Post-translational Modifications Required for Coagulation Factor Secretion and Function

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Introduction

Protein translocation into and transport through the secretory pathway in eukaryotic cells is accompanied by a multitude of covalent modifications that occur on the polypeptide backbone. The sequence of post-translational modifications that occur on a polypeptide is carefully regulated both temporally and spatially, where certain modifications only occur within the endoplasmic reticulum (ER) and others only occur within the Golgi compartment (Fig. 1). In many cases these modifications are required for proper polypeptide folding and secretion. In addition, post-translational modifications can affect the half-life of the protein in the plasma and may be required for functional activity of the polypeptide. The mechanisms that direct post-translational modifications recognize specific structural determinants within the polypeptide backbone. The efficiency of these modifications is determined by the specific host cell enzymatic machinery, the availability of substrates and cofactors, as well as structural properties of the polypeptide. To evaluate the role of post-translational modification in protein function investigators have studied proteins treated with chemicals or enzymes to remove modifications, proteins synthesized in the presence of inhibitors of specific modification reactions, proteins expressed in either different cell types or in cell mutants with defects in specific enzymatic machinery required to perform modifications, or proteins engineered through recombinant DNA technology that contain mutations that prevent modifications. The interpretation of results utilizing different strategies needs to be qualified to consider secondary effects due to the approach utilized. As a consequence, it is desirable to utilize several independent approaches to confirm the importance of any specific modification.

Blood coagulation is regulated by the sequential activation of vitamin K-dependent coagulation proteases within the intrinsic and extrinsic pathways. This involves a complex series of reactions that occur as a cascade that was originally recognized in 1964 (1, 2) and culminates in the generation of thrombin to convert soluble fibrinogen into insoluble fibrin. Maintenance of hemostasis relies on the regulated interaction of the vitamin K-dependent proteases, protease cofactors, membrane surfaces and receptors, calcium ions, and protease inhibitors. Three central and fundamental enzyme complexes in the coagulation cascade are the factor Xa generating complex consisting of factor IXa and the cofactor factor VIIIa, the factor Xa generating complex consisting of factor VIIa and tissue factor, and the thrombin generating complex consisting of factor Xa and the cofactor factor Va (Fig. 2). In addition, anticoagulant pathways involve tissue factor pathway inhibitors (TFPI) 1 and 2, protein C, protein S, thrombomodulin, activated protein C inhibitor, and antithrombin III. All the proteins involved in the coagulation cascade require post-translational modifications for appropriate secretion, plasma half-life, and function that requires specific protein-protein and protein-lipid interactions. This review will summarize the role of post-translational modifications for secretion and activity of the coagulation factors. Particular focus is directed to the coagulation factors VIII and IX since deficiencies in these proteins lead to the common bleeding disorders hemophilia A and hemophilia B, respectively. Recombinant DNA technology has provided the ability to produce safe and efficacious preparations of both factor VIII and factor IX for replacement therapy, and in the process has identified numerous post-translational modifications and their requirement for coagulation factor secretion and function. Gene therapy approaches for these diseases are rapidly approaching (3, 4) and need to consider the requirement for proper post-translational modification in protein secretion and function.

Domain Structure of Coagulation Factors

The domain structures of the vitamin K-dependent coagulation factors factor VII, factor IX, factor X, prothrombin, protein C and protein S deduced from their cDNA sequences demonstrate they contain common structural features (Fig. 3) (5). All contain a signal peptide that is required for translocation into the lumen of the ER. This is followed by a propeptide that directs vitamin-K dependent γ-carboxylation of the mature polypeptide. Upon transist through the trans-Golgi apparatus the propeptide is cleaved away. The amino-terminus of the mature protein contains a γ-carboxy glutamic acid rich region (Gla) that includes a short α-helical stack of aromatic amino acids. Then there are two epidermal growth factor (EGF) like domains. In protein C, factor IX, and factor X, the amino-terminal EGF domain contains β-hydroxyaspartic acid (Hya) at homologous locations. In place of the EGF domains, prothrombin has a small disulfide loop (residues 46-63) followed by two kringle domains. Protein C and factor X are proteolytically processed within the trans-Golgi apparatus after the EGF domains at residues Arg157 and Arg141, respectively, to yield disulfide linked heterodimers. The next region is the activation peptide (12-52 residues) which is glycosylated on asparagine residues and is released by specific proteolysis accompanying activation of protein C, factor IX, factor X, and prothrombin. Activation of factor VII requires cleavage of one peptide bond resulting in two-chain factor VIIa.
without loss of the activation peptide. The remainder of the vitamin K-dependent proteases comprise the serine protease catalytic triad that is absent in protein S.

Factor VIII and factor V are homologous cofactors for proteolytic activation of factor X and prothrombin, respectively, that act to increase the Vmax of substrate activation by four-orders of magnitude. They have a conserved domain organization of A1-A2-B-A3-C1-C2 (6, 7) (Fig. 4). The A domains of factors V and VIII are homologous to the A domains of the plasma copper binding protein ceruloplasmin. Copper has been detected in both factor V and factor VIII and its presence is associated with functional factor VIII activity (8, 9). One mole of reduced Cu(I) was detected in recombinant factor VIII and likely resides within a type I copper ion binding site within the A1 domain (10). The C domains are homologous to phospholipid binding proteins such as milk fat globule protein, suggesting a role in phospholipid interaction. Whereas the amino acid sequences in the A and C domains are 40% identical between factors V and VIII, there is only limited homology between the B domains. However, the B domains of both proteins have conserved the addition of a large number of asparagine-linked oligosaccharides as well as a large number of serine/threonine-linked oligosaccharides, suggesting a role of the carbohydrate in cofactor function. Factors V and VIII contain a signal peptide that is removed upon translocation into the ER. Factor V is secreted from hepatocytes as a single chain polypeptide of 330 kDa (11, 12). Factor VIII is processed within the secretory pathway in the cell to yield a heterodimer comprised primarily of a heavy chain extending up to 200 kDa (primarily two species from residues 1 to 1313 or 1648, where residue 1 is the amino-terminal amino acid after signal peptide cleavage) in a metal-ion dependent association with an 80 kDa light chain (residues 1649 to 2332). This association is stabilized by noncovalent interactions between the amino-terminal and carboxy-terminal ends of the factor VIII light chain with the amino terminus of mature von Willebrand factor (vWF). vWF interaction stabilizes factor VIII upon secretion from the cell, inhibits factor VIII binding to phospholipids, and increases the half-life of factor VIII circulating in plasma (13-15). The ratio of vWF to factor VIII is maintained at 50:1, where an increase or decrease in the plasma vWF level results in a corresponding change in the level of factor VIII.

**Fig. 1** Post-translational modifications within the secretory pathway. The post-translational modifications that occur within the rough endoplasmic reticulum (RER, top) and the Golgi compartment (below) are shown. In addition, the sequence of oligosaccharide processing of N-linked oligosaccharides is shown including the enzymes responsible and the known inhibitors (DNJ, deoxynojirimycin; DMJ, deoxymannojirimycin; SWSN, swainsonine). The point at which N-linked oligosaccharides become resistant to endoglycosidase H is shown. Sugars: □, glucose; ○, mannose; ●, N-acetylglucosamine (GlcNAc); Δ, fucose; ●, galactose (Gal); △, sialic acid (Sial). trans. Transferase. UGT, UDP-glucose:glycoprotein glucosyltransferase. Adapted from Dorner and Kaufman (41), with permission.

**Fig. 2** Protease complexes that mediate hemostasis. The major protease complexes responsible for hemostasis include the intrinsic pathway initiator [(the factor Xla complex with high-molecular-weight-kininogen (HMK)) and the extrinsic pathway initiator [the factor VIIa complex with tissue factor (TF)]. The extrinsic pathway is activated by exposure of plasma factor VII to TF on the surfaces of cells in the extravascular space. Subsequently, two protease complexes composed of factor IXa with factor VIIIa and factor Xa with factor Va act to amplify the low level protease activity by sequential activation of factor X and prothrombin (IIa), respectively. These reactions occur on the surfaces of damaged cells that provide a phospholipid surface (PL) to assemble the protease complexes. Generation of active thrombin (IIa) serves to cleave fibrinogen (FG) to insoluble fibrin (FN), a reaction necessary for hemostasis, and to cleave protein C (PC) to its active form (APC) in the presence of thrombomodulin (TM) on the surface of intact endothelial cells to initiate an anticoagulant response. Anticoagulant pathways are provided by tissue factor pathway inhibitors (TFPI I and 2) that inhibit activation of factor X, antithrombin III with heparin to inhibit IIa cleavage of FG, and APC in the presence of protein S (S) that cleave and inactivate factors Va and VIIIa. In addition, vWF negatively regulates the procoagulant response by preventing factor VIII to bind negatively charged phospholipids and inhibiting factor VIII activation by factor Xa.
Factor V and factor VIII circulate in plasma as inactive precursors that are activated through limited proteolysis by either thrombin or activated factor X (Xa) (Fig. 4). Factor V is cleaved by thrombin first after Arg 709 and then after Arg residues 1018 and 1545 to yield the activated heterodimer consisting of the 94 kDa heavy chain fragment and the 74 kDa light chain fragment (16). Thrombin activation of factor VIII results in cleavage initially after Arg 740 and subsequently after Arg residues 372 and 1689 (17, 18). Cleavage at Arg 372 and 1689 are both required for activation of factor VIII procoagulant activity. The cleavage at 1689 releases activated factor VIII from vWF, thereby relieving the inhibitory activity of vWF on factor VIII permitting the activated form of factor VIII to interact with negatively charged phospholipids. Thrombin-activated factor VIII consists of a heterotrimer of a 50 kDa A1-domain derived polypeptide, a 43 kDa A2-domain derived polypeptide, and a 73 kDa derived light chain fragment (19, 20). Upon thrombin activation, the B-domains of both factors V and VIII are released. Except for the factor V thrombin cleavage site at arginine 1018, the amino-terminal sides of all the thrombin cleavage sites within factors V and VIII are rich in acidic amino acids and contain the post-translationally modified amino acid, tyrosine sulfate.

**Signal Peptide Cleavage**

The precursor forms of proteins that are transported through the ER frequently contain a hydrophobic signal sequence of approximately 30-60 amino acids in length (21) that occurs at the amino-terminus for all the coagulation factors. The signal peptide mediates association of the nascent polypeptide with the cytosolic face of the ER. The signal peptide is composed of three regions: 1) an amino terminal segment of variable length that has a net positive charge, 2) a central hydrophobic core of 6-15 residues, and 3) a C-terminal region that often has a helix breaking residue such as Gly, Pro, or Ser. The cleavage site for signal peptidase is marked by small amino acids (Ala or Gly) in the -3 and -1 positions relative to the cleavage site. Insertion into the ER membrane involves the formation of a loop structure with the amino terminus remaining in the cytoplasm and the growing carboxy terminus being continuously translocated across the membrane through a protein-conducting channel (22). Cleavage of the signal sequence by the signal peptidase then releases the mature amino-terminus into the lumen of the ER and is required for translocation of the coagulation factors into the secretory pathway. Mutations in the signal peptide of factor VIII or factor IX can lead to hemophilia with either undetectable or reduced amounts of antigen in the circulation (23, 24).

**Disulfide Bond Formation**

The vitamin K-dependent coagulation factors, exemplified by factor IX, have conserved disulfide bonds (5). Generally, three disulfide bonds occur within each EGF domain, and several disulfide bonds

![Fig. 3](image-url)  
**Fig. 3** Domain structure of vitamin K dependent coagulation factors. The domains are identified in the key. Sites of proteolytic cleavage that occur within the secretory pathway are indicated by small arrow. The sites of proteolytic cleavage that occur upon zymogen activation are shown by bold arrows. Adapted from Furie and Furie (139), with permission

![Fig. 4](image-url)  
**Fig. 4** Domain structure and proteolytic processing of coagulation cofactors VIII and V. The domain structure of coagulation factors VIII and V are shown. In addition, the location of potential N-linked glycosylation sites, cysteine residues and disulfide bonds, and sites of tyrosine sulfation are depicted for factor VIII. Within the Golgi compartment of the cell, factor VIII is processed to a two-chain molecule by cleavage after Arg residues 1313 and 1648. In plasma, factor VIII is activated by thrombin by cleavage initially after Arg 740 and then after Arg 372 and Arg 1689 to yield a heterotrimer of 50 kDa, 43 kDa, and 73 kDa fragments. The sites required for factor VIII activation by thrombin are shown by **. The sites of proteolytic inactivation by activated protein C (APC) are shown with *. Factor V is secreted into plasma as a single chain polypeptide that requires cleavage at three residues within the B-domain to yield a heterodimer of the 94 kDa and 74 kDa fragments. Sites of proteolytic inactivation by activated protein C are also shown.
occur within the serine protease catalytic domain. In addition, in factors VII, IX, X, and protein C a disulfide bond connects the amino terminal half with the carboxy terminal half of the protein so that after activation, the two portions of the molecule do not dissociate. In factor IX, cysteine residues at 18 and 23 within the Gla domain form a small essential disulfide loop where mutation at either cysteine residue results in severe hemophilia B.

Factor VIII and factor V also have a conserved disulfide bonding pattern in which 2 disulfide bonds occur within the A1 and A2 domains, whereas only the small disulfide loop is present in their A3 domains (Fig. 4). In addition, each C domain in factor V and VIII contains one disulfide bond (25-27). There are a number of non-disulfide bonded cysteine residues within factor VIII; one cysteine residue is not oxidized in each A domain and there are four cysteine residues within the B-domain that are also likely not oxidized. Disulfide bond formation occurs in the oxidizing environment of the ER and it is possible that protein chaperones such as protein disulfide isomerase are important to ensure proper disulfide bond formation and exchange occurs prior to exit from the ER (28).

**Asparagine- and Serine/Threonine-Linked Glycosylation**

Asparagine (N)-linked glycosylation of transmembrane proteins and secreted proteins is an essential process in eukaryotic cells (29).

Addition of N-linked oligosaccharides to many glycoproteins is an obligatory event for the folding and assembly of newly synthesized polypeptides (30). The presence of oligosaccharides is often required for the efficient transport of individual glycoproteins through the secretory pathway (31, 32). In addition, N-linked glycosylation frequently affects the plasma half-life and biological activity of glycoproteins. The luminal enzyme oligosaccharyltransferase catalyzes the transfer of a preassembled high mannos containing oligosaccharide core structure [Glucose (Glc)], Mannose (Man)/N-acetylglucosamine (GlcNAc)3 from a dolichol pyrophosphate precursor onto asparagine acceptor sites within the consensus sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. The mammalian oligosaccharyltransferase is a heterotrimeric transmembrane complex of ribophorin I, ribophorin II, and the oligosaccharyltransferase that has been purified and molecularly cloned (33). The utilization of a particular consensus site for N-linked oligosaccharide attachment is determined by the structure of the growing polypeptide.

As a consequence, proteins expressed in heterologous cells most frequently exhibit occupancy of N-linked sites very similar to that of the native polypeptide (34). To date there is no evidence that the N-linked oligosaccharide addition machinery can be saturated at high expression levels of glycoproteins.

After addition of the high mannos containing oligosaccharide core structure, trimming begins with the removal of the three terminal glucose residues that is mediated by the action of glucosidases I and II (Fig. 1). Glucosidase I removes the terminal α1-3 glucose and glucosidase II subsequently removes the two α1-2 glucose residues. The activities of these enzymes can be inhibited by castanospermine or deoxynojirimycin. Glucose trimming is required for binding to the protein chaperones calnexin and calreticulin within the lumen of the ER (35). Calnexin (IP90, p88, or CNX; an integral transmembrane protein) and calreticulin (CRT; an ER luminal protein) are homologous lectin binding proteins that transiently and selectively bind to overlapping sets of newly synthesized glycoprotein folding intermediates, thereby preventing their transit through the secretory pathway. Prolonged association with CNX and/or CRT is observed when proteins are unfolded, misfolded, or unable to oligomerize. CNX and CRT bind most avidly to mono-glycosylated forms of the N-linked core structure.

Removal of the third glucose from the oligosaccharide core structure correlates with release from CNX and CRT and transport to the Golgi apparatus. The selectivity in binding of unfolded glycoproteins to CRT and CNX is thought to be mediated by reglucosylation of the deglycosylated N-linked oligosaccharide. This reglucosylation activity is performed by a UDP-glucose:glycoprotein glucosyltransferase (UGT). The activity of the UGT is activated by unfolded protein (36). Thereby, only unfolded, mutant, or unassembled proteins are subject to reglucosylation. Reglucosylated proteins can rebind CNX and/or CRT and in this manner, unfolded proteins are retained in the ER through a cycle on CNX/CRT interaction, glucosidase II activity, and UGT activity. Inhibition of glucose trimming by inhibitors of glucosidase I and II, such as deoxynojirimycin or castanospermine, can inhibit this cycle and the secretion of some proteins, for example the vitamin K dependent factor protein C (37). In addition, factor VIII, but not factor V, interacts with CNX and this interaction appears important because inhibition of glucose trimming both prevented the CNX interaction and factor VIII secretion (38) (see Fig. 5 for model for factor VIII processing, folding, and transport). In contrast, inhibition of glucose trimming had no effect on factor V secretion, demonstrating the specificity of CNX-mediated interaction and retention (38). Suprisingly, glucosidase inhibitors are fairly non-toxic and have been used in the treatment of...
AIDS due to their inhibitory effect on the secretion of the HIV envelope protein gp160 (39). Subsequent to glucose trimming in the ER, at least one α-1,2-linked mannose is removed by an ER α-1,2-mannosidase prior to transport out of the ER. Transit out of the ER is the rate limiting step in secretion for the majority of proteins and may vary from 15 min to days, depending upon the rate by which a polypeptide attains a properly folded conformation.

Upon transit through the Golgi apparatus a series of additional carbohydrate modifications occur that are separated spatially and temporally and involve the removal of mannose residues by Golgi mannosidases I and II and the addition of N-acetylglucosamine, fucose, galactose, and sialic acid residues. These reactions occur by specific glycosyltransferases that modify the high mannose carbohydrate to complex forms. Also within the Golgi apparatus, O-linked oligosaccharides are attached to the hydroxyl of serine or threonine residues through an O-glycosidic bond to N-acetylgalactosamine. Serine and threonine residues subject to glycosylation are frequently clustered together and contain an increased frequency of proline residues in the region, especially at positions -1 and +3, relative to the glycosylated residue (40). Galactose, fucose, and sialic acid are frequently attached to the serine/threonine-linked N-acetylgalactosamine. O-glycosylation occurs in the Golgi complex concomitant with processing of complex N-linked oligosaccharides.

Approaches to study the role of N- and O-linked oligosaccharide addition in glycoprotein function include the use of specific enzymatic inhibitors, glycosidases, and mutant cell lines (41). N-linked glycosylation is most easily evaluated by using specific glycosidases. The acquisition of resistance to endo-β-N-acetylgalactosaminidase (Endo-H), which cleaves high-mannose and some hybrid-type oligosaccharides at the GlcNAcβ1-4GlcNAc linkage to leave a single GlcNAc residue attached to the asparagine (42), is frequently used to monitor movement of the protein from the ER to the medial Golgi apparatus. Resistance to Endo-H occurs in the medial Golgi following action of GlcNAc transferase I and mannosidase II. Peptide-N4-(N-acetyl-B-glucosaminyl)asparagine amidase (N-gly) removes all N-linked oligosaccharides, regardless of the complexity of their structure to leave a free aspartic acid residue (43). Also of use are a number of inhibitors that are specific for selective steps in the N-linked oligosaccharide processing pathway (see Fig. 1). In addition, a number of cell lines have been isolated that are defective in specific steps in oligosaccharide processing (44).

In contrast to N-linked glycosylation, there are no specific inhibitors to study the requirement for O-linked glycosylation. It is possible to inhibit O-glycosylation, as well as complex modification of N-linked oligosaccharides, by depletion of the divalent metal ion manganese from the secretory pathway and this may be used as an indication of whether O-linked sugars are present on the polypeptide (45). Of greater utility is a mutant cell line, IdlD, that is deficient in the UDP-galactose and UDP-N-acetylgalactosamine 4-epimerase and cannot synthesize Gal or GalNAc under normal growth conditions in the presence of glucose (46, 47). Finally, it is possible to enzymatically remove O-linked glycans with endo-α-N-acetyl-d-galactosaminidase (O-glycanase) that cleaves the Galβ1→3GalNAc disaccharide unit linked to serine or threonine residues (48). Sialic acid residues on the Gal or GalNAc will inhibit O-glycanase cleavage and therefore need to be removed by prior digestion with neuraminidase.

The majority of N- and O-linked glycosylation on the vitamin-K dependent coagulation factors and in cofactors V and VIII occurs within the activation peptides. Thus, it is tempting to speculate that glycosylation plays a role in regulating the activation of these proteins. Within the activation peptides, N-linked glycosylation occur at residues 157 and 167 in factor IX, at residue 145 in factor VII, and at residues 181 and 191 in factor X. Enzymatic removal of the N-linked oligosaccharides on both recombinant and plasma-derived factor IX dramatically increases the specific activity of both molecules. Whereas enzymatic removal of sialic acid did not reduce the activities of factor IX, factor IXa, and factor X (49), it is likely that the in vivo plasma half-life will be dependent on sialic acid content since the absence of sialic acid will expose the penultimate galactose and lead to clearance via the hepatic galactosyl receptor.

With the development of recombinant factor IX produced in Chinese hamster ovary (CHO) cells for treatment of hemophilia B, a detailed characterization and comparison of the carbohydrate structures was performed between plasma-derived and recombinant-derived factor IX (50). In both plasma- and recombinant- derived factor IX Asn157 and Asn167 within the activation peptide are fully occupied with complex-type N-glycans (51). Recombinant factor IX contains tetra-antennary, tetra-sialylated, core fucosylated glycans at both sites. Plasma-derived factor IX contains bi-, tri-, and tetra-antennary, sialylated glycans, with and without fucose. Both molecules have a range of minor structures, however, the glycans present on plasma-derived factor IX are considerably more heterogeneous and diverse. The diversity may be a consequence of the plasma pool.

Both plasma- and recombinant-derived factor IX contain a number of O-linked oligosaccharides. In the first EGF domain serine residues 53 and 61 in factor IX and residues 52 and 60 in factor VII are uniformly O-glycosylated (52). The EGF1 domain in both recombinant and plasma-derived factor IX contains nonclassical O-linked glycans at Ser53 and Ser61. Ser53 contains Xyl-Xyl-Glc-Ser and Ser61 contains the tetrasaccharide with a terminal sialic acid (NeuAc), NeuAc-Gal-GlcNac-Fuc-Ser61 (53-55). This indicates that CHO cells (the cells used as a host to produce recombinant factor IX) have the enzymatic machinery to produce the structures present on plasma-derived factor IX that is synthesized in human hepatocytes and that it is not saturated at high expression levels. The carbohydrate structure at Ser61 in factor IX contains fucose-linked tetrasaccharide with a terminal sialic acid. Ser61 within the first EGF domain of factor IX has the consensus sequence (C-X-X-G-T/S-C) for fucosyl modification of O-linked sugars and is also found in factor VII, but not in factor X. Although the functional significance of these O-linked residues at Ser53 and Ser61 is not known, this unusual structure may have functional significance since Ser52Ala mutant factor VIIa possesses only 60% coagulant activity of wild-type (52). However, a recent crystal structure of factor IX demonstrated that both these O-linked modifications reside on the face of the EGF domain that apparently does not interact with other components of the factor Xase complex (56). In addition to serine-linked oligosaccharide addition in the first EGF domain, both plasma-derived and recombinant-derived factor IX molecules are partially occupied by O-linked glycans at residues Thr159, Thr160, Thr172, and Thr179 as well as at yet unidentified additional sites (51). The function of these O-linked glycans remains unknown.

Factor V and factor VIII contain a large number of N-linked oligosaccharides. Comparison of the N-linked oligosaccharides present on recombinant factor VIII expressed in mammalian cells to human plasma-derived factor VIII indicated that both proteins display similar occupancy and complexity at the N-linked sites (57). However, a detailed analysis demonstrated that differences in the microheterogeneity of oligosaccharides present on human plasma-derived factor VIII and recombinant factor VIII produced in baby hamster kidney cells do exist (58). The light chains of factor VIIa and factor Va migrate as
doublets upon SDS-PAGE due to differences in the complexity of N-linked oligosaccharides present on the light chain (59). The differences in complexity of the N-linked sugars on the light chain do not affect factor VIII activity, although increased complexity of the N-linked oligosaccharides on the factor Va light chain does increase the interaction with phospholipid and increases functional activity (60). The majority of N-linked oligosaccharides within factor VIII and factor V occur within the B-domain. Inhibition of core high mannose oligosaccharide addition by treatment with tunicamycin inhibited the secretion of factors V and VIII (59). As stated above, factor VIII secretion, but not factor V secretion, from CHO cells requires glucose trimming of glucose residues present on the N-linked oligosaccharide core structure. In contrast, inhibition of N-linked oligosaccharide trimming by treatment with the α-mannosidase inhibitor deoxymannojirimycin (DMJ) did not inhibit secretion of either factors V or VIII. Factor V and factor VIII secreted in the presence of DMJ contain high-mannose containing N-linked oligosaccharides without addition of sialic acid. The activity of factors V and VIII secreted in the presence of DMJ was slightly increased (59), consistent with the increase in activity of factor V after treatment with neuraminidase that would remove negative charge contributed by sialic acid residues (61). These results suggest that the negative charges on oligosaccharides within the B-domains might inhibit interaction between these cofactors and their enzymes and/or substrates. Indeed, the presence of N-linked oligosaccharides on factor V protect from inactivation by activated protein C (62). Finally, both factor V and factor VIII contain a large number of O-linked oligosaccharides within the B-domain (approximately 20 sites in factor VIII). A consensus sequence for O-linked glycosylation is present within a nine amino acid sequence that is tandemly duplicated thirty-one times within the B-domain of factor V. However, to date there is no data regarding the importance of these O-linked sugars for secretion, plasma half-life, or activity. It is possible that differences in carbohydrate content can influence activities of factor VIII or factor V, but to date there are no specific instances where differences in carbohydrate addition in different individuals influence activities of these factors.

Detailed analysis of recombinant factor VIII demonstrated that 3% of the total sugar chains contain a Galα1-3Gal group on some of the outer chains of the bi, tri, and tetra-antennary complex-type sugar chains that is absent on factor VIII derived from human plasma. This structure was present in Kogenate (prepared from baby hamster kidney cells) and not in Recombinate (prepared from CHO cells) (58). The α1-3-galactosyltransferase that produces this structure is expressed in most nonprimate mammalian cells and primates frequently develop antibodies to this structure. Approximately 1% of immunoglobulin in human plasma is directed towards this moiety, so it is expected that antibodies should be detected. A limited clinical trial did not detect any difference in the efficacy and/or half-life of factor VIII that contains the Galα1-3Gal group. Therefore, there is no evidence of detrimental effects of this structure present on recombinant factor VIII.

**Gamma-carboxylation of Glutamic Acid Residues**

The vitamin K-dependent coagulation factors contain the post-translationally modified amino acid γ-carboxy-glycamic acid (Gla). The Gla residues are essential for these proteins to attain a calcium-dependent conformation and for their ability to bind phospholipid surfaces, an essential interaction for their function (63, 64). The precursor of the vitamin K-dependent coagulation factors contain a propeptide that directs γ-carboxylation of up to 12 glutamic acid residues at the amino-terminus of the mature protein (65). The propeptides (residues -18 to -1 in factor IX) of these factors share amino acid similarity by conservation of the γ-carboxylase recognition site and the site for cleavage of the propeptide. NMR structural analysis identified that the propeptide is an amphipathic α-helix with the carboxylase recognition site N-terminal to the helix (66).

The residues that are carboxylated in factor IX are glutamic acid residues 7, 8, 15, 17, 20, 21, 26, 27, 30, 33, 36, and 40. Mutations at residues 6, 7, 17, 21, 27, 30, and 33 result in moderate to severe hemophilia B, indicating their functional importance. The function of Gla residues within the vitamin K-dependent coagulation factors was studied by isolation of proteins from animals treated with inhibitors of γ-carboxylation, such as dicoumarol, by proteolytic removal of the Gla domain, and by site-directed mutagenesis of specific Gla residues. Des-γ-carboxyprothrombin binds Ca$^{2+}$ much more weakly and is defective in procoagulant activity (63). Analysis of partially carboxylated prothrombins demonstrated that their activation rates in coagulation assays are proportional to the number of Gla residues present. Upon cleavage of the Gla domain from vitamin K-dependent factors, there is coincident loss of low affinity Ca$^{2+}$ sites with a greatly reduced biological activity. Several moderate- and low-affinity Ca$^{2+}$ binding sites exist in the Gla domains of factors VII, IX, X, and protein C that are necessary for a conformational change requisite to phospholipid binding and as coordination sites for phospholipid binding. With the identification of the X-ray crystal structure of the prothrombin fragment 1/Ca$^{2+}$ complex (67), and more recent determination of their solution structures (68, 69), a greatly refined model of the Ca$^{2+}$ binding site has been elucidated (70). In the absence of calcium ions, the Gla domain is disordered, whereas in the presence of calcium ions a unique structure is obtained. However, the factor X Gla domain linked to the EGF domain has structure, except in the amino-terminal 10-11 residues (71). It is possible that the structure of the calcium-free Gla domain from factor X is more similar to the structure of the intact protein. The γ-carboxylated calcium containing domain of factor IX that includes the aromatic stack (residues 1-47) forms a functional membrane binding site (72, 73).

The vitamin K-dependent γ-glutamyl carboxylase enzyme converts glutamate residues to Gla residues. In the presence of CO$_2$, O$_2$ and vitamin K hydroquinone (KH$_2$) the enzyme is able to carboxylate a peptide containing glutamic acid residues yielding a Gla containing peptide, vitamin K epoxide and H$_2$O (74). The vitamin K epoxide formed is subsequently reduced to regenerate KH$_2$ by either a thiol or the enzyme vitamin K epoxide reductase. The bovine (75) and human (76) cDNAs encoding the vitamin K-dependent γ-glutamyl carboxylase were isolated to demonstrate the protein is a single chain polypeptide of 94,000 kDa that spans the membrane 3 to 5 times. The over-expressed protein product directed increased carboxylation activity in vitro using isolated microsomes from transfected mammalian or insect cells and a synthetic peptide substrate (75, 77). The ability to express γ-carboxylase activity from the cloned cDNA has permitted identification of the propeptide binding site between residues 50 and 225 (78) and the γ-carboxylase active site for glutamate binding within the amino-terminal 218 residues (79), whereas the vitamin K reactive site is in the carboxy-terminus of the protein (80). Vitamin K epoxidease activity is also catalyzed by the enzyme and the carboxy-terminus of the enzyme is required for this activity.

High level expression of the vitamin K-dependent plasma proteins in transfected mammalian cells is limited by the ability of the mammalian host cell to efficiently perform γ-carboxylation of amino-terminal glutamic acid residues and also to efficiently cleave the propeptide.
Beta-hydroxylation of Aspartic Acid and Asparagine

Blood coagulation factors IX and X, protein C, and protein S contain the modified amino acid erythro-β-hydroxyaspartic acid in the first EGF domain. In addition, one molecule of β-hydroxyasparagine is found in each of the three carboxy-terminal EGF domains in protein S (85). This modification occurs by post-translational hydroxylation of aspartic acid and/or asparagine within the ER. The β-hydroxylase was molecularly cloned and characterized to some extent in recent years (86, 87). β-hydroxylation does not require the propeptide, vitamin K, or concomitant γ-carboxylation. A consensus β-hydroxylation site within EGF domains (Cys-X-Asp/Asn-X-X-Phe/Tyr-X-Cys-X-Cys) was proposed (85). However, human factor VII contains the consensus sequence but is not hydroxylated, suggesting that the hydroxylase has further requirements (88). Hydroxylation of both aspartic acid and asparagine is catalyzed by aspartyl hydroxylase, requires 2-ketoglutarate and Fe^{3+} (89, 90), and is inhibited by agents that inhibit 2-ketoglutarate-dependent dioxygenases (91). The function of β-hydroxyaspartic acid at residue 64 in factor IX is not known. β-hydroxylation is unnecessary for high affinity calcium binding to the first EGF domain (92). In addition, inhibition of β-hydroxylation of factor IX expressed in mammalian cells did not reduce functional activity in factor IX (91). It is interesting that only 0.3 moles/mole of plasma factor IX is modified by β-hydroxylation at Asp64 and this same amount of β-hydroxylation occurs in recombinant factor IX expressed at high levels in CHO cells (91). Thus, the low efficiency of β-hydroxylation appears to be a consequence of the factor IX polypeptide backbone and independent on the cell type used for expression.

Tyrosine Sulfation

Sulfate addition to tyrosine as an O4-sulfate ester is a common post-translational modification of secretory proteins that occurs in the trans-Golgi apparatus (93) and is mediated by tyrosylprotein sulfotransferase that utilizes the activated sulfate donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (94, 95). Although the enzyme has been biochemically characterized (95), to date the enzyme has not been molecularly cloned. This modification occurs on many secretory proteins including a number of proteins that interact with thrombin, such as hirudin (96), fibrinogen (97), heparin cofactor II (98), α2-antiplasmin (99), vitronectin (100), and bovine factor X (101). In addition, both factor V and factor VIII contain multiple sites of tyrosine sulfation (102–104). Comparison of all known tyrosine sulfation sites yielded a consensus sequence that is primarily characterized by a large number of acidic amino acid residues. The consensus tyrosine sulfation site has three aspartic acid and/or glutamic acid residues near the sulfated tyrosine (+5 amino acids), a turn inducing residue within +7/–7, and the absence of cysteine or N-linked glycosylation sites within 7 residues of the sulfated tyrosine (93). Although tyrosine sulfation modification is found on many proteins that transit the secretory apparatus, there are few examples where this modification is required for secretion or functional activity of the molecule, for example tyrosine sulfation in the hormone cholecystokinin is required for its biological activity (105). However, tyrosine sulfation can modulate the biological activity, binding affinities, and secretion of specific proteins (93). For example, tyrosine sulfation at the carboxy terminus of hirudin increases its binding affinity to the anion binding exosite of thrombin (106, 107).

The most direct method to measure sulfation of a particular protein is to measure incorporation of [35S]-sulfuric acid into protein as it is synthesized in cultured cells as described (41). [35S]-sulfuric acid will not be incorporated into methionine and cysteine. Biochemical characterization demonstrated that recombinant factor VIII metabolically labeled with [35S]-sulfuric acid contains six sites of tyrosine sulfation at residues 346, 718, 719, 723, 1664, and 1680 (102) (Fig. 4). All sites were sulfated to near completion, so it does not appear that this modification is inefficient in CHO cells. Sodium chlorate is an inhibitor of ATP sulphurylase, the first of two enzymes involved in the synthesis of PAPS. PAPS is the donor for sulfation of both tyrosine and carbohydrate residues in intact cells (108). Treatment of CHO cells that express factor VIII with sodium chlorate did not affect factor VIII secretion, but reduced the functional activity by 5-fold (102), indicating that sulfation was not required for factor VIII secretion and that at least at one tyrosine residue was required for full procoagulant activity. Site-directed mutagenesis was used to change individual or multiple tyrosine residues to the conserved residue phenylalanine in order to identify their role in factor VIII function. Tyrosine sulfation at all six sites was required for full factor VIII activity. In addition, mutagenesis of Tyr1680 to Phe demonstrated that sulfation at that residue was required for high affinity interaction with vWF (104, 109). In the absence of tyrosine sulfation at 1680 in factor VIII, the affinity for vWF was reduced by 5-fold. In contrast, mutation at residue Tyr1664 did not affect vWF interaction (109). The significance of the Tyr1680 sulfation in vivo is made evident by the presence of a Tyr1680 to Phe mutation that causes a moderate hemophilia A, likely due to reduced interaction.
with vWF and decreased plasma half-life (110). The other sites of tyrosine sulfation within factor VIII affect the rate of cleavage by thrombin at the adjacent thrombin cleavage site. For example, Tyr to Phe mutation at residues 346 and 1664 reduced the rate of thrombin cleavage at residues 372 and 1689, respectively. However, the rate of factor Xa cleavage and activation was not affected. Similarly, thrombin and factor Xa differentially activate non-sulfated recombinant factor V, i.e. the rate of thrombin cleavage and activation of non-sulfated factor V was reduced compared to sulfated factor V and there was no difference in the rate of activation by factor Xa (103). These results suggest that thrombin selectively utilizes the tyrosine sulfate residues adjacent to cleavage sites in factors V and VIII to facilitate interaction and/or cleavage. Most data support that thrombin anion-binding exosites I and II are involved in recognition of factor VIII and factor V. For example, heparin, that interacts with anion-binding exosite II, inhibits thrombin activation of factor VIII (111), although it does not inhibit activation of factor V (112), and hirugen, a peptide that binds the anion-binding exosite I of thrombin inhibits the activation of factor VIII by thrombin (112). In addition, thrombin with a mutation in the anion-binding exosite II that inhibits heparin interaction, is able to activate factor VIII, although poorly, and is also less efficient at cleavage at Arg709 in factor V. These data indicate that both thrombin exosites are involved in the cleavage of factors V and VIII, although factor V and factor VIII significantly differ in their interaction with thrombin.

Plasma-derived and recombinant-derived factor IX are sulfated on Tyr155. Whereas plasma-derived factor IX is mostly sulfated, recombinant factor IX is approximately 15% sulfated (50,55). This is one unusual example where a sulfated tyrosine occurs adjacent to an occupied N-linked glycosylation site (as asparagine residue 157). Plasma-derived factor IX and plasma-derived factor IX differ in their in vivo recovery, where the absolute recovery of plasma-derived factor IX is approximately 50% and the recovery of recombinant factor IX is approximately 30%. Studies suggest Tyr sulfation on factor IX may be responsible for the difference in the recovery of these two sources of factor IX (55) (Personal commun. H. Scoble, Genetics Institute, Andover MA). For example, infusion of recombinant factor IX enriched for full sulfation at Tyr155 demonstrated an equivalent recovery to plasma derived factor IX (approximately 50%). Similarly, removal of the sulfate as well as phosphate from plasma-derived factor IX resulted in a molecule having a recovery similar to recombinant factor IX. Finally, administration of recombinant factor IX to hemophilia B dogs and isolation of the circulating factor IX yielded species that were enriched with tyrosine sulfate compared to the starting material. The sum of these observations suggest that Tyr sulfation at 155 in factor IX can influence in vivo recovery.

Phosphorylation of Serine and Threonine Residues

Phosphate has been observed in factor V, factor VIII, and factor IX, although its significance remains unknown. Plasma-derived factor IX is fully phosphorylated at Ser158 whereas recombinant factor IX contains no phosphate at this position (55). The presence or absence of phosphate or sulfate on factor IX has no effect on the in vitro clotting activity.

Exposure of factors V and VIII to activated platelets results in phosphorylation of serine residues in factor V and primarily threonine residues in factor VIII (113,114). Phosphorylation can occur within both the heavy chain and light chains of factor V and factor VIII, possibly within the acidic acid rich regions. Although the kinase responsible for the phosphorylation remains unknown, it may be related to casein kinase II. Partially phosphorylated factor Va was shown to be more sensitive to APC inactivation, suggesting phosphorylation of these cofactors may downregulate their activity.

Proteolytic Processing

The requirement for propeptide processing for factor IX function was first made apparent by identification mutations resulting in hemophilia B that prevent processing of the factor IX propeptide. Mutations of the Arg at the P1 or P4 positions inhibit propeptide cleavage and the resultant factor IX is secreted into the plasma but is nonfunctional due to the presence of the propeptide (115-117). This mutant is unable to bind phospholipid vesicles and may also display reduced γ-carboxylation of glutamic acid residues (115). It is likely that the presence of the propeptide yields a molecule that is defective in phospholipid interaction due to an inability to undergo a calcium-dependent conformation in the Gla domain.

Early studies demonstrated that the budding yeast S. cerevisiae gene product Kex2 could cleave mammalian precursor polypeptides after paired basic residues (118), suggesting that a human homologue of Kex2 may be the protease required for propeptide processing. Kex2 is a membrane bound Ca<sup>2+</sup>-dependent subtilisin-like serine protease that cleaves substrates, such as alpha mating factor, within the trans-Golgi compartment (119,120). A computer search identified a human homologue of Kex2 upstream of the c-fes/fps proto-oncogene and was subsequently named furin (fes/fps upstream coding region) or PACE, an acronym for paired basic amino acid cleaving enzyme. The cDNA was subsequently cloned and shown to encode a protein that could cleave pro-vWF (121, 122), pro-nerve growth factor (123), proalbumin (124), complement pro-C3 (125), and pro-factor IX (81) after pairs of basic amino acids. PCR-based cloning strategies subsequently identified over a half dozen members of this subtilisin-like serine protease family (126). Expression of some of these enzymes, PC1/PC3, PC2, PC4, is restricted to neuroendocrine tissues and the activities are likely responsible for processing of neuropeptides and endocrine hormones (127,128). In contrast, furin/PACE and PACE4 are ubiquitously expressed, but to a greater extent in the hepatocyte (129,130), and are likely candidates for processing of many other proteins including coagulation factors.

Propeptide cleavage occurs in the trans-Golgi compartment just prior to secretion from the cell. The activity of the propeptide processing enzymes is uniquely regulated where a calcium-dependent induced autocatalytic activation occurs within the trans-Golgi compartment to activate these propeptides to their active form (131-134). In the process of activation, a propeptide is cleaved away from thezymogen. The specific localization of propeptide processing to the trans-Golgi compartment ensures that the propeptide is associated with the mature polypeptide as proteins transit the secretory compartment.

Characterization of the amino acid requirements around the propeptide cleavage site has identified that both the P1 and P4 arginine are important for efficient processing mediated by furin/PACE and PACE4 (135,136). Co-transfection experiments were performed to test whether these enzymes could process pro-factor IX and or pro-vWF. Whereas both furin/PACE and PACE4 were effective at enhancing pro-vWF processing, only furin/PACE was capable of improving pro-factor IX processing. Thus, it appears that different members of this class of enzymes recognize and process overlapping sets of substrates. Over-expression of furin/PACE in transfected cells (124) as well as in transgenic animals (137) improves the processing ability to yield fully processed proteins. Recombinant factor IX is produced by co-

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expression with furin/PACE to ensure complete processing of the propeptide. Similar to factor IX, factor VIII proteolytic processing within the B-domain after arginine residues 1313 and 1648 can saturate the proteolytic machinery of the cell. Both arginine residues at 1313 and 1648 have consensus sites for furin/PACE cleavage. In this case, secretion of heavy chains that extend to residue 1648 and secretion of light chains that extend to 1313 can be detected. In addition, some single chain factor VIII is detected in conditioned medium from transfected mammalian cells and in heparin-treated human plasma (15, 138). However, all analyses to date indicate that these partially processed products of factor VIII have identical activity to fully processed factor VIII. For example, double mutation of Arg1313Ile and Arg1648Ile yields a single chain factor VIII molecule with functional activity similar to wild-type factor VIII (59).

Summary and Perspectives

Eukaryotic cells contain an extensive machinery to modify polypeptides that transit the secretory compartment. In the case of coagulation factors VIII and IX, a large number of post-translational modifications occur, many are required for secretion of the polypeptide and others are required for functional activity of the polypeptide. For factor IX, co-translational translocation into the lumen of the ER occurs concomitantly with signal peptide cleavage and addition of core high mannose oligosaccharides to the polypeptide. In the ER, glucose trimming of the N-linked oligosaccharide core structures, γ-carboxylation of 12 amino terminal glutamic acid residues, and β-hydroxylation of a portion of molecules on residue Asp64 occurs. Upon transit into the Golgi compartment additional modifications occur that include: 1) complex modification of N-linked oligosaccharides, 2) tyrosine sulfation at Tyr155, 3) Ser/Thr glycosylation at residues Ser61 and Ser53 as well as several Thr residues within the activation peptide, and 4) cleavage of the propeptide. In addition, factor IX isolated from human plasma is phosphorylated at Ser158 within the activation peptide. A majority of the modifications within factor IX occur within the activation peptide and may regulate activation of factor IX. Appropriate γ-carboxylation and propeptide cleavage are essential for functional secretion and activity of secreted factor IX. Both of these activities are easily saturated upon expression of factor IX in heterologous cells. The large number of other modifications likely also affect factor IX activity by mechanisms that are not understood to date.

In the case of factor VIII, the primary translation product is also modified by signal peptide cleavage and core high mannose oligosaccharide addition upon translocation into the lumen of the ER. Within the ER, factor VIII requires trimming of glucose residues on the core N-linked glycans for transport to the Golgi compartment. In the Golgi compartment addition modifications occur that include: 1) tyrosine sulfation of six residues that are required for efficient activation by thrombin and for high affinity vWF interaction, 2) extensive addition of oligosaccharides to many Ser/Thr residues within the B-domain, 3) complex modification of N-linked glycans, and 4) cleavage of single chain factor VIII to its heavy and light chain species. To date, there do not appear to be any specific post-translational modifications that significantly limit secretion and/or functional activity of factor VIII, compared to the essential processes of γ-carboxylation and propeptide cleavage in factor IX. Further studies are required to elucidate the effect of factor VIII and factor IX expression in different cell types in order to identify the importance that subtle differences in post-translational modifications may have on their secretion, in vivo half-life, and function. These considerations will be important when considering different cells and tissues as targets for gene therapy.

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