class A antibodies thus have heavy chains of IgG2a subclass of immune cell origin that are scrambled with those produced by P3 × 63Ag8. The other cell lines may also be producing nonimmune IgG, so all subtyping results were confirmed by using vWF-coated plates to capture the immune IgG in ELISA tests.

Each of the recloned antibodies to bovine vWF and antibody H-9 of Meyer et al. (1984a) were labeled with ^{125}I. The ability of unlabeled antibodies to compete with these labeled anti-

Table I: Antibody Characteristics[a]

antibody class	cell line	fusion no.	titer ($\times 10^{-6}$)	subclass	recognition of: human vWF	recognition of: denatured vWF	recognition of: reduced denatured vWF	inhibition of platelet agglutination
A	1	13	3.0	IgG1, IgG2a		+		
	3	13	4.6	IgG1, IgG2a				
	4	13	5.2	IgG1, IgG2a				
	5	13	2.2	IgG1, IgG2a				
	6	13	2.2	IgG1, IgG2a	+	+		
	8	13	2.5	IgG1, IgG2a				
B	10	20	25	IgG1	+	+		
	11	20	20	IgG1				
	12	20	15	IgG1				
	13	20	8.5	IgG1				
	14	20	8.4	IgG1				
	15	20	24	IgG1				
	16	20	14	IgG1		+		
C	2	16	*b*	IgG2b	+			+
D	7	16	3.8	IgG1	+	+	+	
E	9	12	9.0	IgG1		+		

[a] Antibody titer was defined as the dilution of ascites fluid that gave 50% of maximal binding in a solid-phase immunoradiometric assay. Recognition of human vWF was done by solid-phase radioimmunoassay. Recognition of denatured vWF was done on nitrocellulose paper coated with vWF that had been denatured with $NaDodSO_4$ in the absence or presence of 10 mM dithiothreitol. [b] Weak reaction in solid-phase immunoradiometric assay.

Table II: Competitive Binding of Monoclonal Antibodies to vWF[a]

class	labeled antibody	competing unlabeled antibody: 1	6	10	16	2	7	9	H-9
A	1	++	++	++	–	–	–	–	–
	6	++	++	++	–	–	–	–	–
B	10	+/–	+/–	++	+	–	–	–	–
	16	–	–	++	++	–	–	–	–
C	2	–	–	–	–	++	–	–	–
D	7	–	–	–	–	–	++	–	–
E	9	–	–	–	–	–	–	++	–
	H-9	+	+/–	+	+/–	–	–	–	++

[a] Each of the indicated monoclonal antibodies was labeled with ^{125}I and then mixed with buffer or with dilutions of each of the unlabeled monoclonal antibodies. Duplicate aliquots of these mixtures were added to wells of microtiter plates coated with vWF and were incubated overnight at 4 °C. The plates were then washed, and the radioactivity in each well was measured. (++ = complete inhibition of binding of labeled antibody by unlabeled antibody, + = extensive, but not complete, inhibition, +/– = weak inhibition, and – = no inhibition.)

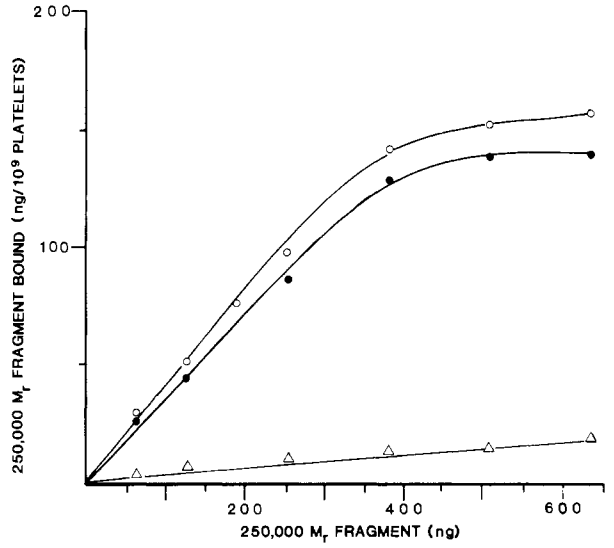

FIGURE 7: Saturable binding of M_r 250 000 fragment to platelets. Formalin-fixed platelets were incubated with the ^{125}I-labeled M_r 250 000 fragment in the presence (△) or absence (○) of 50 μg/mL native vWF for 6 min at 37 °C. Platelets were centrifuged, and the pellets and supernatants were counted to determine the amount of radioactivity bound to platelets. The amount of the M_r 250 000 fragment bound in the presence of excess, native vWF was subtracted from the amount bound in the absence of vWF in order to quantitate specific binding (●). The points represent the average of duplicate determinations.

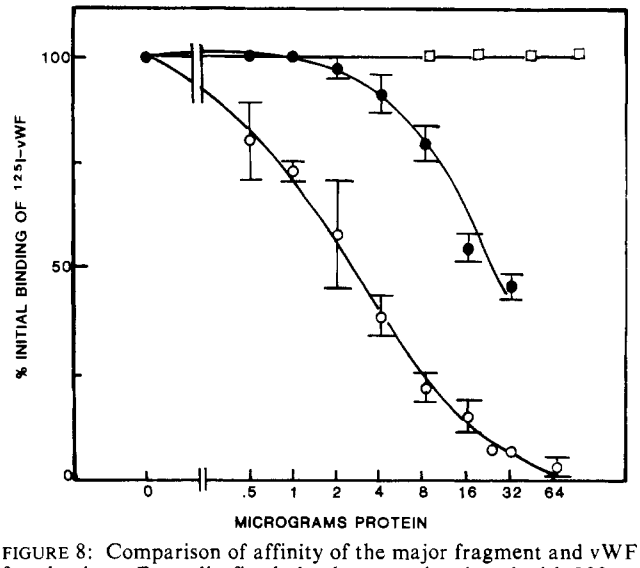

FIGURE 8: Comparison of affinity of the major fragment and vWF for platelets. Formalin-fixed platelets were incubated with 300 ng of ^{125}I-labeled native vWF in the presence of either native vWF (○), M_r 250 000 fragment (●), or human fibrinogen (□) for 6 min at 37 °C. Incubation mixtures were centrifuged, and the platelet pellets and supernatants were counted to determine the amount of ^{125}I-vWF bound to platelets (2.2×10^8 platelets/mL).

bodies for binding to vWF was examined. The competition for binding in classes A and B is shown in Figure 9. Com-

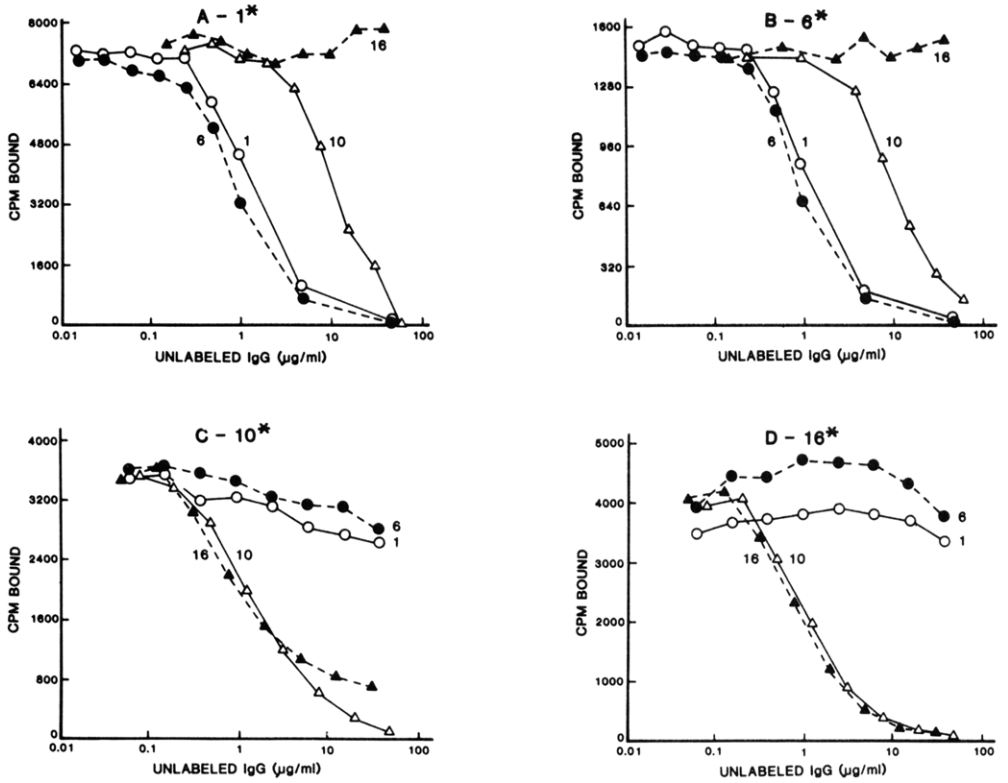

FIGURE 9: Competitive binding of monoclonal antibodies. ^{125}I-Labeled antibodies were mixed with dilutions of unlabeled antibodies and incubated on microtiter plates coated with bovine vWF (2 μg/mL). Plates were then washed, and each well was counted to determine the amount of labeled antibody bound. Competition of unlabeled antibody 1 (○), antibody 6 (●), antibody 10 (△), and antibody 16 (▲) with (A) ^{125}I-labeled antibody 1, (B) ^{125}I-labeled antibody 6, (C) ^{125}I-labeled antibody 10, and (D) ^{125}I-labeled antibody 16 is shown. The other unlabeled monoclonal antibodies tested (2, 7, 9, and H-9) did not compete. Values are averages from duplicate plates.

petition with the other classes was negative (Table II). Antibodies 1 and 6 of class A behaved similarly in competing with either 1 or 6 and not with the class B antibodies 10 and 16. Indeed, in similar experiments (data not shown), all five class A antibodies competed with antibodies 3, 4, and 6, suggesting specificity for the same or closely related epitopes. Antibodies 10 and 16 of class B competed effectively with each other (Figure 9). Only 10 was able to compete with 1 and 6, although it required a 10-fold higher concentration. In separate experiments, all of the class B antibodies competed with labeled antibodies 10, 11, and 14. Competition with other classes was negative (Table II). These data indicate that all of the class A antibodies are directed against the same epitope, or sterically proximate epitopes, and all of the class B antibodies are directed against a different set of epitopes. The partial competition with antibodies 1 and 6 exhibited by antibody 10 suggests that these two epitope sets (A and B) may not be too distant from one another on the vWF molecule. Antibody H-9 to human vWF could be partially displaced by antibodies of both classes A and B, but the binding of H-9 to bovine vWF was sufficiently weak that the reciprocal inhibition could not be demonstrated.

Immunoblotting studies indicated that six out of seven monoclonal antibodies tested recognized the M_r 250 000 fragment (Figure 10). The fragment was not recognized by monoclonal antibody 16, although this antibody recognized native vWF under these conditions. Minor degradation products of the M_r 250 000 fragment that were too faint to be seen by staining with Coomassie Blue were also recognized by some of the monoclonal antibodies. Monoclonal antibody 7, which binds to vWF that has been reduced in the presence of $NaDodSO_4$, recognized both the M_r 69 000 and 49 000 chains of the M_r 250 000 fragment, suggesting that these chains may arise by alternative cleavages of the same region of the

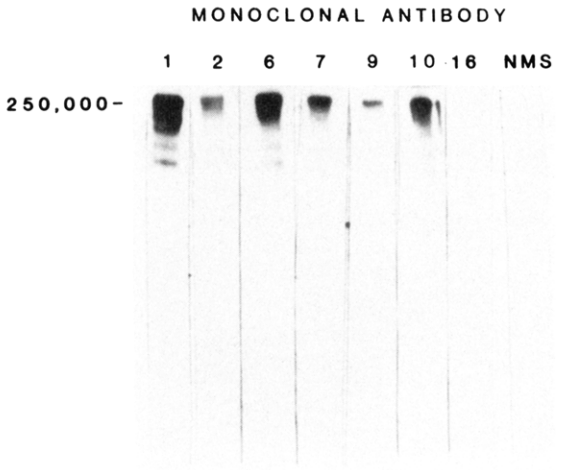

FIGURE 10: Localization of epitopes for monoclonal antibodies on vWF fragment. The purified M_r 250 000 fragment was electrophoresed on a 7.5% nonreduced $NaDodSO_4$–polyacrylamide gel. The protein was transferred to nitrocellulose, and strips were incubated individually with the indicated monoclonal antibodies (1 μg/mL) or normal mouse serum (NMS) (100 μL in 100 mL of SPIRA buffer). The strips were then incubated with ^{125}I-labeled goat-anti-mouse IgG antibody (1×10^6 cpm) in 100 mL of SPIRA buffer, washed, dried, and autoradiographed.

polypeptide chain in the subunits of oligomeric vWF. Monoclonal antibody 2, which inhibits platelet agglutination by bovine vWF, bound to the M_r 250 000 fragment.

Effect of Monoclonal Antibodies on the Interaction of vWF and the M_r 250 000 Fragment with Platelets. Preincubation of the ^{125}I-labeled M_r 250 000 fragment with antibody 2 inhibited its binding to formalin-fixed human platelets in a dose-dependent manner (Figure 2). Monoclonal antibody H-9, which inhibits the ristocetin cofactor activity of human vWF

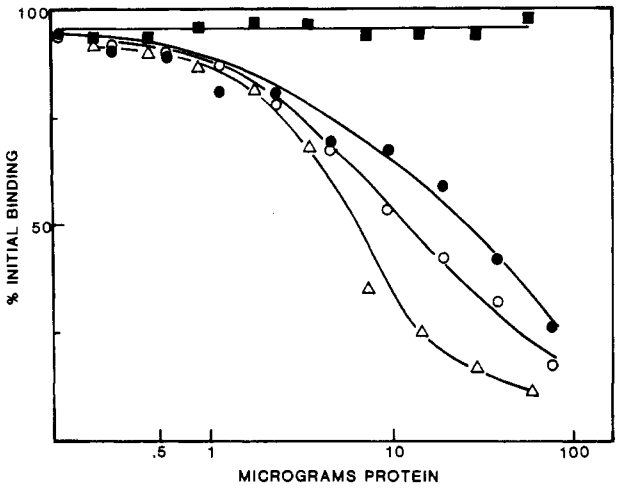

FIGURE 11: Inhibition of binding of M_r 250 000 fragment and vWF to platelets by monoclonal antibodies. The ^{125}I-labeled M_r 250 000 fragment (100 ng) was incubated with monoclonal antibody 2 (○), H-9 (△), or 16 (■), and ^{125}I-vWF (2 μg) was incubated with monoclonal antibody 2 (●) for 16 h at 4 °C. Formalin-fixed platelets (4.4×10^8 platelets/mL) were added, and the mixtures were incubated at 37 °C for 6 min. Platelets were centrifuged, and the pellets and supernatants were counted to determine the amount of radioactivity bound to platelets. In order to determine nonspecific binding, the ^{125}I-labeled M_r 250 000 fragment and ^{125}I-vWF were incubated with unlabeled, native vWF (40 μg) under the same conditions as with the monoclonal antibodies. The amount of either protein that bound in the presence of an excess of vWF was subtracted from the other values. Nonspecific binding was approximately 10–15% of the total binding of the protein to platelets in the absence of monoclonal antibody.

(Meyer et al., 1984b), and which also inhibits platelet adhesion to exposed subendothelium (Meyer et al., 1984b), also inhibited binding of the M_r 250 000 fragment to platelets in a dose-dependent manner (Figure 11) and was more efficient in decreasing binding of the M_r 250 000 fragment than was antibody 2. Monoclonal antibody 16, which does not recognize the M_r 250 000 fragment, did not inhibit binding of the fragment to platelets.

Antibody 2 also inhibited the binding of native vWF to platelets (Figure 11). In experiments where high concentrations of each of the monoclonal antibodies were tested for inhibition of ^{125}I-vWF binding to platelets (Table III), only antibodies 2 and H-9 caused significant inhibition. These two antibodies also inhibited the agglutination of platelets by bovine vWF, although high concentrations were required and inhibition was only partial.

Discussion

The major fragment in a terminal plasmin digest of bovine vWF has a M_r of 250 000 and consists of four disulfide-bonded chains, ranging in M_r from 69 000 to 35 000. It accounts for 60–70% of the total protein in a terminal digest. At physiological ionic strength, the fragment is noncovalently associated into higher molecular weight oligomers, apparently tetramers. The tetramers are dissociated by high ionic strength, suggesting that the forces holding the fragments together are electrostatic. This dissociation is also reversible, since removal of high salt causes the fragments to reassociate. The fragments reassociate at relatively low protein concentration (7 μg/mL), indicating a high affinity of association between fragments. This association may reflect a high affinity between subunits in the native molecule and may play a role in the assembly of subunits in vivo.

Analysis of the effect of plasmin digestion on the functional capacity of vWF to mediate platelet interaction indicates that activity remains after 60 min or more of digestion. A significant portion of the vWF remains as high molecular weight oligomers ($M_r > 1 \times 10^6$) for at least 2 h of digestion. More extensive digestion, however, destroys platelet agglutination activity. This is consistent with the observation that high molecular weight oligomers are necessary for platelet agglutination (Doucet-de Bruine et al., 1978; Gralnick et al., 1981; Ohmori et al., 1983) but is inconsistent with the data of Senogles and Nelsestuen (1983), which suggests that any process that brings the surface membranes of platelets into proximity will allow expression of vWF activity. The large size of vWF may be crucial to span the distance between platelets imposed by the electrostatic repulsion of the platelets (Coller, 1983). The inability of the M_r 250 000 fragment to agglutinate platelets could be due to the presence of only a single, intact binding site. Alternatively, more than one binding site could be present on this fragment, but the intervening distance of these sites may not be large enough to span the separation of two platelets. Even though the M_r 250 000 fragment appears to exist as a tetramer (apparent M_r 1×10^6), the noncovalent forces holding the fragments together may not be strong enough to form a bridge between platelets.

Table III: Effect of Monoclonal Antibodies on Binding of vWF to Platelets[a]

antibody class	antibody no.	antibody concn (μg/mL)	% vWF bound
	H-9	102	6.6
C	2	47	14.1
A	1	45	24.0
	6	44	22.0
D	7	30	23.3
E	9	17	21.7
B	10	58	29.8
	16	36	28.1
nMS			21.4
buffer control			21.1

[a] ^{125}I-Labeled bovine vWF was incubated at 23 °C with the indicated concentrations of each monoclonal antibody or with buffer as a control. After 4 h, samples of each incubation mixture were added to 0.7 mL of formalin-fixed platelets (approximately 2.5×10^8 platelets/mL), incubated at 37 °C for 5 min, and centrifuged to determine the percent of labeled vWF bound to the platelets.

Binding of the M_r 250 000 fragment to platelets was specific, since native vWF could inhibit binding of the fragment but albumin could not. Since binding was measured by using metabolically inactive platelets in phosphate buffer, the binding was probably to the glycoprotein Ib associated vWF receptor. It is not possible to compare the affinity of the M_r 250 000 fragment to that of native vWF by using a direct binding assay. Native vWF is highly multimeric and contains multiple subunits (and therefore multiple binding sites) on one molecule. If one subunit of an oligomer binds to a receptor, all the subunits on that oligomer pellet with the platelets, so it cannot be determined how many subunits are actually interacting with receptors. Association constants and numbers of binding sites also cannot be calculated for native vWF by using this assay. Therefore, in order to compare the affinity of the M_r 250 000 fragment to native vWF, competition studies were performed (Figure 8). The fragment inhibited binding of intact vWF to platelets, but albumin and fibrinogen did not inhibit. However, the fragment's ability to inhibit binding was only one-tenth that of native vWF, suggesting a decreased affinity. This could be due to proteolytic alteration of the binding site or the need for multivalent binding of covalently linked vWF subunits for high affinity.

Monoclonal antibodies 2 and H-9, which inhibit platelet agglutination by bovine vWF, bind to the M_r 250 000 frag-

ment. Both of these antibodies inhibit binding of the M_r 250 000 fragment, as well as native vWF, to platelets. This suggests that the M_r 250 000 fragment carries the platelet binding domain of vWF. The size of the fragment, and the recognition by H-9, suggests that it may be analogous to fragment Sp III of human vWF described by Girma et al. (1986).

The inability of antibodies 2 and H-9 to compete with one another for binding to vWF and their differing capacities to inhibit platelet-associated activities of vWF indicate that these activities may require the functional participation of two different molecular loci on the vWF molecule. Similar distinctions have been made by Bowie et al. (1983), who analyzed several monoclonal antibodies to porcine vWF for inhibition of ristocetin cofactor activity and for their effects on in vitro and in vivo bleeding times. Meyer et al. (1984a) characterized several antibodies to human vWF and found five that had spatially distinct epitopes and inhibited either ristocetin cofactor activity or platelet adhesion to subendothelium. In the studies of Meyer, H-9 inhibited both activities, antibody B2A inhibited only ristocetin cofactor activity, and C8, D3, and D10 inhibited only platelet adhesion. Other authors have also observed differences in the abilities of specific monoclonal antibodies to inhibit the activities of vWF related to its role in platelet adhesion (Stel et al., 1984; Ogata et al., 1983). Such studies have been extended to fragments of human vWF and validate the assignment of functional domains on the basis of binding of monoclonal antibodies. Antibodies that block binding of vWF to subendothelium, to platelet glycoprotein Ib, and to the platelet glycoprotein IIb/IIIa complex have been shown to recognize different structural regions of the vWF molecule (Girma et al., 1986). The value of monoclonal antibodies as structural probes may facilitate the analysis of the differences in structure and organization of bovine vWF that account for its distinctive properties.

This is the first report of the isolation of a discrete fragment of bovine vWF that can bind directly to platelets. This fragment may be useful both for identifying the regions of vWF involved in platelet binding and in identifying the platelet receptor for vWF.

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REFERENCES

Atichartakarn, V., Marder, V. J., Kirby, E. P., & Budzynski, A. Z. (1978) *Blood 51*, 281–297.

Baumgartner, H. R., Tschopp, T. B., & Meyer, D. (1980) *Br. J. Haematol. 44*, 127–139.

Bowie, W. J., Fass, D. N., & Katzmann, J. A. (1983) *Blood 62*, 146–151.

Bruck, C., Portelle, D., Glineur, C., & Bollen, A. (1982) *J. Immunol. Methods 53*, 313–319.

Caen, J. P., Nurden, A. T., Jeanneau, C., Michel, H., Tobelem, G., Levy-Toledano, S., Sultan, Y., Valensi, F., & Bernard, J. (1976) *J. Lab. Clin. Med. 87*, 586–596.

Coller, B. (1983) *Ann. N.Y. Acad. Sci. 416*, 693–708.

Coller, B. S., Peerschke, E. T., & Scudder, L. E. (1983) *Blood 61*, 99–110.

Counts, R. B., Paskell, S. L., & Elgee, S. K. (1978) *J. Clin. Invest. 62*, 702–709.

DeMarco, L., & Shapiro, S. S. (1981) *J. Clin. Invest. 68*, 321–328.

Doucet-de Bruine, M. H. W., Sixma, J. J., Over, J., & Beeser-Visser, N. H. (1978) *J. Lab. Clin. Med. 92*, 96–107.

Engvall, E., & Ruoslahti, E. (1977) *Int. J. Cancer 20*, 1–5.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Forbes, C. D., & Prentice, C. R. M. (1973) *Nature (London) 241*, 149–150.

Fujimura, Y., Titani, K., Holland, L. Z., Russell, S. R., Roberts, J. R., Elder, J. H., Ruggeri, Z. M., & Zimmerman, T. S. (1986) *J. Biol. Chem. 261*, 381–385.

Fulcher, C. A., & Zimmerman, T. S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1648–1652.

Girma, J. P., Kalafatis, M., Pietu, G., Lavergne, J. M., Chopek, M. W., Edgington, T. S., & Meyer, D. (1986) *Blood 67*, 1356–1366.

Gralnick, H. R., Williams, S. B., & Morisato, K. (1981) *Blood 58*, 387–397.

Howard, M. A., & Firkin, B. G. (1971) *Thromb. Diath. Haemorrh. 26*, 362–369.

Jaffe, E. A., Hoyer, L. W., & Nachman, R. L. (1973) *J. Clin. Invest. 52*, 2754–2764.

Katzmann, J. A., Mujwid, D. K., Miller, R. S., & Fass, D. N. (1981) *Blood 58*, 530–536.

Kearney, J. F., Radbruch, A., Liesengang, B., & Rajewsky, K. (1979) *J. Immunol. 123*, 1548–1550.

Kennet, R. H., Denis, K. A., Tung, A. S., & Klinman, N. R. (1978) *Curr. Top. Microbiol. Immunol. 81*, 77–91.

Kirby, E. P. (1982) *J. Lab. Clin. Med. 100*, 963–976.

Kirby, E. P., Martin, N., & Marder, V. J. (1974) *Blood 43*, 629–640.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–698.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Lynch, D. C., Williams, R., Zimmerman, T. S., Kirby, E. P., & Livingston, D. M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2738–2742.

Markwell, M. A. K., & Fox, C. F. (1978) *Biochemistry 17*, 4807–4817.

Martin, S. E., Marder, V. J., Francis, C. W., Loftus, L. S., & Barlow, E. H. (1980) *Blood 55*, 848–858.

Meyer, D., Zimmerman, T. S., Obert, B., & Edgington, T. S. (1984a) *Br. J. Haematol. 57*, 597–608.

Meyer, D., Baumgartner, H. R., & Edgington, T. S. (1984b) *Br. J. Haematol. 57*, 609–620.

Nachman, R. L., & Jaffe, E. A. (1975) *J. Exp. Med. 141*, 1101–1113.

Nachman, R. L., Levine, R., & Jaffe, E. A. (1977) *Thromb. Res. 11*, 433–438.

Ogata, K., Saito, H., & Ratnoff, O. D. (1983) *Blood 61*, 27–35.

Ohmori, K., Fretto, L. J., Harrison, R. L., Switzer, M. E. P., Erickson, H. P., & McKee, P. A. (1982) *J. Cell Biol. 95*, 632–640.

Owen, W. G., & Wagner, R. H. (1972) *Thromb. Diath. Haemorrh. 27*, 502–515.

Ruggeri, Z. M., & Zimmerman, T. S. (1980a) *J. Clin. Invest. 65*, 1140–1143.

Ruggeri, Z. M., & Zimmerman, T. S. (1980b) *J. Clin. Invest. 65*, 1318–1325.

Schullek, J., Jordan, J., & Montgomery, R. R. (1984) *J. Clin. Invest. 73*, 421–428.
Scully, M. F., & Kakkar, V. V. (1982) *Biochim. Biophys. Acta 700*, 130–133.
Senogles, S. E., & Nelsestuen, G. L. (1983) *J. Biol. Chem. 258*, 12327–12333.
Sixma, J. J., Sakariassen, K. S., Stel, H. V., Houdijk, W. P. M., In der Maur, D. W., Hamer, R. J., de Groot, P. G., & van Mourik, J. A. (1984) *J. Clin. Invest. 74*, 261–270.
Sola, B., Avner, P., Sultan, Y., Jeanneau, C., & Maisonneuve, P. (1982) *Proc. Natl. Acad Sci. U.S.A. 79*, 183–187.
Stel, H. V., Sakariassen, K. S., Scholte, B. J., Veerman, E. C. I., van der Kavast, T. H., de Groot, P. G., Sixma, J. J., & van Mourik, J. A. (1984) *Blood 63*, 1408–1415.
Suzuki, K., Nishioka, J., & Hashimoto, S. (1980) *Thromb. Res. 17*, 215–223.
Tobelem, G., Levy-Toledano, S., Bredoux, R., Michel, H., Nurden, A., & Caen, J. (1976) *Nature (London) 263*, 427–428.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Tuszynski, G. P., Knight, L., Piperno, J. R., & Walsh, P. N. (1980) *Anal. Biochem. 106*, 118–122.
Wagner, D. D., Olmstead, J. B., & Marder, V. J. (1982) *J. Cell Biol. 95*, 355–360.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Zimmerman, T. S., & Edgington, T. S. (1973) *J. Exp. Med. 138*, 1015–1020.
Zimmerman, T. S., & Ruggeri, Z. M. (1982) in *Progress in Haemostasis and Thrombosis* (Spaet, T. H., Ed.) pp 203–236, Grune and Stratton, New York.

Molecular Mechanism of Opioid Receptor Selection

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ABSTRACT: Preferred conformations, orientations, and accumulations of 26 opioid peptides on lipid membranes were estimated and compared with pharmacologic and selective binding data taken from the literature. Interaction with μ-receptors was governed by the net positive charge effective at the message domain of the agonist peptides z(eff) as the Boltzmann term $e^{z(\mathrm{eff})}$ that determines relative accumulation on anionic biologic membranes. Selection for δ-receptors was reduced by z(eff) and correlated with $e^{-z(\mathrm{eff})}$. Selection for κ-receptors was governed by the peptide amphiphilic moment $\vec{A}$. A pronounced scalar magnitude A and almost perpendicular orientation of the N-terminal message domain as an α-helix were favorable for κ-site selection. Potencies as κ-agonists and binding affinities correlated with $A \cdot e^{z(\mathrm{eff})}$. The classical site selectivity caused by the receptor requirements for a complementary fit of the agonist to the discriminator site is thus crucially supplemented by a selection mechanism based on peptide membrane interactions (membrane requirements). In the model presented here, the δ-site is exposed to the aqueous compartment surrounding the target cell at a distance comparable to or greater than the Debye–Hückel length and is in a cationic vicinity. The μ-site is exposed to the anionic fixed-charge compartment of the membrane in aqueous surroundings. The κ-site is buried in a more hydrophobic membrane compartment close to the fixed-charge compartment. The relative accumulation of the opioid message domains in these compartments is determined by the address domains and constitutes a major part of the site selection mechanism. The peptide amphiphilic moment, $\vec{A}$, emerged as a new, important parameter for predicting site selectivity and potency and determining peptide quantitative structure–activity relationships (QSAR).

Opioid peptides offer excellent examples for receptor selectivity (Kosterlitz & Paterson, 1985), an important phenomenon in nervous and endocrine regulation. Thus, [Leu]- and [Met]enkephalin prefer opioid δ-receptors, and β-endorphin reacts about equally well with μ- and δ-sites, while dynorphin A is a typical κ-agonist. Opioid peptides have a common N-terminal tetrapeptide sequence, Tyr-Gly-Gly-Phe-, which is the "message" segment triggering the receptor responses, but they have different C-terminal "address" segments, which are responsible for their receptor subtype preferences.

Although peptide structural features that determine site specificity ("receptor requirements") have been recognized in general (Schwyzer, 1963, 1977, 1980) and specific (Chavkin & Goldstein, 1981; Schiller, 1984, 1986; Schiller & DiMaio, 1982) terms, a unified molecular mechanism for site selection has not been advanced. Existing rules for predicting quantitative structure–activity relationships of drugs [QSAR;[1] see Hansch & Leo (1979)] are notoriously unsuccessful in the peptide field, especially for receptor selection.

The discovery of specific interactions between neuropeptides and lipid bilayer membranes (Schoch et al., 1979; Gysin & Schwyzer, 1983, 1984; Gremlich et al., 1983, 1984; Erne et al., 1985) offers a new approach to the problem. Whereas the concept of "receptor requirements" tacitly assumes free accessibility of the receptor recognition site from the aqueous phase surrounding the target cell, the membrane may play an important role as catalyst for peptide–receptor interactions by

[1] Abbreviations: dynorphin$_{1-n}$ and ACTH$_{1-n}$, dynorphin A and adrenocorticotropin peptides, respectively, comprising residues 1–n; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; QSAR, quantitative structure–activity relationships.

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