Hypersulfated Low Molecular Weight Heparin with Reduced Affinity for Antithrombin Acts as an Anticoagulant by Inhibiting Intrinsic Tenase and Prothrombinase*

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In buffer systems, heparin and low molecular weight heparin (LMWH) directly inhibit the intrinsic factor X-activating complex (intrinsic tenase) but have no effect on the prothrombin-activating complex (prothrombinase). Although chemical modification of LMWH, to lower its affinity for antithrombin (LA-LMWH) has no effect on its ability to inhibit intrinsic tenase, N-desulfation of LMWH reduces its activity 12-fold. To further explore the role of sulfation, hypersulfated LA-LMWH was synthesized (sLA-LMWH). sLA-LMWH is not only a 32-fold more potent inhibitor of intrinsic tenase than LA-LMWH; it also acquires prothrombinase inhibitory activity. A direct correlation between the extent of sulfation of LA-LMWH and its inhibitory activity against intrinsic tenase and prothrombinase is observed. In plasma-based assays of tenase and prothrombinase, sLA-LMWH produces similar prolongation of clotting times in plasma depleted of antithrombin and/or heparin cofactor II as it does in control plasma. In contrast, heparin has no effect in antithrombin-depleted plasma. When the effect of sLA-LMWH on various components of tenase and prothrombinase was examined, its inhibitory activity was found to be cofactor-dependent (factors Va and VIIIa) and phospholipid-independent. These studies reveal that sLA-LMWH acts as a potent antithrombin-independent inhibitor of coagulation by attenuating intrinsic tenase and prothrombinase.

Coagulation is initiated when tissue factor, a cell surface protein found on nonvascular cells, is exposed by vascular injury. Tissue factor binds factor VII/VIIa (f.VII/VIIa), forming the extrinsic tenase complex that activates f.X (1). f.Xa generates sufficient thrombin through prothrombinase, the phospholipid membrane-bound complex of f.Xa and f.Va, to induce local aggregation of platelets and activate f.V and f.VIII (2). f.Xa generated via extrinsic tenase is insufficient to sustain hemostasis because tissue factor pathway inhibitor rapidly inactivates tissue factor-bound f.VIIa in a f.Xa-dependent fashion (1, 3). To overcome this limitation, additional f.Xa is generated by intrinsic tenase, the phospholipid membrane-bound complex of f.Ixa and f.VIIIa. f.IXa can be activated by extrinsic tenase or by f.Xia, generated by thrombin cleavage of f.XI (1).

The critical role of intrinsic tenase and prothrombinase in coagulation makes these enzyme complexes attractive targets for inhibition. Prothrombinase and intrinsic tenase share similar properties, with each complex consisting of a vitamin K-dependent serine protease and a nonproteolytic cofactor protein. The reactions are calcium-dependent and require a negatively charged phospholipid surface for optimal expression of activity (4–7).

Heparin and low molecular weight heparin (LMWH) act as anticoagulants by activating antithrombin, which inactivates f.Xa and thrombin (8). In buffer systems, heparin and LMWH also inhibit intrinsic tenase activity in an antithrombin-independent fashion (9, 10). In plasma systems, however, the antithrombin-dependent anticoagulant effects of heparin and LMWH predominate.

The purpose of this study was to investigate methods for modifying heparin so as to maximize its antithrombin-independent effects. Starting with a size-restricted LMWH to capitalize on its decreased propensity to bind to plasma proteins, a property that endows it with pharmacokinetic advantage over unfractionated heparin (11, 12), LMWH was chemically modified to reduce its affinity for antithrombin 1700-fold (from a Kd value of 25 nM to 43 μM) by periodate oxidation (13). Like LMWH, this low affinity LMWH (LA-LMWH), which we termed Vasoflax, inhibited intrinsic tenase but had no effect on prothrombinase, thereby confirming that its ability to inhibit intrinsic tenase is not dependent on its affinity for antithrombin. When LA-LMWH was N-desulfated, however, most of its activity was lost, suggesting that its ability to inhibit intrinsic tenase is charge-dependent. To explore this possibility, LA-
LMWH was progressively sulfated, and the inhibitory activities of these hypersulfated LA-LMWH (sLA-LMWH) compounds against intrinsic tenase and prothrombinase were examined. Herein, we demonstrate that upon sulfation, LA-LMWH becomes a more potent inhibitor of intrinsic tenase and acquires the ability to inhibit prothrombinase.

**Materials**

Human FV, FVIII, and FXa were obtained from Hematologic Technologies Inc. (Essex Junction, VT), whereas FX, FXa, thrombin, and α-thrombin were obtained from Enzyme Research Laboratories (South Bend, IN). Recombinant FVIII (Kogenate) was from Bayer Inc. (Etobicoke, Canada). LMWH was subjected to ultrafiltration using a 3000-Da cut-off membrane and lyophilized. The LA-LMWH was TEMED-sulfated using modifications of the methods of Barenholz et al. (14) and others (16) and assayed using an immunoassay (17).

For glycosaminoglycans, unfractionated grade 1 sodium heparin (184 units/mg) from porcine intestinal mucosa was purchased from Sigma, whereas the LMWH exonaparin was from Rhöne-Poulenc-Rorer Canada (Montreal, Canada).

A series of low affinity low molecular weight heparins (LA-LMWH) and hypersulfated LA-LMWH derivatives (sLA-LMWH) were used in this study (see Table 1). A LMWH fraction (mean molecular weight 5000) was prepared from unfractionated heparin by nitrous acid depolymerization, and its affinity for antithrombin was reduced by sodium periodate oxidation, as previously described (13). The resultant LA-LMWH was subjected to ultrafiltration using a 3000-DA cut-off membrane and lyophilized. The LA-LMWH was O-sulfated using a modification of the method described by Inoue and Nakagawa (19). Individual components of the prothrombinase complex were systematically removed or substituted to examine their susceptibility to inhibition by sLA-LMWH. In all cases, the rate of prothrombin activation was examined in the absence or presence of sLA-LMWH-S5, whereas fibronectin was present in all reactions. The standard conditions were as follows: (a) absence of FV (1.2 μM prothrombin, 100 nM FXa, 24 μM PCPS, and 4 μM CaCl$_2$), (b) absence of phospholipid (1.2 μM prothrombin, 4 μM CaCl$_2$, 12 nM FXa, and 10 nM FXa), (c) absence of cofactor, phospholipid, and calcium (1.2 μM prothrombin and 100 nM FXa), (d) substitution of FV with FV (1.2 μM prothrombin, 4 μM CaCl$_2$, 24 μM PCPS, 8 nM FV, and 0.2 nM FXa), and (e) chromogenic activity of FXa (1 nM FXa and 200 nM S-2222). Assays were performed as described above except for reactions with conditions b and c. Because the rate of generation of thrombin in these systems was slow, even with increased reactant concentrations, subsampling was performed at 15- and 30-min intervals, respectively.

**Methods**

*Effect of Glycosaminoglycans on the Activity of Prothrombinase—To examine the effects of glycosaminoglycans on the activation of prothrombin by the prothrombinase complex, the rate of thrombin generation was measured using a modification of the method of Barrow et al. (9)). Reactions were performed in 10 mM Tris-HCl, pH 7.4, 150 μM NaCl containing 0.1% polyethylene glycol (TSP buffer). Two times concentrated stock solution A was made in TSP buffer so as to give final reaction concentrations of 24 μM PCPS vesicles, 0.24 mM FVa, 1.2 μM prothrombin, and 4 mM CaCl$_2$. mL of stock A was mixed with 30 μl of TSP and 10 μl of glycosaminoglycan at final concentrations ranging from 1 to 1000 μg/ml. Reactions were initiated by addition of 10 μl of 1 nM FXa. Control samples lacking glycosaminoglycan were run in parallel. 10-μl aliquots were removed at 30-s intervals into the wells of a 96-well microtiter plate containing 10 μl of 10 mM EDTA, pH 7.4, to quench the activation reaction. At the end of the time course, the activity of thrombin in each well was determined by adding 190 μl of 200 μM GTP-P-NA containing 0.1 mg/ml polyethylene glycol 5000 (Pierce, Rockville, MD). Reactions were performed in 10 mM Tris-HCl, pH 7.4, 150 μM NaCl containing 0.1% polyethylene glycol (TSP buffer) and monitored at 405 nm at 10-s intervals for 5 min at 23 °C in a SpectraMax 340 plate reader (Molecular Devices, Sunnyvale, CA).

The effects of glycosaminoglycans on the activity of intrinsic tenase were determined in a similar fashion. Two times concentrated stock B was prepared to contain final reaction concentrations of 300 nM FX, 4 mM CaCl$_2$, 24 μM PCPS, 4 μM FXa, and 0.4 nM FXIII. FXIII was activated to FXIIa in stock B by incubation with 10 nM thrombin for 1 min. 50 μl of stock B was mixed with 30 μl of TSP containing glycosaminoglycan at final concentrations ranging from 1 to 1000 μg/ml. Reactions in each well were initiated by the addition of 10 μl of 8 nM FXa, and 10-μl aliquots were removed at 30-s intervals as described above. FXa activity was assayed using S-2222 or Chromozym X.
RESULTS

Effect of Glycosaminoglycans on Intrinsic Tenase—To examine the antithrombin-independent effect of LMWH on intrinsic tenase, a LA-LMWH was prepared as previously described (13). LA-LMWH produced 50% inhibition of the initial velocity of f.X activation (IC_{50}) of 16.3 ± 6.1 μg/ml (Fig. 1A). A commercial LMWH (enoxaparin) with normal antithrombin affinity, inhibited intrinsic tenase with an IC_{50} value of 13.2 ± 7.7 μg/ml (Table I), a value comparable with the IC_{50} value of 6 μg/ml reported for LMWH by other investigators (9). To investigate the influence of sulfation of LA-LMWH on inhibition of tenase activity, LA-LMWH was N-desulfated by solvolysis. N-DS-LA-LMWH had 12-fold lower inhibitory activity, with an IC_{50} value of 166 ± 25 μg/ml. These findings suggest that the ability of LMWH to inhibit intrinsic tenase is independent of its affinity for antithrombin but dependent on its charge.

To further investigate the importance of charge, progressive desulfation of LA-LMWH derivatives was synthesized, and their inhibitory activity was compared with that of the starting material (Table I). The most highly sulfated LA-LMWH, designated S5, was 32-fold more potent than LA-LMWH, inhibiting intrinsic tenase with an IC_{50} value of 0.47 ± 0.2 μg/ml. As illustrated in Fig. 1A, increasing the sulfation of LA-LMWH produces a progressive reduction in IC_{50} values. When a plot of the number of sulfate residues/disaccharide versus IC_{50} is subjected to regression analysis (not shown), the correlation coefficient is = −0.86, a value that on one-way analysis of variance is highly significant (p < 0.001), supporting the concept that the potency of LMWH derivatives is dependent on their degree of sulfation. A dextran sulfate, which contained 3.9 sulfate residues/disaccharide, inhibited intrinsic tenase with an IC_{50} value of 0.4 μg/ml, a value similar to that of S5. This observation provides further evidence that the extent of sulfation is an important determinant of potency in this system.

Effect of Glycosaminoglycans on Prothrombinase—The inhibitory effect of the series of hypersulfated LA-LMWH deriv-
sLA-LMWH Inhibits Intrinsic Tenase and Prothrombinase

**FIG. 2. Influence of sLA-LMWH-S5 on fully and partially reconstructed intrinsic tenase.** The influence of varying concentrations of S5 on the rate of FX activation was determined in series of reactions where the constituents of intrinsic tenase were selectively omitted or replaced. The reactions contained FXa, FVIIa, and PCPS (●), FXa, FVIIIa, and PCPS (●), FXa, FVa, and PCPS (●), and FXa, chromogenic activity (▲). Final reactant conditions are given under "Methods."

**FIG. 3. Influence of sLA-LMWH-S5 on fully and partially reconstructed prothrombinase.** The influence of varying concentrations of S5 on prothrombin activation was determined in series of reactions where the constituents of prothrombinase were selectively omitted or replaced. The reactions were FXa, FVa, and PCPS (●), FXa, FV, and PCPS (●), FXa and PCPS (●), FXa and FVa (▲), FXa (○), and FXa chromogenic activity (▲). Final reactant conditions are given under "Methods."

Activites on prothrombinase activity was examined to determine whether increased inhibitory activity against intrinsic tenase conferred inhibitory properties against prothrombinase (Fig. 1B). At concentrations up to 1000 μg/ml, unfractionated heparin had no effect on prothrombinase function, consistent with the results of Barrow et al. (9). Likewise, neither enoxaparin nor LA-LMWH had inhibitory activity against prothrombinase at these concentrations. In contrast, sLA-LMWH inhibited prothrombinase in a concentration-dependent fashion, and its inhibitory activity increased with progressive sulfation, as reflected by a reduction in IC50 values (Table I). Maximum inhibition was effected by S5, which inhibited prothrombinase with an IC50 value of 30 ± 16 μg/ml. Linear regression analysis of a plot of the number of sulfate residues/disaccharide versus IC50 values yielded a correlation coefficient of −0.92 (not shown), which on one-way analysis of variance was highly significant (p < 0.001). Dextran sulfate inhibited prothrombinase with an IC50 value of 35 μg/ml, further highlighting the importance of sulfation for expression of this activity. The IC50 values against prothrombinase were 2 orders of magnitude higher than those for intrinsic tenase, indicating that all of the hypersulfated carbohydrates have greater inhibitory activity against intrinsic tenase than prothrombinase.

Mode of Disruption of Prothrombinase and Intrinsic Tenase by S5—As the intrinsic tenase and prothrombinase complexes are composed of multiple components, various reactants could serve as targets for modulation by sLA-LMWH. To reveal the susceptible component(s), the influence of S5 on partially reconstructed activation complexes was examined. S5 was used in these experiments because it exhibited the most potent inhibitory effects (Table I).

When all components were present, S5 produced dose-dependent inhibition of both intrinsic tenase (Fig. 2) and prothrombinase (Fig. 3). Similar inhibitory effects were evident in assays devoid of phospholipid. Thus, the IC50 values for S5 on intrinsic tenase in the presence or absence of phospholipid were 0.47 ± 0.2 and 1.0 ± 0.7 μg/ml, respectively. Comparable IC50 values for inhibitory activity of S5 against prothrombinase also were found in the presence or absence of phospholipid (30 ± 16.3 and 68 ± 22 μg/ml, respectively). In contrast, in systems devoid of cofactor (FVa/FVIIa), S5 had no inhibitory effect, suggesting that S5 interferes with the cofactor activity of FVa and FVIIa in their respective enzyme complexes. S5 also had no inhibitory activity in prothrombinase systems devoid of cofactor and phospholipid or of cofactor, phospholipid, and calcium. For intrinsic tenase, despite increases in reactant concentrations, rates of activation remained slow in a system lacking FVIIa and PCPS and were unmeasurable in a system devoid of FVIIa, PCPS, and calcium (data not shown). The data obtained in partially reconstructed systems support the concept that the cofactor-enzyme interaction is the predominant target of S5 in both prothrombinase and intrinsic tenase.

When FV or FVIII was substituted for FVa or FVIIa, an initial lag phase was seen on plots of thrombin or FXa generation versus time, reflecting the positive feedback effect of thrombin or FXa on FXa or FVII activation. When the linear part of the curve was analyzed to give an apparent rate of activation, S5 had a similar inhibitory effect in systems using FV and FVIII as it did in those using their activated counterparts, suggesting that S5 has no effect on cofactor activation.

The effect of S5 on the chromogenic activity of FXa and FXa was also examined. The hydrolysis of S-2222 by FXa is unaffected by S5. Although S5 produced some inhibition of FXa-mediated hydrolysis of Pefi FXa, this assay is of limited value because FXa has almost no activity against chromogenic amide substrates (21–23).

Affinity of LA-LMWH and sLA-LMWH for FXa and FXa—To begin to explore why S5 has greater activity against intrinsic tenase than prothrombinase, its affinity for FXa and FXa was determined and compared with that of LA-LMWH, which only has inhibitory activity against intrinsic tenase. Binding affinities were determined from competition experiments where fluorescein-labeled LA-LMWH-S5 was displaced from FXa or FXa by unlabeled S5 or LA-LMWH (Fig. 4). LA-LMWH displaced Fl-S5 from FXa and FXa with Kd values of 1000 and 9300 nM, respectively. In contrast, S5 displaced Fl-S5 from FXa and FXa with Kd values of 115 and 555 nM, respectively, consistent with its more potent inhibitory effect. The greater than 5-fold higher affinity of S5 for FXa relative to FXa could explain why S5 inhibits intrinsic tenase more effectively than prothrombinase.

Effect of S5 on Coagulation Assays—To determine whether the inhibitory activity of S5 observed in buffer systems also occurs in plasma systems, the effects of S5 on the APTT and FXa clotting time were examined (Fig. 5). The APTT was used as a measure of both intrinsic tenase and prothrombinase,
or S5. The initial fluorescence intensity ($I_0$) of 50 nM Fl-S5 in the presence of 80 nM f.IXa (■) or 100 nM f.Xa (●) was monitored at λem of 492 nm and λex of 535 in a fluorimeter. Subsequent intensity values ($I/I_0$) were determined as the sample was titrated with LA-LMWH (A) or S5 (B). Values for $I/I_0$ are plotted versus the concentration of the titrant. Displacement data were used to calculate $K_I$ values for LA-LMWH or S5 binding to f.IXa or f.Xa, as described under “Methods.”

whereas the f.Xa clotting time was used as an index of prothrombinase activity. To verify that S5 was acting independent of plasma protease inhibitors, its anticoagulant activity in plasma fractions immunodepleted of antithrombin and/or of both antithrombin and heparin cofactor II was compared with that in control plasma. S5 produces concentration-dependent prolongation of the APTT and f.Xa clotting time in control plasma, plasma immunodepleted of antithrombin, and plasma depleted of both antithrombin and heparin cofactor II. These data confirm that S5 inhibits coagulation in an antithrombin-and heparin cofactor II-independent manner and are consistent with its inhibitory activity against intrinsic tenase and prothrombinase. In contrast, concentrations of heparin that produce similar prolongations of the APTT and f.Xa clotting time in control plasma have no effect in either immunodepleted plasma, indicating that the anticoagulant effects of heparin are antithrombin-dependent.

**DISCUSSION**

Intrinsic tenase and prothrombinase complexes are critical for thrombin generation in the process of blood coagulation and, as such, are ideal targets for inhibitors of blood coagulation. Because these multicomponent complexes are assembled from intrinsic and activated components, several approaches can be used for their inhibition. These include direct inactivation of the enzyme or cofactor or disruption of the capacity of the complex to assemble productively. Our results demonstrate that unfractionated heparin and LMWH directly inhibit intrinsic tenase, consistent with the results of Barrow et al. (9). However, we have extended their findings in two important ways. First, we demonstrate that reducing the affinity of LMWH for antithrombin does not affect its ability to inhibit intrinsic tenase. Second, we demonstrate that progressive hypersulfation of LA-LMWH increases its potency of inhibition of intrinsic tenase and enables inhibition of prothrombinase. This endows sLA-LMWH with greater activity because it acts at two critical sites in the coagulation system.

**Mechanism**—The kinetics of f.X activation in the presence of unfractionated heparin have established that heparin inhibits intrinsic tenase in a noncompetitive fashion (9). The data reported here demonstrate that inhibition of the activation complexes involves the cofactor and enzyme but not the phospholipid surface. Because intrinsic tenase and prothrombinase are homologous complexes, it is conceivable that the site of action of sLA-LMWH is the same for both systems. Our data suggest that sLA-LMWH disrupts the interaction of the enzyme with its cofactor, inferring that one or both of these components bind sLA-LMWH. That heparin binds to f.IXa is well known (24–26). Likewise, calcium-dependent heparin binding to f.Xa has recently been described (27). The competitive binding studies reported here reveal that S5 binds over 5-fold more tightly to f.IXa than to f.Xa, consistent with the greater inhibitory activity of all glycosaminoglycans tested against tenase rather than prothrombinase. These results suggest that the enzymes within the activation complexes represent at least part of the target for glycosaminoglycan binding and subsequent disruption of complex assembly. The binding site for heparin on f.IXa has not been formally identified but is presumed to be at a site homologous to the heparin-binding domain of f.Xa and thrombin because arrangements of basic residues in the protease domains are largely preserved (21, 28–31). It is notable that the putative heparin-binding sites on f.Xa and f.IXa also comprise respective f.Va and f.VIIIa binding sites (32–35). In addition to the enzyme component of the activation complexes, f.V and f.VIII also bind heparin, providing additional targets for interference by glycosaminoglycans (10, 36).
Although previous studies also suggested that the interaction of fIXa and fVIIa were the likely target for glycosaminoglycan inhibition, direct binding studies did not reveal disruption of binding of the two factors on phospholipid surfaces (9). One possible explanation for this paradox is that the glycosaminoglycan may interfere with interaction of the substrate (either fX or prothrombin) with an exosite that is only expressed on the enzyme in the presence of its cofactor. The existence of such an exosite, or alternate substrate binding mode, has been suggested for prothrombinase (37–39) and tenase (33, 40–42). Further studies will be necessary to reveal the precise mechanism by which glycosaminoglycans inhibit these complexes.

Heparin also directly inhibits activation of the fVIII-von Willebrand factor complex by thrombin (10). This may reflect competition between heparin and fVIII for thrombin binding because, in addition to exosite 1, exosite 2, the heparin-binding domain, has also been implicated in fVIII recognition by thrombin (43). Although inhibition of fVIII activation by heparin may cause additional attenuation of intrinsic tenase activity, it is not the predominant mechanism because sLA-LMWH had the same effect regardless of whether fVIII or fVIIa was used.

Role of Sulfation—A series of variably sulfated LA-LMWH derivatives was used to investigate the structural requirements for inhibition of intrinsic tenase so that more potent inhibitors could be identified. In our assay of intrinsic tenase activity, LA-LMWH produced 50% inhibition of the initial velocity of fX activation at 16.3 μg/ml. A LMWH with normal antithrombin affinity inhibited intrinsic tenase to a similar extent, with an IC50 value of 13.2 μg/ml (Table I). In contrast, N-DS-LMWH had less inhibitory effect, with a 12-fold increase in the IC50 value to 166 μg/ml. These findings indicate that the inhibition of intrinsic tenase by LMWH is independent of the antithrombin-binding pentasaccharide sequence. That the inhibitory activity is dependent on the charge of the glycosaminoglycan is supported by the observation that the potency for inhibition of intrinsic tenase and prothrombinase by LA-LMWH and sulfated derivatives thereof correlates with the number of sulfate residues/disaccharide. Furthermore, a dextran sulfate whose sulfate content is equivalent to that of S5 has similar inhibitory activity against intrinsic tenase and prothrombinase, indicating that this activity does not require the heparin backbone. In contrast, Barrow et al. (9) demonstrated that less sulfated carbohydrates such as dermatan sulfate, chondroitin sulfate, or keratin sulfate inhibit intrinsic tenase with lower potency, as indicated by the IC50 values for these substrates (46). This finding is consistent with increased affinity for the enzyme and/or the cofactor.

The results obtained in the present study are similar to those reported for DHG, a depolymerized glycosaminoglycan from the sea cucumber (48). DHG was reported to inhibit both tenase and prothrombinase activities in a dose-dependent manner that involved the respective cofactors. It was observed that both fIXa and fVIIa bound to immobilized DHG. However, DHG also accelerated thrombin inhibition by heparin cofactor II (49). DHG displays a higher molecular weight than S5 (12, 500, and 5000, respectively) and a lower degree of sulfation (2.6 and 3.9 sulfate residues/disaccharide, respectively) (50). A phosophorothioate oligonucleotide that inhibits intrinsic tenase and activates heparin cofactor II has recently been described (36). Our observation that S5 retains its anticoagulant activity in plasma immunodepleted of antithrombin and heparin cofactor II indicates that serpin activation is not the means by which S5 exerts its inhibitory effects on coagulation.

As a potent inhibitor of intrinsic tenase and prothrombinase, sLA-LMWH inhibits coagulation in a novel fashion. sLA-LMWH can be added to a growing list of enzyme complex inhibitors, which include active site blocked fXa and fIXa (51, 52), fIXa antibodies (53), and active site directed fXa inhibitors (54–57). sLA-LMWH may have advantages over other agents because it simultaneously attenuates fXa and thrombin generation, with selectively greater inhibition of fXa generation by intrinsic tenase.

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REFERENCES

1. Broze, G. J. (1995) Annu. Rev. Med. 46, 103–112
2. Rapaport, S. I., and Rao, L. V. M. (1995) Trends Haemostasis 74, 7–17
3. Broze, G. J., and Miletić, J. P. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1886–1890
4. Mann, R. G. (1987) Trends Biochem. Sci. 12, 229–233
5. Mann, K. G., Jenny, R. J., and Krishnaswamy, S. (1988) Annu. Rev. Biochem. 57, 915–956
6. Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P., and Krishnaswamy, S. (1990) Blood 76, 1–16
7. Nesheim, M. E. (1984) Surv. Synth. Path. Res 3, 219–232
8. Rosenberg, R. D., and Bauer, K. A. (1993) in. Hemosis and Thrombosis: Basic Principles and Clinical Practice (Colman, R. W., Hirsh, J., Marder, J. V., and Salzman, E. W., eds) pp. 837–960. J. B. Lipincott Company, Philadelphia, PA
9. Barrow, R. T., Parker, E. T., Krishnaswamy, S., and Lollar, P. (1994) J. Biol. Chem. 269, 26796–26800
10. Barrow, R. T., Healey, J. F., and Lollar, P. (1994) J. Biol. Chem. 269, 593–598
11. Hirsh, J., and Levine, M. N. (1992) Blood 79, 1–17
12. Weitz, J. I. (1987) N. Engl. J. Med. 317, 688–688
13. Weitz, J. I., Young, E., Johnstone, M. A., Stafford, R. A., Frederen, J. C., and Hirsh, J. (1999) Circulation 99, 682–689
14. Weitz, J. I., Young, E., Johnstone, M. A., Stafford, R. A., Frederen, J. C., and Hirsh, J. (1999) Circulation 99, 682–689
15. Laemmli, U. K. (1970) Nature 227, 680–685
16. Benarolz, Y., Gibbs, D., Litman, B. J., Goll, J., Thompson, T. E., and Carlson, F. D. (1977) Biochemistry 16, 2806–2810
17. Bloom, J. W., Nesheim, M. E., and Mann, K. G. (1979) Biochemistry 18, 4419–4425
18. Ames, B. N. (1996) Methods Enzymol. VIII, 115–118
19. Nagasawa, K., Uchiyama, H., and Wajima, N. (1986) Carbohydr. Res. 158, 183–190
20. Inoue, Y., and Nagasawa, K. (1976) Carbohydr. Res. 46, 87–95
21. Frederen, J. C., Stafford, R. A., and Weitz, J. I. (1997) J. Biol. Chem. 272, 25493–25499
22. Brandstetter, H., Bauer, M., Huber, R., Lollar, P., and Bode, W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9796–9800
23. Bode, W., Brandstetter, H., Mathér, T., and Stubbs, M. T. (1997) Thromb. Haemostasis 78, 501–511
24. Sturzebecher, J., Kopetzki, E., Bode, W., and Hopfner, K. P. (1997) FEBS Lett. 412, 295–300
25. Pepper, D. S., and Prowse, C. (1977) Thromb. Res. 11, 687–692
26. Jordan, R. E., Oosta, G. M., Gardner, W. T., and Rosenberg, R. D. (1980) J. Biol. Chem. 255, 10073–10080
27. Reznik, A. R. (1998) J. Biol. Chem. 273, 16824–16827
28. Gan, Z., Liu, Y., Chen, Z., Lewis, S. D., and Shaffer, J. A. (1994) J. Biol. Chem. 269, 1303–1305
29. Bajaj, S. P., and Birktoft, J. J. (1993) Methods Enzymol. 222, 96–128
30. Brandstetter, H., Kuhne, A., Bode, W., Huber, R., von der Saal, W., Wirthsen, K., and Engh, R. A. (1996) J. Biol. Chem. 271, 20988–20992
31. Reznik, A. R. (2000) J. Biol. Chem. 275, 3320–3327
32. Rudolph, A. E., Porche-Sorbet, R., and Miletić, J. P. (2000) Biochemistry 39, 2861–2867
33. Chang, J., Jin, J., Lollar, P., Bode, W., Brandstetter, H., Hamaguchi, N., Straugh, D. L., and Stafford, D. W. (1998) J. Biol. Chem. 273, 28129–28134
34. Mathur, A., and Raja, S. P. (1999) J. Biol. Chem. 274, 14877–14886
35. Kolkmann, J. A., Lenting, P. J., and Mertens, K. (1999) Biochem. J. 339, 217–221
36. Sheehan, J. P., and Lan, C. C. (1998) Blood 92, 1617–1625

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37. Betz, A., and Krishnaswamy, S. (1998) J. Biol. Chem. 273, 10709–10718
38. Betz, A., Vlasuk, G., Bergum, P. W., and Krishnaswamy, S. (1997) Biochemistry 36, 181–191
39. Mao, S. S., Przysiecki, C. T., Krueger, J. A., Cooper, C. M., Lewis, S. D., Joyee, J., Lelis, C., Garsky, V. M., Sardana, M., and Shafer, J. A. (1998) J. Biol. Chem. 273, 30086–30091
40. Fay, P. J., and Koshibu, K. (1998) J. Biol. Chem. 273, 19049–19054
41. Fay, P. J., Koshibu, K., and Mastri, M. (1999) J. Biol. Chem. 274, 15401–15406
42. Rolldan, J. A., and Mertens, K. (2000) Biochemistry 39, 7398–7405
43. Esmon, C. T., and Lollar, P. (1996) J. Biol. Chem. 271, 13882–13887
44. Desai, U. R., Petitou, M., Bjork, I., and Olson, S. T. (1998) Biochemistry 37, 13033–13041
45. Schoen, P., Wielders, S., Petitou, M., and Lindhout, T. (1999) Thromb. Res. 57, 415–423
46. Olson, S. T., Halvorson, H. R., and Bjork, I. (1991) J. Biol. Chem. 266, 6342–6352
47. Gretenhuis, P. D., Westerduin, P., Meuleman, D., Petitou, M., and van Boeckel, C. A. (1995) Nat. Struct. Biol. 2, 736–739
48. Nagase, H., Enjoji, K., Minamiguchi, K., Kitazato, K. T., Kitazato, K., Saito, H., and Kato, H. (1995) Blood 85, 1527–1534
49. Nagase, H., Kitazato, K. T., Sasaki, E., Hattori, M., Kitazato, K., and Saito, H. (1997) Thromb. Haemostasis 77, 399–402
50. Nagase, H., Enjoji, K., Kamikubo, Y., Kitazato, K. T., Kitazato, K., Saito, H., and Kato, H. (1997) Thromb. Haemostasis 78, 864–870
51. Benedict, C. R., Ryan, J., Wolitzky, B., Ramos, R., Gerlach, M., Tijburg, P., and Stern, D. (1991) J. Clin. Invest. 88, 1760–1765
52. Wong, A. G., Gunn, A. C., Ku, P., Hellenbach, S. J., and Sinha, U. (1997) Thromb. Haemostasis 77, 1143–1147
53. Feuerstein, G. Z., Patel, A., Toomey, J. R., Bugelski, P., Nichols, A. J., Church, W. R., Valocik, R., Koster, P., Baker, A., and Blackburn, M. N. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 2554–2562
54. Tuszynski, G. P., Gasic, T. B., and Gasic, G. J. (1987) J. Biol. Chem. 262, 9718–9723
55. Vlasuk, G. (1993) Thromb. Haemostasis 70, 212–216
56. Tanisuchi, Y., Sakai, Y., Hisamichi, N., Kayama, M., Mano, Y., Sata, K., Hirayama, F., Kosho, H., Matsumoto, Y., and Kawasaki, T. (1998) Thromb. Haemostasis 79, 543–548
57. Cappello, M., Vlasuk, G. P., Bergum, P. W., Huang, S., and Hotez, P. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6152–6156
