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Initiation of the Extrinsic Pathway of Blood Coagulation: Evidence for the Tissue Factor Dependent Autoactivation of Human Coagulation Factor VII†

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ABSTRACT: Previous studies demonstrated proteolytic activation of human blood coagulation factor VII by an unidentified protease following complex formation with tissue factor expressed on the surface of a human bladder carcinoma cell line (J82). In the present study, an active-site mutant human factor VII cDNA ($Ser_{344} \rightarrow Ala$) has been constructed, subcloned, and expressed in baby hamster kidney cells. Mutant factor VII was purified to homogeneity in a single step from serum-free culture supernatants by immunoaffinity column chromatography. Mutant factor VII was fully carboxylated, possessed no apparent clotting activity, and was indistinguishable from plasma factor VII by SDS–PAGE. Cell binding studies indicated that mutant factor VII bound to J82 tissue factor with essentially the same affinity as plasma factor VII and was cleaved by factor Xa at the same rate as plasma factor VII. In contrast to radiolabeled single-chain plasma factor VII that was progressively converted to two-chain factor VIIa on J82 monolayers, mutant factor VII was not cleaved following complex formation with J82 tissue factor. Incubation of radiolabeled mutant factor VII with J82 cells in the presence of recombinant factor VIIa resulted in the time-dependent and tissue factor dependent conversion of single-chain mutant factor VII to two-chain mutant factor VIIa. Plasma levels of antithrombin III had no discernible effect on the factor VIIa catalyzed activation of factor VII on J82 cell-surface tissue factor but completely blocked this reaction catalyzed by factor Xa. These results are consistent with an autocatalytic mechanism of factor VII activation following complex formation with cell-surface tissue factor, which may play an important role in the initiation of extrinsic coagulation in normal hemostasis.

There is considerable evidence that blood coagulation is initiated by the formation of a bimolecular complex between circulating blood coagulation factor VII and its high-affinity, cell-surface receptor, tissue factor, presented by many cell types in extravascular tissues. The factor VII–tissue factor complex possesses little, if any, proteolytic activity toward its protein substrates, factors IX and X. Consequently, it is widely believed that single-chain factor VII is converted to two-chain factor VIIa by cleavage of the Arg_{152}–Ile peptide bond in zymogen factor VII in order to express any significant proteolytic activity. The factor VIIa–tissue factor complex then rapidly activates factors IX and X by limited proteolysis, which eventually leads to thrombin formation and a fibrin clot.

The identity of the protease responsible for the conversion of the factor VII–tissue factor complex to a factor VIIa–tissue factor complex in vivo is unknown. Several proteases including factor Xa (Radcliffe & Nemerson, 1976; Bajaj et al., 1981; Nemerson & Repke, 1985; Rao & Rapaport, 1988; Wildgoose & Kisiel, 1989), factor IXa (Masys et al., 1982; Wildgoose & Kisiel, 1989), thrombin (Broze & Majerus, 1980), and factor XIIa (Kisiel et al., 1977; Broze & Majerus, 1981) are efficient activators of factor VII in the test tube. More recently, Pedersen et al. (1989) have demonstrated that recombinant human factor VII is autoactivated in vitro in a reaction

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that is greatly potentiated on a positively charged surface. Using a recombinant site-specific mutant of human factor VII in which the active-site serine residue has been replaced with an alanine residue (S344A),[1] we demonstrate here that factor VII is autoactivated under physiological conditions following complex formation with either soluble tissue factor or cell-surface tissue factor provided by a human bladder carcinoma cell line. Furthermore, plasma levels of antithrombin III effectively blocked the conversion of factor VII to factor VIIa on the cell surface catalyzed by factor Xa but had little effect on the factor VIIa mediated activation of factor VII on the cell surface. Our data suggest that trace levels of circulating factor VIIa trigger the conversion of factor VII to factor VIIa in complex with cell-surface tissue factor.

Experimental Procedures

Materials. Tissue culture flasks and 12-well plates were purchased from Corning. Microtitration plates (96 well) were obtained from Dynatech Laboratories. Dulbecco's modified medium was obtained from Gibco. Minimum essential medium (Eagle's) and nonessential amino acids were purchased from Mediatech. Bovine serum albumin (fatty acid free), ovalbumin (grade V), trypsin (1× solution; tissue culture grade), vitamin K, and penicillin–streptomycin were obtained from Sigma. Iodo-gen was purchased from Pierce. Sodium [^{125}I]iodide was obtained from DuPont–New England Nuclear. Fetal bovine serum was a product of Hyclone Laboratories. Protein A–Sepharose 4 FF was obtained from Pharmacia. Affi-Gel 10 was purchased from Bio-Rad. All other reagents were of the highest purity commercially available.

Proteins. Human plasma-derived factor VII, factor Xa, and antithrombin III were purified to homogeneity essentially as described (Kondo & Kisiel, 1987; Mahoney et al., 1980). Recombinant wild-type human factor VIIa was purified from BHK cell culture medium as described (Thim et al., 1988). Recombinant human tissue factor apoprotein was generously provided by Dr. Gordon Vehar, Genentech, Inc., South San Francisco, CA. The tissue factor apoprotein was produced in *Escherichia coli* and purified to homogeneity by immunoaffinity chromatography using a tissue factor specific monoclonal antibody column (Paborsky et al., 1989). Recombinant tissue factor apoprotein preparations were relipidated essentially as described (Broze et al., 1985) with the exception that small unilamellar phospholipid vesicles (PC:PS 75:25) were used in place of rabbit brain phospholipids. The effective tissue factor apoprotein concentration was assumed to be 50% of the total tissue factor apoprotein concentration in relipidated samples (Bach et al., 1986). Phosphatidylcholine–phosphatidylserine (75:25 mol:mol) vesicles were prepared as described (Barenholz et al., 1977). Rabbit anti-human tissue factor apoprotein IgG was prepared as previously described (Sakai et al., 1989). A calcium-dependent murine anti-human factor VII monoclonal antibody (McAbCaVII20) was produced in Balb/c mice essentially according to Kohler and Milstein (1975) and purified from ascites fluid by protein A–Sepharose 4 FF column chromatography. Monoclonal antibody CaVII20 was coupled to Affi-Gel 10 according to the manufacturer's recommendations.

General Methods. SDS–polyacrylamide slab gel electrophoresis (SDS–PAGE)[2] was performed according to Laemmli (1970) using 10% polyacrylamide separating gels. Following electrophoresis, the proteins were visualized by staining with Coomassie Brilliant Blue or by autoradiography. The coagulant activities of plasma and S344A factor VII were assessed in a one-stage clotting assay using hereditary factor VII deficient plasma (<1% factor VII antigen) and purified, relipidated human brain tissue factor apoprotein (Pedersen et al., 1990). Factor VII concentrations were determined by an ELISA method (Engvall, 1980) essentially as described (Wildgoose et al., 1990). The γ-carboxyglutamic acid (Gla) content of plasma and S344A factor VII preparations were determined according to Smalley and Preusch (1988). Standard DNA techniques were carried out as described (Maniatis et al., 1982). Synthetic oligonucleotides were prepared by solid-phase phosphoramidite chemistry on an automated synthesizer (Applied Biosystems Model 380). Nucleotide sequence determinations were performed by the dideoxy chain-termination technique (Sanger et al., 1977). Radioiodination of factor VII preparations was carried out using Iodo-gen according to Fraker and Speck (1978) essentially as described (Sakai et al., 1989).

Binding of Factor VII to J82 Cells. The methodology employed for the culturing of the human bladder carcinoma J82 cell line (ATCC HTB-1) and for measuring the association of ^{125}I-labeled factor VII and ^{125}I-labeled S344A factor VII to J82 cell monolayers was essentially that previously described (Sakai et al., 1989).

Mutagenesis of Factor VII. A 651-bp *Xba*I–*Kpn*I fragment of the human factor VII cDNA which contains a portion of the factor VII heavy chain was subcloned in a 3′ to 5′ orientation in M13mp19. The S344A mutation in this fragment was then prepared by the two-primer oligonucleotide-directed mutagenesis technique (Zoller & Smith, 1984). The mutant was sequenced by the dideoxy chain-termination technique to verify the mutation and the fidelity of the remaining sequence. RF DNA was prepared from mutant plaques, and a mutated 140-bp *Pst*I–*Kpn*I fragment was used to replace the corresponding fragment of the wild-type factor VII cDNA in the pDX expression vector (Wildgoose et al., 1990).

Transfection/Selection. BHK cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and vitamin K (5 μg/mL). Cells were cotransfected with the factor VII expression plasmid and a plasmid containing the DHFR selectable marker cDNA by the calcium phosphate procedure (Graham & van der Eb, 1973). For selection of stable colonies, the cells were divided 48 h after transfection into medium containing 150 nM methotrexate. Colonies were screened for factor VII production and grown individually for protein analyses. One clone (1656-6) was selected for its stability and expression level and initially grown in serum-containing medium and subsequently in serum-free medium in a cell factory (Nunc). Serum-free cell culture supernatants were collected, made 50 mM in benzamidine, and employed for the isolation of S344A factor VII.

Purification of S344A Factor VII. Recombinant S344A factor VII was purified from ~1 L of serum-free BHK medium in a single step by immunoaffinity chromatography. Prior to chromatography, the BHK medium was filtered through a 0.22-μm filter and subsequently made 10 mM in $CaCl_2$. The calcified, filtered medium was then applied to a column (1.6 × 10 cm) of monoclonal antibody Ca VII 20–

[1] Mutant factor VII is designated according to the notation described by Shapiro and Vallee (1989), in which the single-letter code for the original amino acid is followed by its position in the sequence and the single-letter code for the new amino acid.

[2] Abbreviations: ELISA, enzyme-linked immunosorbent assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS/BSA, Tris-buffered saline/0.1% bovine serum albumin; EDTA, ethylenediaminetetraacetic acid.

Affi-Gel 10 equilibrated at 4 °C with TBS/10 mM $CaCl_2$ at a flow rate of ~30 mL/h. Following sample application, the column was washed extensively with TBS/10 mM $CaCl_2$. S344A Factor VII was then eluted from the column with TBS/30 mM EDTA. Fraction containing S344A factor VII were combined, concentrated by ultrafiltration (Amicon YM-10 membrane) to ~1 mg/mL, and stored at −80 °C.

Electrophoretic Analysis of Cell-Associated Factor VII. Wells of a 12-well plate were seeded with 5×10^5 J82 cells and grown to confluence. Each well was washed once with buffer A [10 mM Hepes (pH 7.45) containing 137 mM NaCl, 4 mM KCl, and 11 mM glucose] supplemented with 10 mM EDTA and subsequently washed three times with buffer A. Wells were incubated with either 10 nM ^{125}I-labeled factor VII (plasma) or 10 nM ^{125}I-labeled S344A factor VII in buffer A supplemented with 0.5% BSA and 5 mM $CaCl_2$ (buffer A+) for varying times at 37 °C with constant oscillation (50 rpm) on an orbit shaker (Lab-Line). At the desired incubation time, the well was rapidly washed six times with buffer A+. The cell-associated radioligand was then eluted with 100 μL of 20 mM Tris-HCl (pH 7.5)/10 mM EDTA. Samples were reduced (10% 2-mercaptoethanol), subjected to SDS–PAGE (Laemmli, 1970), and developed by autoradiography. In some experiments, the J82 cells were preincubated with 1 mL of buffer A+ containing either preimmune rabbit IgG or neutralizing rabbit IgG against human tissue factor apoprotein (final IgG concentration = 1 mg/mL) for 1 h at 37 °C. At this point, the cells were offered 10 nM ^{125}I-labeled factor VII or ^{125}I-labeled S344A factor VII and incubated at 37 °C for varying periods of time and eluted for SDS–PAGE and autoradiography as described above.

Results

S344A Factor VII was purified to apparent homogeneity in a single step from serum-free culture supernatants of BHK cells stably transfected with a plasmid containing the $Ser_{344} \rightarrow Ala$ factor VII sequence using a calcium-dependent anti-human factor VII monoclonal antibody column. S344A Factor VII migrated with the same mobility as plasma-derived factor VII in SDS–PAGE under both nonreducing and reducing conditions (Figure 1A). In addition, radioiodinated preparations of both plasma and S344A factor VII were indistinguishable by SDS–PAGE and autoradiography (Figure 1B). In a one-stage clotting assay, S344A factor VII exhibited no detectable coagulant activity, consistent with a dysfunctional active-site catalytic triad. Analysis of plasma and S344A factor VII for γ-carboxyglutamic acid content following alkaline hydrolysis indicated that both preparations were fully carboxylated with 10.3 and 10.7 mol of γ-carboxyglutamic acid/mol of protein, respectively. S344A Factor VII was converted to two-chain S344A factor VIIa by catalytic amounts of factor Xa in the presence of calcium and phospholipid at approximately the same rate as plasma factor VII (data not shown). Furthermore, the binding isotherms for ^{125}I-labeled factor VII and ^{125}I-labeled S344A factor VII on J82 cell-surface tissue factor were virtually identical (data not shown), indicating that the epitope (or epitopes) in S344A factor VII responsible for its interaction with tissue factor was not affected by the mutagenesis.

Previous studies demonstrated that plasma and recombinant single-chain factor VII were converted to factor VIIa on the J82 cell surface in a time-dependent and tissue factor dependent reaction (Fair & MacDonald, 1987; Sakai et al., 1989). This reaction was inhibited by benzamidine but was not affected by polyclonal antibodies directed against human factor X, factor IX, or prothrombin. The protease responsible

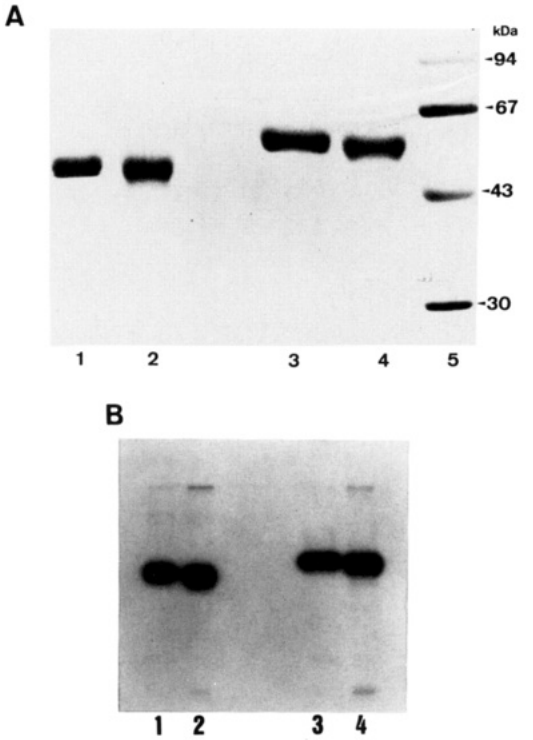

FIGURE 1: SDS–PAGE (A) and autoradiography (B) of purified S344A factor VII. (A) Lanes: 1, 10 μg of unreduced plasma factor VII; 2, 10 μg of unreduced S344A factor VII; 3, 10 μg of reduced plasma factor VII; 4, 10 μg of reduced S344A factor VII; 5, molecular weight standards (phosphorylase *b*, 94 000; bovine serum albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000). (B) Autoradiography of radioiodinated plasma and S344A factor VII. Approximately 1 μg of protein was loaded in each well. Lanes: 1, unreduced plasma factor VII; 2, unreduced S344A factor VII; 3, reduced plasma factor VII; 4, reduced S344A factor VII.

for the cleavage of single-chain factor VII in complex with J82 cell-surface tissue factor was not identified in these studies, although one possibility included that of a tumor-derived protease. In light of this, we incubated ^{125}I-labeled factor VII and ^{125}I-labeled S344A factor VII with monolayers of J82 cells and temporally examined the structure of the offered radioligand by SDS–PAGE in the presence of reducing agent followed by autoradiography. In agreement with our previous findings, ^{125}I-labeled factor VII was progressively converted to two-chain factor VIIa on J82 monolayers in a reaction that was blocked by pretreatment of the J82 cells with rabbit anti-human tissue factor IgG (data not shown). In marked contrast, ^{125}I-labeled S344A factor VII was not cleaved under identical incubation conditions (Figure 2A). This finding suggested that a protease present in the plasma factor VII preparation, and not a tumor-derived protease, was responsible for its conversion to factor VIIa on the J82 cell monolayer. Moreover, this putative contaminating protease was not present in our S344A factor VII preparations. Inasmuch as plasma factor VII was devoid of detectable factor X, factor IX, and prothrombin coagulant activity and its conversion to two-chain factor VIIa on the cell surface was not affected by polyclonal antibodies against these putative contaminants, we speculated that the most likely contaminant in our plasma factor VII preparation was factor VIIa, which, in turn, converted factor VII to factor VIIa autocatalytically. To test this hypothesis, we incubated ^{125}I-labeled S344A factor VII with J82 cell monolayers in the presence of recombinant factor VIIa (offered radioligand:enzyme molar ratio = 20:1). As shown in Figure 2B, ^{125}I-labeled S344A factor VII was converted to two-chain S344A factor VIIa under these conditions. While it is not

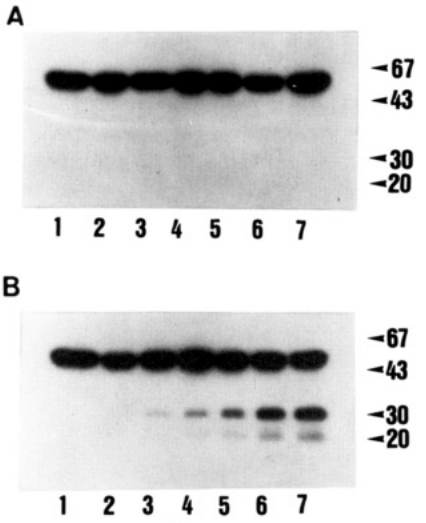

FIGURE 2: Autoradiography of ^{125}I-labeled S344A factor VII recovered from the cells. After the cells were washed and eluted with 10 mM EDTA, the recovered ^{125}I-labeled S344A factor VII/VIIa was reduced with 2-mercaptoethanol and subjected to SDS–PAGE and autoradiography. (A) Lanes 1–7 contain aliquots of ^{125}I-labeled S344A factor VII recovered from the cell incubation mixture at 0, 5, 15, 30, 60, 90, and 120 min, respectively. (B) Same as in (A), but ^{125}I-labeled S344A factor VII (offering concentration, 10 nM) was incubated with the cells in the presence of 0.5 nM recombinant factor VIIa.

known with certainty, the apparent lag phase in S344A factor VIIa formation observed in this experiment in all likelihood reflects the time-dependent association of ^{125}I-labeled S344A factor VII with cell-surface tissue factor (Fair & MacDonald, 1987; Sakai et al., 1989). Substitution of recombinant factor VIIa with an equivalent concentration of factor Xa also produced conversion of S344A factor VII to factor VIIa, although at a slightly higher rate (data not shown). Consistent with the results obtained with plasma factor VII, no conversion of ^{125}I-labeled S344A factor VII to factor VIIa was observed on the J82 cell surface in the copresence of recombinant factor VIIa and rabbit anti-human tissue factor IgG.

To further rule out the participation of a cellular protease in these reactions, we next investigated whether these reactions could be demonstrated in fluid phase using purified relipidated recombinant tissue factor apoprotein. Incubation of ^{125}I-labeled S344A factor VII with equimolar amounts of relipidated tissue factor apoprotein produced results qualitatively similar to that observed on the cell surface (Figure 3A), whereas incubation of ^{125}I-labeled factor VII with relipidated tissue factor apoprotein resulted in a relatively rapid conversion to factor VIIa in the absence of an exogenous protease (Figure 3B). Similar to the results obtained on the cell surface tissue factor, incubation of ^{125}I-labeled S344A factor VII with relipidated tissue factor apoprotein in the presence of catalytic amounts of recombinant factor VIIa resulted in its time-dependent conversion to two-chain S344A factor VIIa (Figure 3C).

The above results suggested the possibility that trace, or "idling", concentrations of circulating factor VIIa could serve as the catalyst to rapidly convert a functionally inactive factor VII–tissue factor complex to a proteolytically active factor VIIa–tissue factor complex at a site of vascular injury. Alternatively, unregulated circulating factor Xa could also catalyze this reaction on the basis of its ability to rapidly activate factor VII in the test tube. Accordingly, we investigated the relative abilities of factor VIIa and factor Xa to convert ^{125}I-labeled S344A factor VII to factor VIIa in complex with J82 cell-surface tissue factor in the presence and

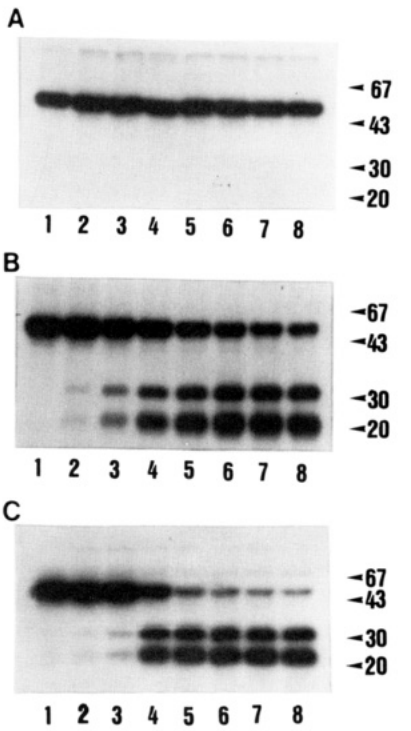

FIGURE 3: Autoradiography of ^{125}I-labeled S344A factor VII and ^{125}I-labeled factor VII (plasma-derived) with relipidated recombinant human tissue factor apoprotein. (A) ^{125}I-Labeled S344A factor VII (25 nM) was incubated with relipidated tissue factor apoprotein (effective concentration, 25 nM) for varying times at 37 °C and subjected to SDS–PAGE and autoradiography. (B) Same as (A), but ^{125}I-labeled factor VII (plasma-derived) was employed in place of ^{125}I-labeled S344A factor VII. (C) Same as (A) but in the presence of 1 nM recombinant factor VIIa. Lanes 1–8 contain aliquots of the incubation mixture at 0, 1, 5, 15, 30, 60, 90, and 120 min, respectively.

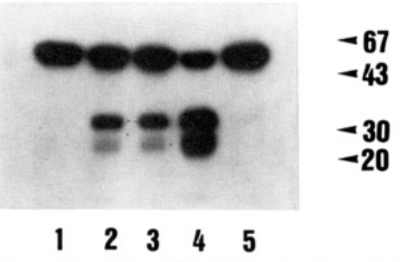

FIGURE 4: Effect of antithrombin III on the factor VIIa or factor Xa mediated activation of ^{125}I-labeled S344A factor VII on J82 cells. Cells were incubated with 10 nM S344A factor VII with either factor VIIa or factor Xa in the presence or absence of 4 μM antithrombin III for 2 h at 37 °C and processed for autoradiography as described under Experimental Procedures. Lanes: 1, S344A factor VII alone; 2, S344A factor VII plus 0.5 nM recombinant factor VIIa; 3, same as lane 2 but in the presence of antithrombin III; 4, S344A factor VII plus 0.5 nM factor Xa; 5, same as lane 4 but in the presence of antithrombin III.

absence of plasma levels of the serine protease inhibitor antithrombin III. As shown in Figure 4 (lanes 2 and 3), the ability of recombinant factor VIIa to cleave S344A factor VII was essentially the same in the presence and absence of 4 μM antithrombin III. In the absence of antithrombin III, factor Xa was clearly more efficient than factor VIIa in cleaving S344A factor VII (Figure 4, lane 4). However, in the presence of antithrombin III, essentially no factor Xa dependent activation of S344A factor VII was observed (Figure 4, lane 5).

Discussion

In the current study, an active-site $Ser_{344} \rightarrow$ Ala human factor VII mutant was constructed, expressed, and purified

to homogeneity from BHK cell serum-free culture supernatants. The S344A factor VII was subsequently used to investigate the mechanism of the conversion of single-chain zymogen factor VII to two-chain factor VIIa on monolayers of a human bladder carcinoma cell line, J82, that expresses abundant amounts of cell-surface tissue factor. This reaction was previously shown to be inhibited by 10 mM benzamidine but was unaffected by polyclonal antibody preparations against human factor Xa, human factor IXa, human thrombin, bovine factor XIIa, and bovine factor Xa, all potential catalysts of this reaction and derived either from tumor cell synthesis and secretion or from adsorption from the serum-containing culture medium. Whether a unique tumor-derived protease was responsible for this reaction was not addressed in previous studies. Evidence is presented herein that the proteolytic activation of recombinant and plasma-derived single-chain factor VII on J82 cells following complex formation with tissue factor observed in this and previous studies occurs not as a result of a tumor-derived protease but rather via a factor VIIa catalyzed reaction. Conversion of factor VII to factor VIIa on the J82 cell surface was absolutely dependent on the association of factor VII with cell-surface tissue factor, as no activation was observed on cell monolayers pretreated with anti-human tissue factor apoprotein IgG in spite of a small percentage (~10%) of the offered radioligand binding at a site distinct from tissue factor on the cell surface in a calcium-dependent manner (Sakai et al., 1989; this study). Furthermore, unbound radiolabeled factor VII in the cell supernatant was not cleaved following a 3-h incubation at 37 °C (data not shown).

Our experimental findings confirm and extend the earlier findings of Pedersen et al. (1989) concerning the autoactivation of single-chain recombinant factor VII in vitro. In that study, factor VII was activated in a sigmoidal manner in a reaction that was enhanced 2 orders of magnitude by a positively charged surface provided by either an anion-exchange matrix (Q Sepharose) or poly(D-lysine). The surface-enhanced reaction was abolished at high ionic strength (0.5 M), strongly suggesting that binding of factor VII to these matrixes was obligatory for autoactivation to occur. Interestingly, negatively charged matrixes such as S-Sepharose, under conditions that allow for factor VII binding, failed to promote factor VII autoactivation. Accordingly, it is very probable that the anion-exchange resin interacts with surface epitopes in the factor VII molecule that also participate in the interaction of factor VII with the extracellular domain of tissue factor apoprotein. The likely consequence of this interaction is a conformational change in factor VII that renders it highly susceptible to proteolytic activation by trace amounts of factor VIIa, as well as several other coagulation proteases.

Our data support a factor VII autoactivation mechanism in which trace amounts of factor VIIa activate tissue factor bound factor VII. Whether this reaction is physiologically meaningful in normal hemostasis is unknown, although several lines of investigation would suggest that it is. Miller et al. (1985) have previously shown that factor VII in hemophilic plasma is less activated than in normal plasma. The reason (or reasons) for the depressed levels of factor VIIa in hemophiliacs is not known with certainty, although it is highly probable that this phenomenon is associated with decreased production of circulating factor Xa as a result of a dysfunctional intrinsic tenase complex. Furthermore, the circulatory lifetime of factor VIIa (~2.5 h) is almost as long as that of factor VII, suggesting that inhibitors do not regulate factor VIIa levels in blood, in sharp contrast to all other blood coagulation proteases. In this regard, we show here that plasma levels of antithrombin III, a potent inhibitor of several coagulation serine proteases, had no effect on the factor VIIa catalyzed activation of factor VII on J82 cell-surface tissue factor while completely blocking the factor Xa dependent activation of factor VII under comparable conditions. Taken together, our data strongly suggest that the initiation of the extrinsic pathway of blood coagulation occurs as a result of the proteolytic activation of zymogen factor VII in complex with extravascular cell-surface tissue factor by circulating factor VIIa. Given that the circulating factor VIIa concentration may be in the picomolar (or femtomolar) range, and thus cannot effectively compete for cellular tissue factor binding by plasma levels of zymogen factor VII (~10 nM), plasma factor VIIa would presumably function as a fluid-phase catalyst for this important reaction.

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Expression and Initial Characterization of Five Site-Directed Mutants of the N-Terminal Half-Molecule of Human Transferrin†

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ABSTRACT: Five site-directed mutants of the N-terminal half-molecule of human serum transferrin have been expressed in baby hamster kidney cells and purified to homogeneity. Expression levels and overall yields varied considerably from the wild-type protein, depending on the mutant in question. The mutants are D63S, D63C, G65R, K206Q, and H207E and are based on mutations observed in a variety of transferrins of known sequence. Their molecular masses, determined by electrospray mass spectrometry, agree with theory, except for the D63C mutant, which appears to be cysteinylated. All mutants bind iron but with varying affinities; qualitatively, in increasing order D63S ≈ D63C ≈ G65R ≪ wild type ≤ H207E ≪K206Q. In general, reduction of formal negative charge within the binding cleft shifts the visible spectral maximum of the iron complex toward the blue and reduces the affinity for iron, and increasing the formal negative charge shifts the visible maximum toward the red and increases the affinity for iron. The K206Q mutant is exceptional inasmuch as its visible maximum shows a blue shift, but its affinity for iron is the greatest of all of the mutants studied. All mutants reported, in addition to the wild-type protein, exhibit very similar visible molar extinction coefficients for the iron complex and very similar changes in extinction coefficients at 240 nm on binding Fe(III) or Ga(III). These results suggest that in all cases the bound metal ion is coordinated by two tyrosyl side chains.

The transferrins (siderophilins) comprise a group of ca. 80-kDa glycoproteins found in the blood plasma and body fluids of vertebrates (Harris & Aisen, 1989; Chasteen & Woodworth, 1990) and recently found in the larvae of an insect (Bartfeld & Law, 1990). To date, the X-ray crystal structures have been reported for lactoferrin from human milk (Anderson et al., 1989, 1990) and transferrin from rabbit blood serum (Bailey et al., 1988). These structures reveal that both proteins consist of two lobes of approximately equal size connected by a short peptide. Each lobe consists of two domains defining a deep cleft in which resides a binding site for iron or many other tri- and divalent metal ions. The cleft also binds a synergistic anion, usually carbonate, but it can adapt to many other small carboxylic acids containing a second electron-donor group (Schlabach & Bates, 1975; Woodworth et al., 1975; Dubach et al., 1991). The crystallographic studies reveal that the ligands to the bound Fe(III) or bound Cu(II) (Smith et al., 1991) are the phenolate oxygens of two tyrosyl side chains, a ring nitrogen of the imidazole side chain of a histidine, the carboxylate side chain of an aspartyl residue, and two oxygens from the bound carbonate. Previously reported waters of hydration (Koenig & Schillinger, 1969; Villafranca et al., 1976) appear to be absent from the bound metal ions.

The physiological functions of the plasma transferrins appear to be to shuttle iron in a soluble nontoxic form among the organs of the body, e.g., intestine, liver, bone marrow, and reticuloendothelial system, and to serve a general immunological role by depriving microorganisms of essential iron (Bullen et al., 1990). Physical–chemical studies of these proteins include UV-vis, epr, NMR, CD, and Raman spectroscopies of their complexes with various metal ions and synergistic anions (Harris & Aisen, 1989) and microcalorimetric and reaction kinetic measurements of complex for-

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