Tyrosine Sulfation of the Glycoprotein Ib−IX Complex: Identification of Sulfated Residues and Effect on Ligand Binding†

Jing-fei Dong, Chester Q. Li, and José A. López*,‡

Gladstone Institute of Cardiovascular Disease, Cardiovascular Research Institute, and Department of Medicine, University of California, San Francisco, California 94141-9100

Received July 1, 1994; Revised Manuscript Received August 31, 1994⊗

ABSTRACT: Here, we present evidence that glycoprotein (GP) Ibα, one of three polypeptides that make up the GP Ib−IX complex—the receptor for von Willebrand factor (vWf) on the surface of unactivated platelets—is modified by sulfation of tyrosine residues. Only GP Ibα was found to incorporate ^{35}S when the GP Ib−IX complex was immunoprecipitated from [^{35}S]sulfate metabolically labeled L and CHO cells that express the recombinant complex. The occurrence of sulfation on tyrosine residues of the polypeptide backbone was determined by removing O- and N-linked oligosaccharides. Limited proteolytic digestion of metabolically labeled GP Ibα revealed that sulfated tyrosine residues are located in the 45-kDa globular region containing the vWf binding site. By mutating potentially sulfated tyrosine residues to phenylalanine and comparing the stoichiometry of sulfate incorporation of these mutants to the incorporation in wild-type GP Ibα, three clustered tyrosine residues—Tyr-276, Tyr-278, and Tyr-279—were identified that undergo the modification. Culturing cells in sulfate-depleted medium containing sodium chlorate and guaiacol completely inhibited GP Ibα sulfation but did not decrease GP Ib−IX expression on the cell surface. Similarly, transiently transfected CHO cells expressed the mutant GP Ibα polypeptide on their surfaces at the same levels as they expressed wild-type GP Ibα. These results suggest that tyrosine sulfation of GP Ibα has little or no effect on the synthesis, assembly, and surface expression of the GP Ib−IX complex. Nevertheless, inhibiting sulfation of GP Ibα reduced the binding of ^{125}I-labeled vWf in the presence of ristocetin by up to 37%. These data strongly suggest that sulfation of tyrosines in the ligand-binding region of GP Ibα is necessary for optimal binding of vWf.

Platelets play several important roles in hemostasis and thrombosis. Each requires participation of receptors on the platelet surface, a major one being the glycoprotein (GP)[1] Ib−IX complex. This receptor mediates initial attachment of platelets to the blood vessel wall at sites of injury by binding von Willebrand factor (vWf) in the subendothelium. It also has crucial roles in two other platelet functions important in thrombosis: (a) aggregation of platelets induced by high shear in regions of arterial stenosis and (b) platelet activation induced by low concentrations of thrombin [for review, see López (1994)].

The GP Ib−IX complex comprises three membrane-spanning polypeptides—GP Ibα, GP Ibβ, and GP IX—with molecular masses of 135−155, 24, and 17−20 kDa, respectively (Berndt et al., 1985; Clemetson, 1985). The three subunits are each products of distinct genes (Lopez et al., 1987, 1988; Hickey et al., 1989), are present in equimolar amounts on the platelet membrane (Berndt et al., 1985), and are absent from the platelets of patients with the hereditary bleeding disorder Bernard−Soulier syndrome (George et al., 1984). All three must be present for efficient cell-surface expression of GP Ibα (López et al., 1992), indicating that proper assembly of the three subunits into a complex is required for full expression on the plasma membrane.

Several lines of evidence indicate that the vWf- and thrombin-binding domains of the complex reside in a 45-kDa globular region that encompasses approximately 300 amino acids at the amino terminus of GP Ibα. The vWf-binding region probably lies between residues 221 and 318 and likely requires a disulfide loop (Katagiri et al., 1990; Cruz et al., 1992), although the precise sequences involved in vWf binding remain to be determined: different sequences have been identified using ristocetin and botrocetin as inducers of vWf binding (Vicente et al., 1988; Katagiri et al., 1990). What does appear clear is that there is an electrostatic component to the GP Ibα−vWf interaction, as both natural and *in vitro* mutations that change the charge of either GP Ibα or vWf affect their interaction. For example, the mutations of negative charges between residues 251 and 279 of GP Ibα have been reported to disrupt vWf binding (Murata et al., 1991). However, whether electrostatic interactions account for all of the binding of ligand to receptor is not resolved because mutations that cause disorders of increased receptor affinity [pseudo-von Willebrand disease (Miller et al., 1991; Russell & Roth, 1993)] or of increased ligand affinity [type IIb von Willebrand disease (Ginsburg & Sadler, 1993)] often do not involve changes in charged amino acids.

The interaction of GP Ibα with both vWf and thrombin also requires more than the simple linear amino acid sequence of GP Ibα: the concentrations of GP Ibα synthetic peptides that block these interactions are much greater than would

† This work was supported by NIH Grants HL02463 and HL46416.

* To whom correspondence should be addressed at Gladstone Institute of Cardiovascular Disease, P.O. Box 419100, San Francisco, CA 94141-9100. Telephone: (415) 826-7500. Fax: (415) 285-5632.

‡ University of California, San Francisco.

⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1994.

[1] Abbreviations: vWf, von Willebrand factor; GP, glycoprotein; CHO, Chinese hamster ovary.

0006-2960/94/0433-13946$04.50/0

be anticipated based on the affinities between the intact components (Katagiri et al., 1990; Vicente et al., 1990). This discrepancy may exist because the synthetic peptides do not faithfully mimic the three-dimensional structure of the receptor or because they lack posttranslational modifications present in the native receptor. Known posttranslational modifications of proteins include phosphorylation (Edelman et al., 1987), methylation (Adler, 1979), glycosylation (Kornfeld & Kornfeld, 1985), modification by lipid (fatty acylation and prenylation) (Linder et al., 1993), and sulfation (Kornfeld & Kornfeld, 1985; Huttner, 1987, 1988). These modifications in many instances make important contributions to the functions of the altered proteins (Huttner, 1987, 1988). The sequence of the charged region of GP Ibα suggests sulfation as the most likely of these modifications to affect the interaction of this polypeptide with its ligands.

Protein sulfation is a widespread posttranslational modification that involves enzymatic covalent attachment of sulfate, either to sugar side chains or to the polypeptide backbone (Huttner, 1987; Hille et al., 1990; Hille & Huttner, 1990). This modification occurs in the trans-Golgi compartment and, therefore, affects only proteins that traverse this compartment: secretory proteins, proteins targeted for granules, and the extracellular regions of plasma membrane proteins (Baeuerle & Huttner, 1987). Tyrosine is the only amino acid residue known to undergo sulfation. A consensus sequence for tyrosine sulfation has been suggested (Hortin et al., 1986a; Huttner, 1987), the key feature of which is the presence of acidic amino acids at defined positions relative to the tyrosine residue that becomes sulfated.

GP Ibα contains three tyrosine residues (Tyr-276, Tyr-278, Tyr-279) that fit the consensus sequence for tyrosine sulfation, all within the acidic region implicated in vWf and thrombin binding. Determining whether these tyrosines become sulfated and, if so, the functional and biosynthetic consequences of the modification were the aims of this study.

MATERIALS AND METHODS

Cell Lines. Cell lines expressing the full GP Ib−IX complex or incomplete complexes have been described (López et al., 1992, 1993, 1994). The cell lines used in this study were: L2H cells, which are transfected L cells expressing the entire complex at high levels; CHO αβIX cells, which also express the full complex; and CHO βIX cells, which express GP Ibβ and GP IX only. L2H cells were cultured in a mixture of Dulbecco's modified Eagle's medium and F12 medium (DMEM/F12, 1:1) (Life Technologies, Inc., Grand Island, NY); CHO cells were grown in minimum essential medium (MEM) (Life Technologies, Inc.). Both media were supplemented with 10% heat-inactivated fetal bovine serum (FBS). To maintain high expression of the recombinant polypeptides, the media were supplemented with the following selection agents: 400 μg/mL G418 (Life Technologies, Inc.) for CHO αβIX cells; 400 μg/mL G418 and 80 μM methotrexate (Sigma Chemical Co., St. Louis, MO) for CHO βIX cells; and 1 mM sodium hypoxanthine, 4 μM aminopterin, and 160 μM thymidine (HAT, Life Technologies, Inc.) for L2H cells. The cell lines were maintained in an atmosphere of 5% CO_2 and 99% humidity.

Metabolic Labeling and Immunoprecipitation. To determine if sulfate is incorporated into GP Ibα, L2H cells in 100-mm culture dishes at 80% confluence were first incubated with sulfate-free MEM containing 2% of normal concentrations of cysteine and methionine and 10% dialyzed FBS for 15 min and then switched to the same medium containing [^{35}S]sulfate (0.1 mCi/mL, carrier-free; ICN, Irvine, CA). After a 4-h incubation, cells were lysed in 1 mL of lysis buffer composed of 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P (NP)-40 (Sigma), 0.5% sodium deoxycholate (Sigma), 0.1% SDS, 1 mM phenylmethanesulfonyl fluoride (PMSF) (Boehringer-Mannheim, Indianapolis, IN), 1 mg/mL leupeptin (Boehringer-Mannheim), 1 mg/mL DNase 1 (Boehringer-Mannheim), 0.1 mg/mL soybean trypsin inhibitor (Boehringer-Mannheim), and 1.6 μg/mL benzamidine (Boehringer-Mannheim). The cell lysate was centrifuged at 10000*g* for 20 min to remove debris, and the supernatant was incubated with a rabbit polyclonal GP Ibα antibody (López et al., 1992) (1.4 μg/mL) overnight at 4 °C followed by a 3-h incubation with 50 μL of a suspension of fixed *Staphylococcus areus* cells (Pansorbin, Calbiochem Corp., La Jolla, CA). The Pansorbin was then pelleted and washed twice in lysis buffer, and the immunoprecipitate was released by boiling for 5 min in SDS sample buffer. To generate the amino-terminal 45-kDa fragment of GP Ibα, the immunoprecipitated sample was diluted in 20 μL of phosphate-buffered saline (PBS) containing 100 μg/mL chymotrypsin and 50 μg/mL trypsin and incubated for 12 h at 37 °C.

Glycanase Treatment. N-Glycan chains were removed from immunoprecipitated proteins by treating the immunoprecipitate with N-glycanase. An aliquot of the immunoprecipitate was mixed with an equal volume of N-glycanase solution [400 mM sodium phosphate, pH 8.3, 3.5% NP-40, 2.5 mM PMSF, 0.5 U N-glycanase (Genzyme Corp., Cambridge, MA)] and incubated at 37 °C overnight. To remove O-glycans, the immunoprecipitate was treated sequentially with neuraminidase and O-glycanase. An aliquot of the immunoprecipitate was first mixed with an equal volume of neuraminidase solution [3.5% NP-40, 20 mM calcium acetate, 40 mM Tris−maleate, pH 6.0, 2.5 mM PMSF, and 0.25 U neuraminidase (Sigma)] and incubated for 1 h at 37 °C. O-Glycanase (Genzyme) was then added to a final concentration of 200 U/mL and incubated overnight at 37 °C. The reaction was terminated by mixing the solution with an equal volume of 2× SDS sample buffer.

SDS−Polyacrylamide Gel Electrophoresis. Immunoprecipitated samples were subjected to electrophoresis on 0.75 mm thick, 5−15% gradient SDS−polyacrylamide gels under reducing conditions (2% β-mercaptoethanol) with 5−7 mA/gel for 12 h at room temperature. The dried gels were exposed to a phosphorimager plate for 48−72 h at room temperature. Incorporation of ^{35}S was detected with a phosphorimager system (Fuji Model BAS 1000).

Mutagenesis and Transfection. Mutagenesis was performed directly on the GP Ibα cDNA cloned into the *Eco*RI site of the expression vector pDX (López et al., 1992) using the Transformer site-directed mutagenesis kit (K1600-1, Clontech, Palo Alto, CA). Three mutants were generated for these studies with either Tyr-276, Tyr-278, or Tyr-279 mutated to phenylalanine residues. Purified plasmid DNA from the desired mutants was then transiently transfected into CHO βIX cells by lipofection (LipofectAMINE, BRL Life Sciences, Grand Island, NY). Briefly, the cells were harvested with 0.25% trypsin and 1 mM EDTA, transferred to a 35-mm culture dish, and plated to a density of 1×10^5 cells/well. Cells were grown overnight and typically reached

60−80% confluence before transfection. For transfection, 1 μg of purified DNA was mixed with 6 μL of liposome suspension in 200 μL of MEM without antibiotics or serum. The mixture was incubated for 15 min at room temperature, then mixed with 800 μL of MEM, and layered onto the monolayer of cells. The transfecting medium was removed after a 5-h incubation. The transfected cells were labeled metabolically with [^{35}S]sulfate 48 h after transfection. Immunoprecipitation, SDS−PAGE, and detection of ^{35}S incorporation were performed as described above.

Stoichiometry of Sulfation. CHO βIX cells that transiently expressed wild-type or mutant GP Ibα were metabolically labeled with both [^{3}H]tyrosine and [^{35}S]sulfate in tyrosine- and sulfate-free MEM supplemented with 1% dialyzed FBS for 4 h. The cells were then lysed, and the complex was immunoprecipitated and subjected to SDS−PAGE. The GP Ibα band that incorporated ^{3}H and ^{35}S was cut out of the gel and subjected to liquid scintillation counting on two channels to determine the incorporation of the two isotopes. The level of sulfate incorporation in the mutants was compared to that in wild-type GP Ibα by first normalizing the amount of protein present in each band based on the levels of [^{3}H]-tyrosine radioactivity. The relative levels of [^{35}S]sulfate incorporated into equivalent amounts of wild-type or mutant GP Ibα were then determined by counting in the ^{35}S channel. The results obtained from three separate transient transfections were averaged to obtain the final results, which were expressed as a relative ratio of sulfate incorporated into the mutant polypeptides as compared to wild-type GP Ibα.

Inhibition of Tyrosine Sulfation. L2H cells were grown in 100-mm culture dishes to 80% confluence and then switched to sulfate-free medium with 2% of the normal concentrations of methionine and cysteine and supplemented with 10% dialyzed FBS. Sodium chlorate and guaiacol were then added to the medium to final concentrations of 5 and 0.2 mM, respectively. Cells were maintained under these conditions for periods of 2−24 h before further assays. Control cells were grown in complete medium.

Immunostaining and Flow Cytometry. The effect of sulfation on surface expression was assessed by flow cytometry in both L2H cells grown in sulfate-depleted or sulfate-replete medium and CHO βIX cells transiently expressing mutant or wild-type GP Ibα. Cells were harvested with 0.53 mM EDTA and resuspended in PBS. Nonspecific binding sites were first blocked by incubating the cells with PBS containing 5% FBS and 1% bovine serum albumin for 20 min. The cells were then incubated with the fluorescein isothiocyanate (FITC)-conjugated GP Ibα monoclonal antibody AN51 (DAKO, Carpinteria, CA) (1.4 μg/10^{6} cells) for 1 h at room temperature followed by three washes in PBS. Cell-surface expression of GP Ib−IX was determined by flow cytometry analysis on a FACScan (Becton Dickinson), stimulating at 488 nm and collecting at 530 nm (fluorescein window).

Iodination of vWf and Assay of Ligand Binding. Purified human vWf (kindly provided by Dr. Zaverio Ruggeri of the Scripps Research Institute, La Jolla, CA) was iodinated with Na^{125}I by the Iodogen method (Fraker & Speck, 1978; Ruggeri et al., 1992) and purified on a Sephadex G25 column (Pharmacia Laboratories, Piscataway, NJ). The specific activity of ^{125}I-labeled vWf was 0.27 mCi/mg of protein.

Ligand binding was assessed as previously described (López et al., 1992) with minor modifications according to the method of Ruggeri et al. (1992). Briefly, L2H cells cultured for 24 h either in sulfate-free medium with sodium chlorate or in complete medium were detached with EDTA and resuspended in Tyrode's buffer to a final concentration of 4 × 10^{6} cells/mL. Twenty-five microliters of the cell suspension was placed into each of the 12 1-mL Eppendorf tubes. ^{125}I-Labeled vWf was added to duplicate sets of six tubes at final concentrations of 1, 2, 4, 8, 16, and 32 mg/mL. A 32-fold excess of unlabeled vWf was then added to one set of tubes, and the equivalent volume of Tyrode's buffer was added to the other set of tubes. Ristocetin was then added to both sets of tubes to a final concentration of 1.4 mg/mL. The mixture was incubated at room temperature for 30 min and then layered onto 20% sucrose. Membrane-bound ^{125}I-labeled vWf was separated from free ^{125}I-labeled vWf by centrifugation at 12000*g* for 5 min. The capillary tips of the tubes containing the cell pellet were cut off, and the radioactivity was counted in a gamma counter. The specificity of binding was determined by incubating the cells in AN51 (1.4 μg/10^{6} cells) for 30 min prior to adding vWf. All direct binding data were corrected for nonspecific binding (defined as the amount of radioactivity bound to untransfected cells under identical conditions), which was less than 15% of the total binding at the highest vWf concentration used, and were linearized by performing a Scatchard-type analysis. The dissociation constant (K_d) and the maximum amount of ^{125}I-labeled vWf bound (B_{max}) were calculated based on the Scatchard plot.

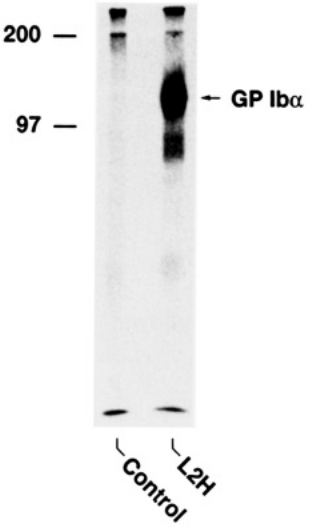

FIGURE 1: Sulfation of GP Ibα. L2H cells and control untransfected L cells were metabolically labeled for 4 h with [^{35}S]sulfate and lysed with a buffer containing 0.1% SDS, and the lysates were immunoprecipitated with the GP Ibα monoclonal antibody AN51. The immunoprecipitates were separated by SDS−PAGE under reducing conditions, and the dried gel was subjected to autoradiography. The only specific band that incorporated ^{35}S was GP Ibα.

RESULTS

Tyrosine Sulfation of GP Ibα. To determine if any part of the GP Ib−IX complex undergoes sulfation, L2H cells were metabolically labeled with [^{35}S]sulfate, and the complex was immunoprecipitated from lysed cells with a rabbit polyclonal GP Ibα antibody (López et al., 1992). The immunoprecipitated complex was then analyzed by SDS−PAGE under reducing conditions. The only detectable radioactive band corresponded to GP Ibα (Figure 1), suggesting that, of the three GP Ib-IX complex polypeptides, only GP Ibα was sulfated.

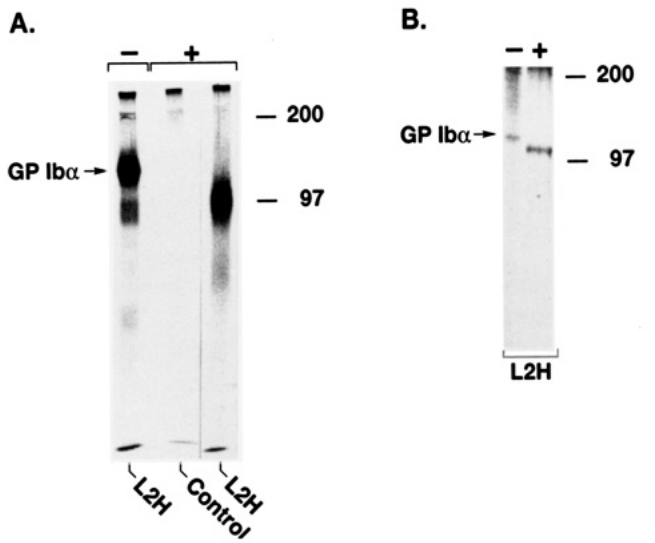

FIGURE 2: Sulfation of GP Ibα occurs on the polypeptide backbone. L2H cells were metabolically labeled with [^{35}S]sulfate, and GP Ib−IX immunoprecipitated with AN51 from the detergent lysate. One aliquot of the immunoprecipitates from each labeling experiment was treated with glycanases to remove sugar side chains. (A) Treatment with neuraminidase followed by *O*-glycanase to remove *O*-glycans: (−) untreated; (+) treated. (B) Treatment with *N*-glycanase to remove *N*-glycans. In both cases, the radioactivity in the deglycosylated band was quantitated by densitometry and corrected for the amount of each sample loaded. Following both treatments, the radioactivities in the deglycosylated and native bands were equivalent.

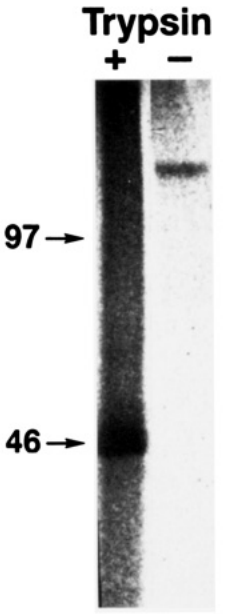

FIGURE 3: Tyrosine sulfation occurs in the amino-terminal globular region of GP Ibα. AN51 immunoprecipitates of [^{35}S]sulfate L2H cell lysates were treated with trypsin (50 μg/mL) and chymotrypsin (100 μg/mL) for 12 h at 37 °C. Enzyme-digested (+) or undigested (−) immunoprecipitates were evaluated by SDS−PAGE and gel autoradiography. (Arrows mark the positions of molecular weight markers.)

Proteins can be sulfated either on their polypeptide backbones (where tyrosine is the only amino acid known to undergo this modification) or on their oligosaccharide side chains. To distinguish between these two possibilities, the oligosaccharides were removed by treating portions of the immunoprecipitate with glycanases. To remove N-glycans (of which GP Ibα is likely to contain four) (López et al., 1987), the immunoprecipitate was treated with N-glycanase. This treatment yielded the expected reduction in molecular mass but caused no reduction in [^{35}S]sulfate incorporation (Figure 2B). Likewise, removing O-glycans with neuraminidase and O-glycanase caused no decrease in sulfate incorporation (Figure 2A), indicating that sulfation occurs on tyrosine residues in the polypeptide backbone of GP Ibα.

Identification of Sulfated Tyrosines in GP Ibα. To identify the sites on GP Ibα that undergo sulfation, the immunoprecipitate was first subjected to limited proteolytic digestion with trypsin and chymotrypsin, which releases the 45-kDa globular N terminus of GP Ibα from the remainder of the molecule (Andrews et al., 1989). After this treatment, the only distinct radioactive band migrated at 45 kDa, indicating that the globular N-terminal region contains sulfated tyrosines (Figure 3).

Although this region contains seven tyrosine residues, only three of them—Tyr-276, Tyr-278, and Tyr-279—are within sequences with characteristics believed necessary for tyrosine sulfation (Hortin et al., 1986a) (Figure 4). We therefore used site-directed mutagenesis to determine whether any of these residues are sulfated. Each tyrosine residue was individually mutated to phenylalanine, which differs from tyrosine only in its lack of a hydroxyl group. None of the mutations yielded sequences unfavorable for sulfate addition to the remaining tyrosines. The GP Ibα tyrosine mutants were then transiently transfected into CHO βIX cells and evaluated for incorporation of [^{35}S]sulfate by metabolic labeling. Each of the three mutants still incorporated [^{35}S]sulfate (Figure 5)—which could mean that sulfation occurs on all of the tyrosines, on two of the three, or on none of them. To distinguish between these possibilities, the levels of sulfate incorporation in each of the mutants were compared to the level in wild-type GP Ibα by metabolically labeling cells expressing the polypeptides simultaneously with both [^{3}H]-tyrosine and [^{35}S]sulfate. The [^{3}H]tyrosine labeling was used as an internal standard to compare the relative amounts of mutant and wild-type GP Ibα from immunoprecipitated samples. Because wild-type GP Ibα contains 14 tyrosines, mutating one to phenylalanine would cause only a slight underestimation of the amount of polypeptide present, and therefore the data were not corrected. After immunoprecipitation and SDS−PAGE, the GP Ibα bands were cut from the dried gel, and their radioactivity was measured by scintillation counting using the ^{35}S channel and the ^{3}H channel. The ratio of the two isotopes was determined in the mutant molecules and compared to the ratio in wild-type GP Ibα. This comparison is expressed as the "relative ratio" of sulfate incorporation (Table 1). Each of the three mutants incorporated about two-thirds as much sulfate as did wild-type GP Ibα, indicating that, within a population of GP Ibα polypeptides, an equivalent fraction of each of the candidate tyrosines is sulfated. Taken together, these results indicate that GP Ibα—and not the two other GP Ib−IX subunits—is tyrosine-sulfated, and they are the most consistent with the modification occurring on each of the three tyrosines clustered in the acidic region of GP Ibα.

Cell-Surface Expression of Unsulfated GP Ib−IX Complex. To determine whether synthesis or cell-surface expression of the GP Ib−IX complex can be affected by tyrosine sulfation, L2H cells were grown for up to 24 h in sulfate-free medium supplemented with sodium chlorate and guaiacol, a condition that has been reported to inhibit up to 95% of sulfation without affecting synthesis or other posttranslational modifications of proteins (Baeuerle & Huttner, 1986;

GP Ibα (269-287)	D	E	G	D	T	D	L	Y*	D	Y*	Y*	P	E	E	D	T	E	G	D
Human C4α (732-749)	M	E	A	N	E	D	Y^{SO3}	E	D	Y^{SO3}	E	Y^{SO3}	D	E	L	P	A	K	
Hirudin (53-65)	D	G	D	F	E	E	I	P	E	E	Y^{SO3}	L	Q						

FIGURE 4: Sequence of potentially sulfated region of GP Ibα. The sequence surrounding potentially sulfated tyrosines of GP Ibα is compared here to similar sequences of two other proteins known to undergo sulfation (Hortin et al., 1986b; López et al., 1987; Rydel et al., 1990). (*) potential site of sulfation of GP Ibα.

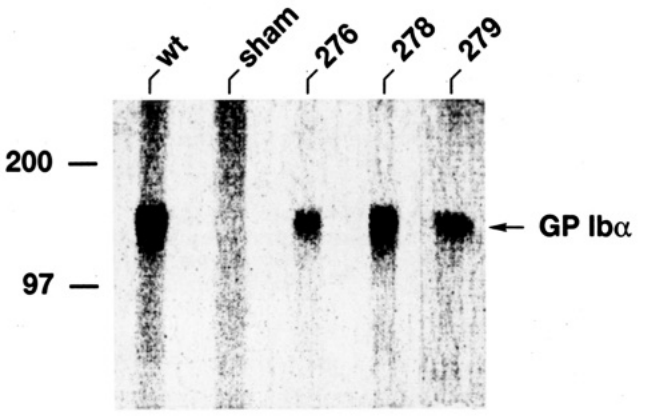

FIGURE 5: [^{35}S]Sulfate incorporation into GP Ibα tyrosine → phenylalanine mutants. By site-directed mutagenesis, each of three tyrosine residues (Tyr-276, Tyr-278, Tyr-279) was replaced by phenylalanine. The wild-type and tyrosine mutants of GP Ibα were then expressed transiently in CHO βIX cells. The cells were then metabolically labeled with [^{35}S]sulfate, lysed, and GP Ibα immunoprecipitated with AN51. The immunoprecipitate was resolved by SDS−PAGE, and the gel was evaluated by autoradiography. The numbers above the lanes indicate the amino acid residue number of the mutated tyrosine. Sham, cells transfected with the expression plasmid only; wt, wild-type GP Ibα.

Table 1: Stoichiometry of Tyrosine Sulfation of GP Ibα

		tyrosine mutants of GP Ibα		
	wild type	Tyr-276	Tyr-278	Tyr-279
relative ratio (^{35}S/^{3}H)[a]	3.0 ± 0.1	1.9 ± 0.3	2.1 ± 0.1	1.9 ± 0.3

[a] These ratios were from three separate experiments and have been corrected such that the ratio from the wild-type GP Ibα was converted to 3.0 by an artificial factor with which the other ratios were compared.

Imai et al., 1993). This treatment essentially abolished GP Ibα sulfation (Figure 6B) and showed no significant effect on cell morphology (not shown).

L2H cells that had been subjected to sulfate starvation for 2−24 h were immunostained with the GP Ibα monoclonal antibody AN51 and analyzed for surface expression of GP Ib−IX by flow cytometry. The immunofluorescence profiles obtained from these cells showed no decrease in the levels of complex on the surface relative to that on sulfate-replete cells (and perhaps even showed a modest increase) (Figure 6A), suggesting that the synthesis and cell-surface expression of the GP Ib−IX complex is not dependent on tyrosine sulfation.

Global inhibition of sulfation might nonspecifically alter surface expression of GP Ib−IX and thereby yield results that do not reflect a specific effect of sulfation inhibition on the complex. We therefore also examined the surface expression of GP Ibα tyrosine mutants transiently expressed in CHO βIX cells. As shown in Figure 7, the levels of cell-surface expression in these tyrosine mutants were similar to each other and to the level of surface expression of wild-type GP Ibα, further evidence that inhibition of GP Ibα tyrosine sulfation does not decrease the synthesis or cell-surface expression of the complex.

Role of Tyrosine Sulfation of GP Ibα in vWf Binding. The potential effect of tyrosine sulfation on the ligand-binding function of the complex was determined by comparing the binding of ^{125}I-labeled vWf to sulfate-depleted and sulfate-replete cells expressing the complex. Aliquots of L2H and CHO αβIX cells were incubated with ^{125}I-labeled vWf and ristocetin for 30 min in the presence or absence of excess unlabeled vWf. The binding affinity of vWf for wild-type recombinant GP Ib−IX was first determined by incubating the cells with ^{125}I-labeled vWf over a concentration range of 1−32 μg/mL, a range over which the K_d for vWf binding to platelets has been determined in several previous studies (Fujimura et al., 1987; Vicente et al., 1988; Federici et al., 1989). The binding isotherms for vWf binding to cells in which sulfation had been globally inhibited were obtained and compared to those of cells grown in normal conditions. The binding of vWf to the unsulfated GP Ib−IX complex expressed on the cell surface was reduced by 25−37% compared to the binding of vWf to cells expressing the sulfated receptor complex (Student's t test, $p < 0.01$) (Figure 8). The calculated K_d and B_{max} for specific binding of ^{125}I-labeled vWf to unsulfated GP Ib−IX complex in the presence of ristocetin were 2.2 μg/mL and 8.1 μg, respectively, as compared to 2.9 μg/mL and 11.4 μg for the sulfated wild-type GP Ib−IX complex (Figure 8).

DISCUSSION

Sulfation of tyrosine residues has been identified in many proteins (Huttner, 1982) since it was first reported in the 1950s (Bettelheim, 1954). This covalent modification of protein occurs in the trans-Golgi compartment and is catalyzed by a membrane-bound enzyme, tyrosylprotein sulfotransferase, which recognizes tyrosine residues in exposed acidic domains (Lee & Huttner, 1983; Hortin et al., 1986a; Huttner, 1987; Han & Martinage, 1992). Tyrosine sulfation has been reported on various classes of secreted and membrane proteins and in extracellularly exposed as well as in intracellularly retained proteins (Hille & Huttner, 1990). Here, we present evidence that the α subunit of the GP Ib−IX complex is one of the membrane proteins that undergo tyrosine sulfation and that this modification significantly enhances what is probably the most important function of the complex: binding von Willebrand factor. We studied the process in CHO and L cells because of the ease with which conditions can be manipulated in these cells and because the recombinant complex expressed in these cells possesses most, if not all, of the structural and functional properties of the platelet complex (López et al., 1992). It seems reasonable to assume that platelet GP Ibα is sulfated to a similar extent as the recombinant complex, given that platelets are very rich in tyrosylprotein phosphotransferase (Sane & Baker, 1993).

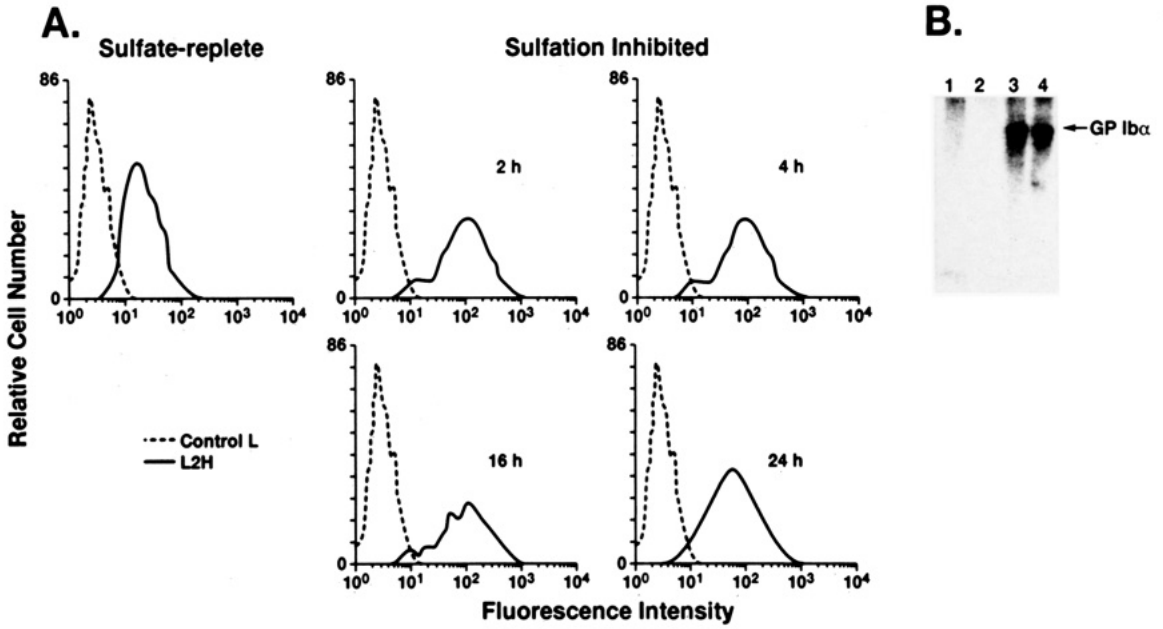

FIGURE 6: Inhibiting tyrosine sulfation does not diminish GP Ib−IX complex assembly or cell-surface expression. (A) L2H cells were cultured for 2−24 h in sulfate-free medium containing 5 mM sodium chlorate and 0.2 mM guaiacol before determining the cell-surface expression of the GP Ib−IX complex. Flow cytometry was performed after incubating the cells with FITC-labeled AN51. Expression of GP Ibα on the surface of sulfate-depleted cells was similar to expression in sulfate-replete cells. Untransfected L cells were used as a negative control. (B) L2H cells were either starved of sulfate for 24 h or grown in complete medium and then labeled for 4 h with [^{35}S]sulfate. GP Ibα was immunoprecipitated with AN51 and subjected to SDS−PAGE. Growth in the sulfate-free medium with inhibitors (sodium chlorate and guaiacol) virtually abolished the sulfation of GP Ibα. Lanes 1 and 2, sulfate-starved cells; lanes 3 and 4, cells grown in complete medium.

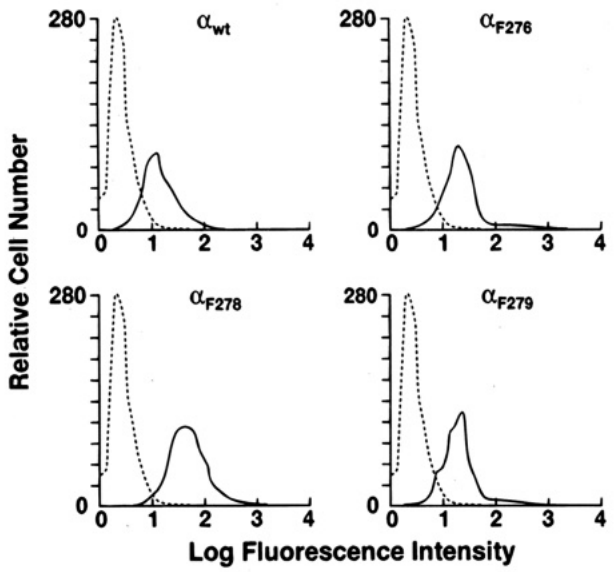

FIGURE 7: Cell-surface expression of GP Ib−IX complex containing GP Ibα tyrosine mutants. Flow cytometry analysis performed 48 h after CHO βIX cells were transiently transfected with wild-type GP Ibα or any of the three tyrosine mutants and labeled with FITC-AN51. The different tyrosine mutants displayed profiles similar to that of wild-type GP Ibα. (− −) sham-transfected control; (−) transfected with GP Ibα.

We determined that only GP Ibα is sulfated by demonstrating that radioactivity was incorporated into a band corresponding to this polypeptide when the entire complex was immunoprecipitated. We then showed that sulfation occurs on tyrosine residues and not on carbohydrate since the amount of radioactivity incorporated into GP Ibα is not reduced upon removal of the N- and O-linked sugars. Our results indicate that at least three clustered tyrosine residues in the globular region of GP Ibα—Tyr-276, Tyr-278, and Tyr-279—are sulfated. This conclusion is supported by two lines of evidence. First, [^{35}S]sulfate was still incorporated into polypeptides containing single-residue mutations of each of the tyrosine residues. Second, the stoichiometry data showed that each tyrosine mutant incorporated less ^{35}S than did wild-type GP Ibα, but no mutation abrogated sulfation altogether. The finding that each mutant incorporated approximately two-thirds as much sulfate as did wild-type GP Ibα suggests that each residue is equivalently sulfated and, therefore, likely fully sulfated. Whether sulfation of these clustered tyrosines is sequential or whether sulfation of one tyrosine influences that of another remains to be determined.

The presence of three or more sulfated tyrosines in a single polypeptide is rare. So far, the only other proteins that have been reported to have three sulfated tyrosines on one polypeptide chain are the fourth component of complement (Hortin et al., 1986b) and P-selectin (Sako et al., 1993).

Although a number of proteins have been found to be tyrosine-sulfated (Hortin et al., 1989), the functional role of the modification has been determined only in a few cases. It was suggested early on that tyrosine sulfation may affect the secretory pathways of modified proteins (Huttner, 1987, 1988), but subsequent evidence supported this notion in only a few instances (Hortin et al., 1986b; Cardelli et al., 1990). In fact, *in vitro* inhibition of tyrosine sulfation did not affect the synthesis (Pittman et al., 1992), secretion (Pittman et al., 1992), or other posttranslational modifications (Imai et al., 1993) of several proteins that have been studied. Similarly, we found that tyrosine sulfation of GP Ibα did not have a major effect on the synthesis and cell-surface expression of the GP Ib−IX complex. Neither global inhibition of tyrosine sulfation nor selective mutation of sulfated tyrosine residues decreased the cell-surface expression of the complex. This result also indicates that tyrosine sulfation does not interrupt the assembly of the complex because efficient cell-surface

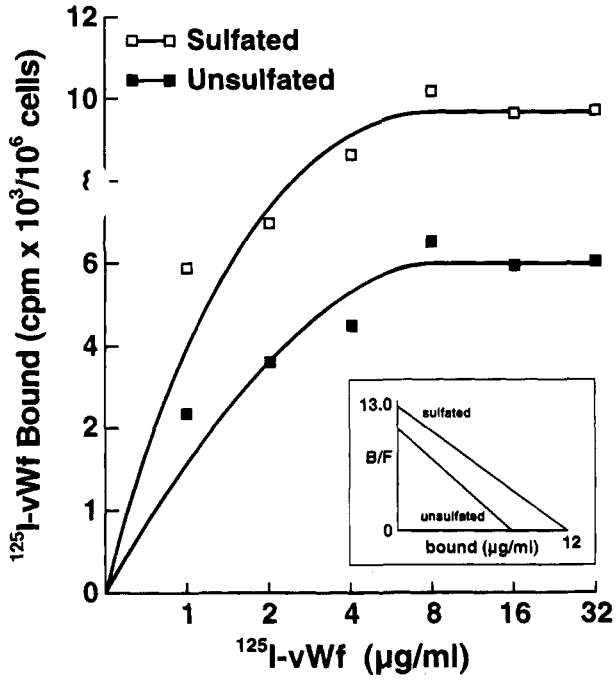

FIGURE 8: Binding of ^{125}I-labeled vWf to the cell-surface GP Ib–IX complex of L2H cells. ^{125}I-Labeled vWf (0.27 mCi/mg of protein) was incubated with L2H cells at room temperature for 30 min in the presence of ristocetin (1.4 mg/mL). Membrane-bound radioactivity for unsulfated and sulfated L2H cells was detected in a gamma counter. The specific binding was determined by subtracting the amount of radioactivity bound to untransfected cells treated identically. Each experimental point represents the mean of three separate experiments. The coefficient of variation of each point was less than 11.5%. The data from the direct-binding experiments were linearized by plotting the ratio of bound-to-free versus bound. The K_d was then determined from the slope of the resulting straight line, and the maximum vWf binding was obtained from the *x*-intercept.

expression of GP Ibα requires the presence of GP Ibβ and GP IX (López et al., 1992).

The sulfated tyrosine residues in GP Ibα are clustered in a highly acidic region of the globular amino terminus of the polypeptide. This location contains both the necessary amino acid sequences for tyrosine sulfation (Hortin et al., 1986a; Huttner, 1987) and the appropriate secondary structure (Hortin et al., 1986a). The primary structure of this segment of GP Ibα has numerous negative charges accounted for by multiple aspartic and glutamic acid residues. Such a highly acidic and hydrophilic environment is thought to be a prerequisite for sulfation because tyrosylprotein sulfotransferase specifically recognizes and sulfates tyrosines adjacent to acidic amino acid residues (Lee & Huttner, 1983; Vargas et al., 1985; Niehrs & Huttner, 1990). The presence of this modification also indicates that these tyrosine residues are by necessity located at the surface of the protein since the sulfate is added in the Golgi apparatus after protein folding occurs (Baeuerle & Huttner, 1987) and must be accessible to the enzyme. The suggestion that sulfated regions are exposed is supported by the finding that the sulfate-containing peptide of the fourth component of complement can be released by trypsin without denaturing the protein (Hortin et al., 1986b). Full sulfation of the acidic region of GP Ibα yields a region with a remarkable density of negative charge—13 negative charges within a 19 amino acid stretch—and an obvious candidate site for electrostatic interactions with other proteins.

Such a role for tyrosine sulfation of GP Ibα is suggested by our finding that despite the normal expression of unsulfated GP Ib–IX on the cell surface, the vWf-binding capacity of the unsulfated complex in the presence of ristocetin was reduced 25–37%. The necessity of this posttranslational modification for proper ligand-binding function of the GP Ib–IX complex probably explains the ineffectiveness of synthetic peptides in blocking the interaction (Katagiri et al., 1990; Vicente et al., 1990). The negative charges in the sulfated region have already been implicated as important for botrocetin-induced binding of vWf (Murata et al., 1991); the current findings suggest that the charges contributed by sulfate further increase vWf binding, possibly by directly contributing negative charges to the ligand-binding site and thereby consolidating electrostatic interactions with vWf.

Addition of sulfate may stabilize ionic interactions because the sulfate groups are known to be capable of strong interaction with basic residues of other proteins (Payne et al., 1991). Furthermore, sulfate groups may form well-defined planar salt bridges with arginine residues that are more stable than unoriented ionic interactions (Ichimura et al., 1982), suggesting an important role for sulfation in either stabilizing protein conformation (Jenne et al., 1989; Mikkelsen et al., 1991) or enhancing protein–protein interactions (Payne et al., 1991). For example, heparin can bind tightly to specific regions of proteins using ionic interactions almost exclusively (DeAngelis & Glabe, 1988). Tyrosine sulfation of hirudin also probably increases the overall binding energy of hirudin to thrombin (Stone et al., 1989; Rydel et al., 1990) through ionic interactions with lysine residues in thrombin (Payne et al., 1991; Skrzypczak-Jankun et al., 1991). Similarly, tyrosine sulfation may increase the strength of the interaction between GP Ib–IX and vWf.

Another mechanism by which sulfation may affect ligand binding is by inducing global conformational changes to binding domains. This does not appear to be the case with tyrosine sulfation of GP Ibα, since binding of the conformation-sensitive antibody AN51—which blocks vWf binding—is unaffected by inhibiting the modification.

Other studies of secretory and membrane proteins (Hortin et al., 1989) suggest that tyrosine sulfation may be important for protein–protein interactions. For example, two identified sulfated tyrosine residues in the light chain of factor VIII, Tyr-1664 and Tyr-1680, reside in the region (residues 1649–1689) that binds vWf (Foster et al., 1988; Lollar et al., 1988; Leyte et al., 1989). Sulfation of one of these tyrosine residues, Tyr-1680, has since been proven essential for the interaction (Leyte et al., 1991). In addition, the procoagulant activity of factor VIII decreased 5-fold when its sulfation was inhibited (Leyte et al., 1991).

The clinical and pharmaceutical potential of modifying the sulfation process has been suggested by a few studies. Giorgi and Meek (1985) noted that the sulfation of cholecystokinin is inhibited in the brains of rats treated with dichloronitrophenol. In the case of the GP Ib–IX complex, pharmacologically blocking sulfation of GP Ibα in megakaryocytes and platelets could lead to a defective interaction of platelets with vWf, with the potential for long-lasting therapeutic benefits.

ACKNOWLEDGMENT

We thank Bill Hyun for his invaluable help in flow cytometry analysis, Liliana Jach for graphics, Amy Corder

for photography, Drs. Robert Mahley and Karl Weisgraber for carefully reading the manuscript, Dr. Perumal Thiagarajan for helpful discussions, Dawn Levy and Lewis DeSimone for editorial assistance, and Alana Koehler for assistance in preparing the manuscript.

REFERENCES

Adler, J. (1979) in *Transmethylation* (Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds.) pp 505−509, Elsevier/North-Holland, New York.

Andrews, R. K., Booth, W. J., Gorman, J. J., Castaldi, P. A., & Berndt, M. C. (1989) *Biochemistry 28*, 8317−8326.

Baeuerle, P. A., & Huttner, W. B. (1986) *Biochem. Biophys. Res. Commun. 141*, 870−877.

Baeuerle, P. A., & Huttner, W. B. (1987) *J. Cell Biol. 105*, 2655−2664.

Berndt, M. C., Gregory, C., Kabral, A., Zola, H., Fournier, D., & Castaldi, P. A. (1985) *Eur. J. Biochem. 151*, 637−649.

Bettelheim, F. R. (1954) *J. Am. Chem. Soc. 76*, 2838−2839.

Cardelli, J. A., Bush, J. M., Ebert, D., & Freeze, H. H. (1990) *J. Biol. Chem. 265*, 8847−8853.

Clemetson, K. J. (1985) in *Platelet Membrane Glycoproteins* (George, J. N., Nurden, A. T., & Phillips, D. R., Eds.) pp 51−85, Plenum Press, New York.

Cruz, M. A., Petersen, E., Turci, S. M., & Handin, R. I. (1992) *J. Biol. Chem. 267*, 1303−1309.

DeAngelis, P. L., & Glabe, C. G. (1988) *Biochemistry 27*, 8189−8194.

Edelman, A. M., Blumenthal, D. K., & Krebs, E. G. (1987) *Annu. Rev. Biochem. 56*, 567−613.

Federici, A. B., Bader, R., Pagani, S., Colibretti, M. L., De Marco, L., & Mannucci, P. M. (1989) *Br. J. Haematol. 73*, 93−99.

Foster, P. A., Fulcher, C. A., Houghten, R. A., & Zimmerman, T. S. (1988) *J. Biol. Chem. 263*, 5230−5234.

Fraker, P. J., & Speck, J. C., Jr. (1978) *Biochem. Biophys. Res. Commun. 80*, 849−857.

Fujimura, Y., Holland, L. Z., Ruggeri, Z. M., & Zimmerman, T. S. (1987) *Blood 70*, 985−988.

George, J. N., Nurden, A. T., & Phillips, D. R. (1984) *N. Engl. J. Med. 311*, 1084−1098.

Ginsburg, D., & Sadler, J. E. (1993) *Thromb. Haemostasis 69*, 177−184.

Giorgi, O., & Meek, J. L. (1985) *Biochem. Pharmacol. 34*, 45−49.

Han, K.-K., & Martinage, A. (1992) *Int. J. Biochem. 24*, 1349−1363.

Hickey, M. J., Williams, S. A., & Roth, G. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6773−6777.

Hille, A., & Huttner, W. B. (1990) *Eur. J. Biochem. 188*, 587−596.

Hille, A., Braulke, T., von Figura, K., & Huttner, W. B. (1990) *Eur. J. Biochem. 188*, 577−586.

Hortin, G., Folz, R., Gordon, J. I., & Strauss, A. W. (1986a) *Biochem. Biophys. Res. Commun. 141*, 326−333.

Hortin, G., Sims, H., & Strauss, A. W. (1986b) *J. Biol. Chem. 261*, 1786−1793.

Hortin, G. L., Farries, T. C., Graham, J. P., & Atkinson, J. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1338−1342.

Huttner, W. B. (1982) *Nature 299*, 273−276.

Huttner, W. B. (1987) *Trends Biochem. Sci. 12*, 361−363.

Huttner, W. B. (1988) *Annu. Rev. Physiol. 50*, 363−376.

Ichimura, S., Mita, K., & Zama, M. (1982) *Biochemistry 21*, 5329−5334.

Imai, Y., Lasky, L. A., & Rosen, S. D. (1993) *Nature 361*, 555−557.

Jenne, D., Hille, A., Stanley, K. K., & Huttner, W. B. (1989) *Eur. J. Biochem. 185*, 391−395.

Katagiri, Y., Hayashi, Y., Yamamoto, K., Tanoue, K., Kosaki, G., & Yamazaki, H. (1990) *Thromb. Haemostasis 63*, 122−126.

Kornfeld, R., & Kornfeld, S. (1985) *Annu. Rev. Biochem. 54*, 631−664.

Lee, R. W., & Huttner, W. B. (1983) *J. Biol. Chem. 258*, 11326−11334.

Leyte, A., Verbeet, M. P., Brodniewicz-Proba, T., Van Mourik, J. A., & Mertens, K. (1989) *Biochem. J. 257*, 679−683.

Leyte, A., van Schijndel, H. B., Niehrs, C., Huttner, W. B., Verbeet, M. P., Mertens, K., & van Mourik, J. A. (1991) *J. Biol. Chem. 266*, 740−746.

Linder, M. E., Middleton, P., Hepler, J. R., Taussig, R., Gilman, A. G., & Mumby, S. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3675−3679.

Lollar, P., Hill-Eubanks, D. C., & Parker, C. G. (1988) *J. Biol. Chem. 263*, 10451−10455.

López, J. A. (1994) *Blood Coagulation Fibrinolysis 5*, 97−119.

López, J. A., Chung, D. W., Fujikawa, K., Hagen, F. S., Papayannopoulou, T., & Roth, G. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5615−5619.

López, J. A., Chung, D. W., Fujikawa, K., Hagen, F. S., Davie, E. W., & Roth, G. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2135−2139.

López, J. A., Leung, B., Reynolds, C. C., Li, C. Q., & Fox, J. E. B. (1992) *J. Biol. Chem. 267*, 12851−12859.

López, J. A., Sae-Tung, G., Li, C. Q., & Weisman, S. M. (1993) *Thromb. Haemostasis 69*, 1153 (abstr).

López, J. A., Weisman, S., Sih, T., Li, C. Q., & Sanan, D. A. (1994) *Blood Coagulation Fibrinolysis 5*, 479−486.

Mikkelsen, J., Thomsen, J., & Ezban, M. (1991) *Biochemistry 30*, 1533−1537.

Miller, J. L., Cunningham, D., Lyle, V. A., & Finch, C. N. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4761−4765.

Murata, M., Ware, J., & Ruggeri, Z. M. (1991) *J. Biol. Chem. 266*, 15474−15480.

Niehrs, C., & Huttner, W. B. (1990) *EMBO J. 9*, 35−42.

Payne, M. H., Kratenansky, J. L., Yates, M. T., & Mao, S. J. T. (1991) *J. Med. Chem. 34*, 1184−1187.

Pittman, D. D., Wang, J. H., & Kaufman, R. J. (1992) *Biochemistry 31*, 3315−3325.

Ruggeri, Z. M., Zimmerman, T. S., Russell, S., Bader, R., & De Marco, L. (1992) *Methods Enzymol. 215*, 263−275.

Russell, S. D., & Roth, G. J. (1993) *Blood 81*, 1787−1791.

Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., & Fenton, J. W., II. (1990) *Science 249*, 277−280.

Sako, D., Chang, X.-J., Barone, K. M., Vachino, G., White, H. M., Shaw, G., Veldman, G. M., Bean, K. M., Ahern, T. J., Furie, B., Cumming, D. A., & Larsen, G. R. (1993) *Cell 75*, 1179−1186.

Sane, D. C., & Baker, M. S. (1993) *Thromb. Haemostasis 69*, 272−275.

Skrzypczak-Jankun, E., Carperos, V. E., Ravichandran, K. G., Tulinsky, A., Westbrook, M., & Maraganore, J. M. (1991) *J. Mol. Biol. 221*, 1379−1393.

Stone, S. R., Dennis, S., & Hofsteenge, J. (1989) *Biochemistry 28*, 6857−6863.

Vargas, F., Frerot, O., Tuong, M. D. T., & Schwartz, J. C. (1985) *Biochemistry 24*, 5938−5943.

Vicente, V., Kostel, P. J., & Ruggeri, Z. M. (1988) *J. Biol. Chem. 263*, 18473−18479.

Vicente, V., Houghten, R. A., & Ruggeri, Z. M. (1990) *J. Biol. Chem. 265*, 274−280.