J Biomed. 2016 June; 1(2):e6077. doi: 10.17795/jmb-6077.

Published online 2016 June 12. **Review Article**

The Effects of Influencing Factors on γ-carboxylation and Expression of Recombinant Vitamin K Dependent Coagulation Factors

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Received 2016 March 16; **Accepted** 2016 June 01.

Abstract

Vitamin K-dependent coagulation factors (VKD) have an important role in the treatment of bleeding disorders. Any disturbance in production, processing, or post-translational modifications (PTM) of these factors inhibitsthe production of functional coagulation proteins. Among the required PTMs, γ-carboxylation is a unique PTM which presents a major challenge in obtaining high expression of fully functional recombinant proteins. Studies have shown that the rate of γ-carboxylation directly relates to the amount of produced functional proteins and that it can be influenced by several factors. Here, we investigate the effects of influencing factors on γ-carboxylation and expression of VKD recombinant proteins in expression systems.

Keywords: Coagulation Factors, Expression Systems, VKD Proteins, γ-carboxylation

1. Context

The vitamin K-dependent (VKD) blood coagulation proteins are components of the calcium-binding proteins family, which includes prothrombin, factors VII, IX, and X, and proteins C, S and Z. These seven proteins have an essential role in the initiation and regulation of blood coagulation. The VKD plasma proteins are primarily synthesized as preproprotein in hepatocytes, and for functional activity, they require extensive post-translational modifications (PTMs), including the removal of the signal peptide and propeptide, γ-carboxylation of N-terminal glutamic acid residues, β-hydroxylation, N- and O-linked glycosylation, and in some cases, cleavage at internal basic residues (1). Among the required PTMs, γ-carboxylation of glutamate residues is a necessary PTM for VKDs (2). γ-carboxylation happens primarily in the liver by the γ-glutamyl carboxylase enzyme (3), and γ-carboxylase converts multiple glutamic acid residues (Glu) to γ-carboxylated glutamic acid (Gla) in a progressive manner in the Gla domains of VKD proteins (4, 5). The following are the two essential enzymes of the system: 1) the VKD γ-carboxylase, which requires reduced vitamin K (Vit.K1H2) as a cofactor and 2) the warfarin-sensitive enzyme vitamin K 2,3-epoxide reductase (VKOR), which produces the cofactor (6, 7). Recycling is accomplished by the combined actions of VKOR (8, 9) and a redox protein that supplies the electrons to VKOR (10). In the absence of vitamin K or in the presence of vitamin K antagonists, the synthesis of active VKD proteins is reduced (11).

In the ER, VKD precursors are targeted to γ-carboxylase by their γ-carboxylase-recognition site, which is a propeptide cleaved by the paired basic in most cases. Propeptide intervenes between the signal sequence (prepeptide) and the amino terminal end of the mature protein. Following the cleavage of prepeptide and γ-carboxylation in the ER, propeptide sequence is cleaved by the paired basic amino acid cleaving enzyme (PACE/furin) in the Golgi apparatus (11, 12).

2. Evidence Acquisition

A major problem with the production of recombinant VKD coagulation factors has been the poor yield of functional γ-carboxylated proteins produced by and isolated from the transfected cells (13, 14). Here we review strategies to increase γ-carboxylation and expression of functional VKD coagulation factors in expression systems.

3. Results

3.1. The γ-carboxylase

The γ-carboxylase is an integral membrane enzyme measuring 92 kDa and is localized in the ER, where VKD proteins are γ-carboxylated during their secretion (10). To date, γ-carboxylase activity has only been detected in multicellular organisms, i.e., mammals, drosophila, and the marine snail conus (15, 16); which suggests the importance of VKD proteins to intercellular interactions. In humans, one γ-carboxylase is responsible for modifying all VKD proteins and is the product of an autosomal gene (17). The VKD

γ-carboxylase is a bi-functional enzyme that catalyzes the oxygenation of vitamin K hydroquinone to vitamin K epoxide and carboxylation of multiple Glu in VKD proteins to Gla (18).

Since poor carboxylation has been observed in mammalian cell lines expressing recombinant VKD proteins (19), it has been suggested that increase of γ-carboxylase level may improve VKD protein γ-carboxylation. Wajih et al. in 2002 engineered recombinant human factor IX (r-hFIX), producing BHK cells to stably overexpress γ-carboxylase. They indicated that overexpression of γ-carboxylase inhibits production of functional r-hFIX (6). Rehemtulla et al. in 1993 also showed that overexpression of γ-carboxylase in cells stably expressing recombinant human factor IX (r-hFIX), inhibits synthesis of functional r-hFIX (20). The same results have been observed by Hallgren et al., in 2002, and have shown that cells overexpressing recombinant γ-carboxylase produce less functional r-hFIX (19). Hallgren et al. in 2002 have suggested that an excess of γ-carboxylase in the ER inhibits release of γ-carboxylated proteins by forming intracellular complexes with the VKD protein precursors (19). γ-carboxylation of FIX-carboxylase complexes leads to full γ-carboxylation of FIX but not to release of FIX from the complex unless excess substrate- and VKD product release require binding of a second VKD protein. Since most of the γ-carboxylase enzymes bind to FIX and result in full FIX carboxylation, so overexpressed γ-carboxylase is functional. On the other hand, low vitamin K level may then explain why overexpression of γ-carboxylase did not improve γ-carboxylation and secretion of FIX that was expressed at high level (20). If vitamin K is limiting, then the total rate of Glu-to-Gla conversion would not increase with increased γ-carboxylase expression (19). Therefore, because γ-carboxylase turnover is limited by dissociation of propeptide-containing substrates, high-level expression often leads to the secretion of poorly γ-carboxylated VKD proteins (13, 21). It appears from this data that overexpression of γ-carboxylase alone is not sufficient for increased production of recombinant VKD proteins.

Because of requirements of VKD proteins to the γ-carboxylation in the Gla domain, mammalian expression systems have been previously reported to be the most suitable host for the production of VKD and several other recombinant therapeutics (13). However, high-level expression of biologically active VKD proteins by these cells is hard to achieve due to inefficient c-carboxylation (20). This is exemplified by theobservation that FIX expressed by CHO cells was incompletely γ-carboxylated (22). Vatandoost et al. demonstrated the ability of Drosophilia S2 cells in recognition and γ-carboxylation of coagulation factor IX (23). They also showed that γ-carboxylation in this system is much more efficient compared to CHO mammalian cells. Moreover, Bandyopadhyay et al. showed that, using equivalent amounts of dγC and hγC in vitro, the yield of c-carboxylated products was about five-fold higher for dγC as relative to hγC (24). Consequently, alternative expression systems with an efficient γ-carboxylation system may be exploited to increase the production of fully active VKD.

3.2. Propeptide

Evidence from a number of different approaches has now clearly demonstrated that the primary gene product of VKD proteins contains a “propeptide” region between the signal peptide and the amino terminus of the mature protein that is involved in the interaction of these proteins with the γ-carboxylase (25). Propeptide is a signal for the correct γ-carboxylation of the adjacent γ-carboxyl region (Gla domain) (26). Since propeptide sequences of the VKD coagulation factors serve as a recognition site for the enzyme γ-carboxylase (2), many investigators have suggested that the propeptide must be a specific structural requirement for γ-carboxylase (26). Other studies indicated that propeptide contains two recognition elements: γ-carboxylase recognition site (CRS) located towards the N-terminus, and pro-peptidase recognition elements located near the C-terminus (27). This suggested that γ-carboxylase binds directly to amino acids of the CRS and this site (28) is required for γ-carboxylation (29).

Disruption of CRS in coagulation factors yields a mature protein that either lacks or is deficient in, γ-carboxyl glutamic acids (7). Deletion of the propeptide (residues -18 to -1) inhibited factor IX γ-carboxylation (2). Mutation in the propeptide of factor IX results in reduced affinity of γ-carboxylase to the factor IX precursor (2). A series of experiments involving deletion of parts of the propeptide within the precursor form of protein C also demonstrated the requirement of the protein C propeptide for γ-carboxylation (30). Meanwhile, extensive conservation of sequence was not seen in the propeptide sequences of the VKD proteins. Two highly conserved residues, Phe -16 and Ala -10, have been proven critical for γ-carboxylation (2). Substitution of Ala for Phe -16, or Glu for Ala -10 almost completely inhibited γ-carboxylation (11). Mutation of His to Gly at residue -18, Val to Ser at residue -17, Leu to Gly or Asp at residue - 15, and Ala to Asp at residue -10 resulted in partial inhibition of γ-carboxylation of prothrombin. Substitution of Ala for Ser -14 or Ser for Val -8 also did not inhibit γ-carboxylation (31). Handford et al. (1991) have introduced two novel mutations into the propeptide of human factor IX at positions -17 (Val-Asp) and - 6 (Leu-Asp) to study the effect of these changes on γ-carboxylation and proteolysis processing (27). Both mutations reduce the expression

of a calcium-dependent epitope in the Gla domain; however, only - 6 (Leu-Asp) shows reduced binding to barium sulfate (27). These results indicate that residues -18, -17, -16, -15 and -10 define the γ-carboxylation recognition site (27).

Since the affinity of γ-carboxylase for propeptides of VKD proteins varies with the lowest affinity for prothrombin,this can explain a high efficient carboxylation of the human prothrombin (hProt) (3, 32). So, replacement of the prepro-leader peptide of VKD proteins with those of the hProt, or mutation in specific residues, was thought to be worth examining. Exchanging the propeptide of the hFX, expressed in the HEK293T cell line, with that of the hProt substantially enhanced γ-carboxylation (33). In addition, replacement of the hFIX prepro sequence with the porcine prothrombins led to increased levels of transcription of the chimeric transgenes, as compared to the native clone (34). Vatandoost et al. (2015) mutated the factor IX cDNA in specific residue -13 (H to P) and -14 (D to A) on the basis of prothrombin propeptide. The results showed that the concentrations and activity of factor IX and so of γ-carboxylation in mutant factor IX, are more than native factor IX (35).

Studies have shown that some C-terminal propeptide residues are required for proteolysis of the propeptide from the mature protein. Mutations of the Arg at positions -1 or -4 inhibit propeptide processing and the result in nonfunctional factor IX. In addition, change of Ala to Glu at position -10 does not prevent propeptide cleavage. It also was confirmed that Arg -4 is required for correct propeptide processing. Galeffi et al. (1987), after expression of mutant factor IX in dog kidney cells, found that it is secreted into the medium in a precursor form containing the propeptide and is inefficiently γ-carboxylated compared to wild-type recombinant factor IX as control (26). This result supports the hypothesis that the propeptide region is required for efficient γ-carboxylation of VKD proteins.

3.3. VKOR

Vitamin K is essential for blood coagulation but must be enzymatically activated. This enzymatically-activated form of vitamin K is a reduced form required for γ-carboxylation of glutamic acid residues in VKD coagulation proteins. The vitamin K epoxide reductase (VKOR) as an integral membrane enzyme in the ER is responsible for reducing vitamin K to the activated form. Its C1 subunit (VKORC1) is sensitive to warfarin and is the target of anticoagulant therapy with coumarin inhibitors such as warfarin (36). Fatal bleeding may be caused by vitamin-K deficiency and by the vitamin K antagonist warfarin. Mutation in the VKOR gene can be associated with γ-carboxylation deficiencies in the VKD coagulation factors.

Since γ-carboxylase requires reduced vitamin K (K1H2) as a cofactor (37), this warfarin-sensitive enzyme recycles vitamin K to support the activation of VKD proteins. Concomitant with γ-carboxylation, vitamin K1H2 is converted to vitamin K 2,3-epoxide (7), and this epoxide is reduced by VKORC1 (37, 38) to the vitamin K1H2 cofactor (7). In a functional γ-carboxylation system, it is necessary to VKOR preserve reduced Vit.KH2 cofactor for γ-carboxylase. In this case, VKOR and γ-carboxylase must reside close together in the ER membrane so that VKOR can provide K1H2 to the γ-carboxylase through a direct interaction between the active sites of the two enzymes. Alternatively, γ-carboxylase and VKOR may be physically distinct, with vitamin K shuttling between the enzymes by migration through the membrane, analogous to how the structurally similar ubiquinone functions in mitochondrial respiration (38).

There are two sensitive cysteines in the active site of the γ-carboxylase enzyme with catalytic activity. One cysteine in thiol form is a weak base and catalyzes deprotonization of KH2, and with binding oxygen to anionic KH2, it creates the intermediate epoxide vitamin K. Another cysteine makes binding CO_2 to Glu and forms Gla. This suggested that the presence of VKOR is essential for providing γ-carboxylase cofactor.

The γ-carboxylase converts VKD proteins to Gla-containing proteins by adding CO_2 to Glu residues in newly synthesized proteins (7). Each Glu to Gla conversion requires one molecule of K1H2, and so γ-carboxylation of an individual VKD protein depends upon VKOR recycling of several vitamin K molecules (38). This modification generates a calcium-binding module required for the activities of VKD proteins (18).

It was shown that r-hFIX-producing BHK cells, stably transfected with a VKORC1 cDNA construct, produce 2.9-fold more fully γ-carboxylated functional r-hFIX than r-hFIX-producing BHK cells using the endogenous system to γ-carboxylate the protein (6). Hallgren et al. (2006) have shown that r-VKORC1 expression resulted in a 2.2-fold increase in the rate of factor IX γ-carboxylation in low-producing FIX BHK cells, and a similar increase in extent of γ-carboxylation of secreted FIX in high-producing FIX BHK cells (39).

On the other hand, it has been suggested that the redox protein is critical to efficiency of γ-carboxylation and increases in γ-carboxylation may not be possible unless it is co-expressed with VKORC1 (39). In addition, Wajih et al. (2005) stably double transfected BHK cells with VKORC1 and γ-carboxylase cDNA constructs in a bicistronic vector and indicate that activities of VKOR and γ-carboxylase significantly enhanced all transfected cell lines with the highest activities (14).

This data suggest that overexpression of VKORC1 can be utilized for increased cellular production of recombinant VKD proteins (6). Moreover, consistent with the notion that VKORC1 is the rate limiting step in the system and is a key regulatory protein in synthesis of active VKD proteins.

3.4. Calumenin

Calumenin is a water-soluble acidic protein (40) and belongs to the Ca-reticulocalbin calumenin (CREC) subfamily of Ca-binding proteins, where an EF-hand motif binds to metal (41, 42). The CREC proteins are found in the secretary pathway and have been shown to have chaperone functions (42). Consistent with electron microscopy studies of the ER membrane (43), it was demonstrated that calumenin is strongly associated with lipid-detergent micelles derived from the ER membrane (40). This result showed that calumenin, despite being a hydrophilic protein, is associated with ER membrane proteins. Since calumenin is physically associated with γ-carboxylase in ER (43), it is proposed that calumenin has an important regulatory function in the γ-carboxylation system and may inhibit γ-carboxylase activity (41).

The expression of calumenin in COS-1 cells has been shown to inhibit VKOR activity and confer warfarin resistance to the VKOR enzyme complex (40). Stable cell lines transfected with only factor VII cDNA had a 9% production of functional rFVII, but in rFVII-producing cells that calumenin stably suppressed by shRNA, functional rFVII produced up to 68% (44). So, calumenin appears to be a chaperone that regulates the capacity of the VKD γ-carboxylation system in production of functional VKD proteins. Moreover, this regulatory role puts calumenin in a central position regarding the maintenance of a normal blood-coagulation system.

3.5. PACE/Furin

VKD proteins are synthesized as a precursor polypeptide which requires proteolytic cleavage of the N-terminal pre-propeptide for functional activity (1). The prepeptide or signal sequence is cleaved by a signal peptidase cotranslationally during entrance to ER. In the Golgi apparatus and prior to secretion from the cell, the propeptide sequence is cleaved by the PACE/furin enzyme (11, 12). Furin is a protein that is encoded by the FUR gene in humans. It was named FUR because it is in the upstream region of FES oncogene. Furin is also known as paired basic amino acid cleaving enzyme (PACE) (1).

The protein encoded by this gene is an enzyme which belongs to the subtilisin-like proprotein convertase family. The members of this family are proprotein convertases that process latent precursor proteins into their biologically active products. This encoded protein is a calcium-dependent serine endoprotease that can efficiently cleave precursor proteins at their paired basic amino acid processing sites. Furin is enriched in the Golgi apparatus, where it functions to cleave other proteins into their mature/active forms (45). Furin cleaves proteins just downstream of a basic amino acid target sequence (Arg-X-(Arg/Lys)-Arg').

A strategy to improve production of VKD proteins is to co-transfect its gene with the PACE/furin gene. Studies have shown that overexpression of PACE improved specific activity of rhFIX approximately two to three-fold, an increase expected from the two to three-fold increase in propeptide cleavage (1). So cleavage of the prepropeptide has been known as one of the biochemical limitations in the production of γ-carboxylated proteins (3, 46).

3.6. Gla Domain

Gla domain in VKD proteins contains many glutamate residues to form Gla. Proteins with this domain are known informally as Gla proteins. Human proteins containing this domain include the blood coagulation factors II (prothrombin), VII, IX, and X, the anticoagulant proteins C, S, and the factor-X targeting protein Z. Other Gla proteins include the bone Gla protein osteocalcin (BGLAP), matrix Gla protein (MGP), GAS6, Transthyretin (TTR), inter-alpha-trypsin inhibitor heavy chain H2 (ITIH2), Periostin and 4 Proline rich Gla (PRRG1-4) (47). The Gla domains in the VKD proteins contain 9 - 12 Gla residues; of these, the nine N-terminal Gla residues are fully conserved and the tenth is highly conserved (48).

Multiple Glu carboxylation is required for the activity of VKD proteins. Gla formation results in creation of a cluster of bidentate ligands with calcium binding affinity. In the presence of calcium, Gla clusters form a binding network involving multiple calcium ions with a number of different proteins (18). Calcium ions induce conformational changes in the Gla domain and are necessary for the Gla domain to fold properly. A common structural feature of functional Gla domains is the clustering of N-terminal hydrophobic residues into a hydrophobic patch that mediates interaction with the cell surface membrane (49).

The Gla domain binds calcium ions by chelating them between two carboxylic acid residues. These residues are part of a region that starts at the N-terminal extremity of the mature form of Gla proteins, and that ends with a conserved aromatic residue (49). This results in a conserved Gla-x(3)-Gla-x-Cys motif (50) that is found in the middle of the domain, and which seems to be important for substrate recognition by the carboxylase.

Since the discovery of Gla in 1974, several different approaches have been used to study the importance of individual Gla residues in the function of the VKD proteins (48). Studies with VKD proteins that lack the CRS/propeptide show that it defines a new tethering site on the carboxylase distinct from the CRS/propeptide-binding site (51).

A consensus sequence within the Gla domain of the VKD proteins was identified by Price et al. which suggested that this domain may play a role in the binding of the substrate to the carboxylase (50). Mutational analysis showed that this region is important for carboxylation and epoxidation (11). Mutation of Gla at residue 33 in prothrombin to aspartic acid yielded a protein with reduced coagulant activity (52). In addition, earlier studies have shown that γ-carboxylation at Glu-36 and Glu-40 was not important for FIX function, suggesting that the γ-carboxylation of rhFIX may be sufficient for high clotting activity (48, 53). The studies also show that the importance of some homologous Gla residues differs among these proteins. For instance, disruption of Gla at residue 14 and 19 in prothrombin decreases functional activity, but has no effect on the activity of protein C. Likewise, disruption of Gla at residue 7 and 20 in protein C eliminates activity, but the same mutations in prothrombin inhibit function only partially (48). So mutation effects are dependent on mutant position and protein type. The replacement of FX Gla with other VKDs Gla domain revealed implicated in the assembly to FVIIa/ TF. Thus Gla mediates protein-protein interactions and may consequently be implicated in the assembly of the protein complexes of blood coagulation.

4. Conclusions

A major problem in production of recombinant coagulation proteins is low expression and weak functional activity of produced proteins. Expression and activity of recombinant coagulation proteins are affected by PTMs such as γ-carboxylation of N-terminal glutamic acid residues, removal of the signal peptide and propeptide, N- and O-linked glycosylation and in some cases, cleavage at internal basic residues. Some efforts have been made for change in the affecting factors on PTMs to improve expression and activity of recombinant coagulation factors. These studies have demonstrated that influence of some factors are significant and sometimes have the opposite effect. Therefore, it seems to be necessary to check these factors for production of recombinant VKDs.

An essential PTM for the expression and activity of coagulation factors is γ-carboxylation of glutamic acid residues. It has been shown that γ-carboxylation has a direct correlation with functional protein expression and can be increased by different factors. Studies on different VKDs have shown the effect of different factors on γ-carboxylation, so that overexpression of γ-carboxylation enzyme in VKD-producing cell lines has been associated with decrease of γ-carboxylation level and expression reduction. On the other hand, increase of VKORC1 enzyme as a γ-carboxylation cofactor has been effective in enhancement of γ-carboxylation speed and secretion of VKDs. At the same time increase of these two factors also increases γ-carboxylation in the balance of substrate and enzyme. Moreover, Calumenin reduction, as an inhibitor, caused significant increase of recombinant coagulation factors expression.

Overexpression of only the prepeptide-processing enzymes called PACE/furin in cells producing recombinant coagulation factors has been shown to increase the expression of coagulation factors but co-expression with γ-carboxylase does not cause improvement in carboxylation rate. Mutation or replacement of the prepeptide and propeptide has different effects on the γ-carboxylation, expression and activity of clotting factors. Although replacement of factors X and IX with the prothrombin propeptide was associated with expression increase of these factors, mutation in this domain has different effects on carboxylation and expression of clotting factors. Alteration of the expression system also caused higher increases in post-translational modifications in HEK293 cells than CHO and COS1, and increase of expression and activity in Drosophila S2 cells compared to mammalian cells.

Considering the above mentioned, it appears that co-expression of factors that increase full γ-carboxylation of coagulation factors is an effective method for efficient production of recombinant coagulation factors, however, the ability of used expression systems must be considered in amount and number tolerance of expressed external factors.

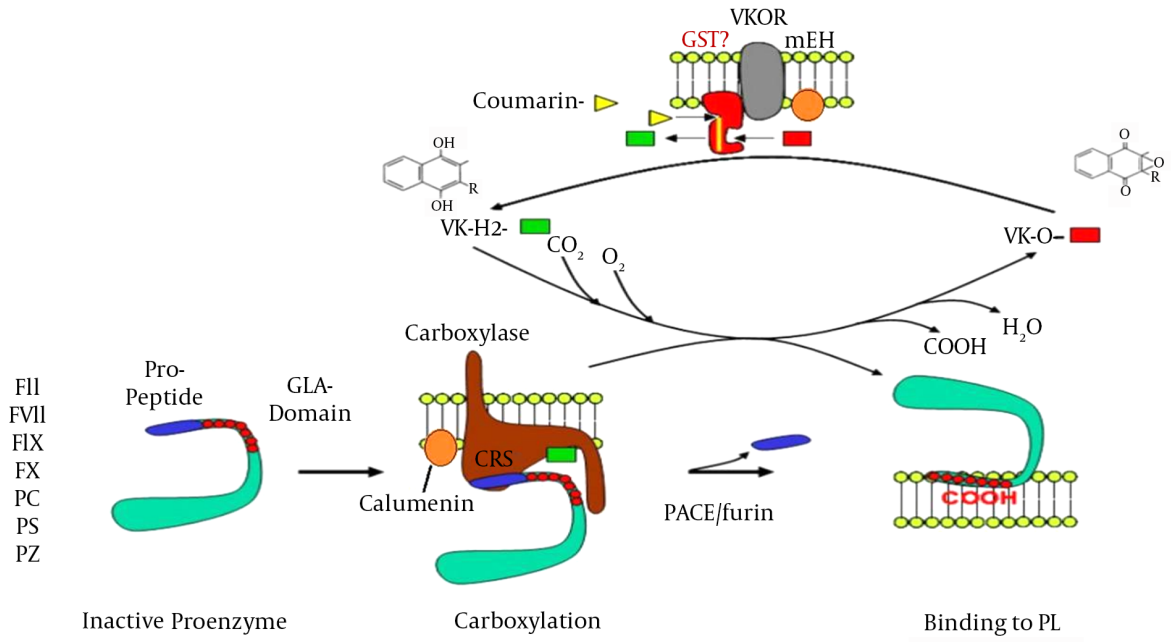

Figure 1. The γ-carboxylation System

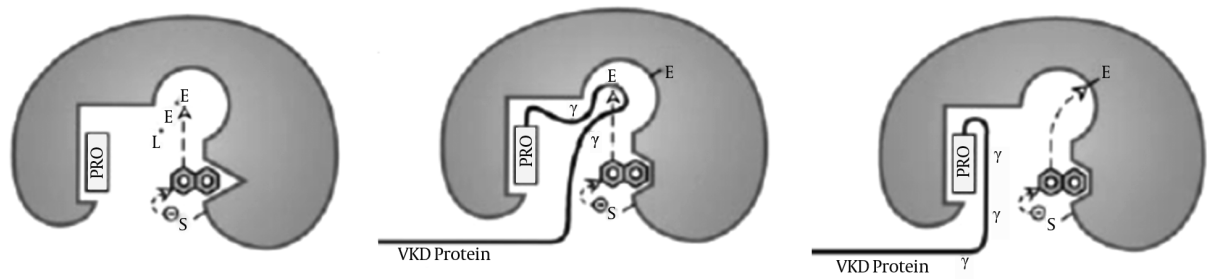

Figure 2. The Carboxylase Active Site, most vitamin K-dependent (VKD) proteins bind to the carboxylase through the propeptide (PRO), and there may also be a second point of contact for the glutamyl (glu; E) residues, as illustrated by the pocket.

References

1. Wasley LC, Rehemtulla A, Bristol JA, Kaufman RJ. PACE/furin can process the vitamin K-dependent pro-factor IX precursor within the secretory pathway. *J Biol Chem.* 1993;**268**(12):8458–65. [PubMed: 8473289].
2. Chu K, Wu SM, Stanley T, Stafford DW, High KA. A mutation in the propeptide of Factor IX leads to warfarin sensitivity by a novel mechanism. *J Clin Invest.* 1996;**98**(7):1619–25. doi: 10.1172/JCI118956. [PubMed: 8833911].
3. Blostein M, Cuerquis J, Landry S, Galipeau J. The carboxylation efficiency of the vitamin K-dependent clotting factors: studies with factor IX. *Haemophilia.* 2008;**14**(5):1063–8. doi: 10.1111/j.1365-2516.2008.01828.x. [PubMed: 18680528].
4. Paquin A, Jaalouk DE, Galipeau J. Retrovector encoding a green fluorescent protein-herpes simplex virus thymidine kinase fusion protein serves as a versatile suicide/reporter for cell and gene therapy applications. *Hum Gene Ther.* 2001;**12**(1):13–23. doi: 10.1089/104303401450924. [PubMed: 11177538].
5. Larson PJ, Camire RM, Wong D, Fasano NC, Monroe DM, Tracy PB, et al. Structure/function analyses of recombinant variants of human factor Xa: factor Xa incorporation into prothrombinase on the thrombin-activated platelet surface is not mimicked by synthetic phospholipid vesicles. *Biochemistry.* 1998;**37**(14):5029–38. doi: 10.1021/bi972428p. [PubMed: 9538022].
6. Wajih N, Hutson SM, Owen J, Wallin R. Increased production of functional recombinant human clotting factor IX by baby hamster kidney cells engineered to overexpress VKORC1, the vitamin K 2,3-epoxide-reducing enzyme of the vitamin K cycle. *J Biol Chem.* 2005;**280**(36):31603–7. doi: 10.1074/jbc.M505373200. [PubMed: 16030016].

7. Wallin R, Hutson SM. Warfarin and the vitamin K-dependent gamma-carboxylation system. *Trends Mol Med.* 2004;**10**(7):299–302. doi: 10.1016/j.molmed.2004.05.003. [PubMed: 15242675].
8. Li T, Chang CY, Jin DY, Lin PJ, Khvorova A, Stafford DW. Identification of the gene for vitamin K epoxide reductase. *Nature.* 2004;**427**(6974):541–4. doi: 10.1038/nature02254. [PubMed: 14765195].
9. Rost S, Fregin A, Ivaskevicius V, Conzelmann E, Hortnagel K, Pelz HJ, et al. Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature.* 2004;**427**(6974):537–41. doi: 10.1038/nature02214. [PubMed: 14765194].
10. Berkner KL. The vitamin K-dependent carboxylase. *Annu Rev Nutr.* 2005;**25**:127–49. doi: 10.1146/annurev.nutr.25.050304.092713. [PubMed: 16011462].
11. Furie B, Furie BC. Molecular basis of vitamin K-dependent gamma-carboxylation. *Blood.* 1990;**75**(9):1753–62. [PubMed: 2184900].
12. Bristol JA, Ratcliffe JV, Roth DA, Jacobs MA, Furie BC, Furie B. Biosynthesis of prothrombin: intracellular localization of the vitamin K-dependent carboxylase and the sites of gamma-carboxylation. *Blood.* 1996;**88**(7):2585–93. [PubMed: 8839851].
13. Kaufman RJ, Wasley LC, Furie BC, Furie B, Shoemaker CB. Expression, purification, and characterization of recombinant gamma-carboxylated factor IX synthesized in Chinese hamster ovary cells. *J Biol Chem.* 1986;**261**(21):9622–8. [PubMed: 3733688].
14. Wajih N, Sane DC, Hutson SM, Wallin R. Engineering of a recombinant vitamin K-dependent gamma-carboxylation system with enhanced gamma-carboxyglutamic acid forming capacity: evidence for a functional CXXC redox center in the system. *J Biol Chem.* 2005;**280**(11):10540–7. doi: 10.1074/jbc.M413982200. [PubMed: 15640149].
15. Bandyopadhyay PK, Garrett JE, Shetty RP, Keate T, Walker CS, Olivera BM. gamma -Glutamyl carboxylation: An extracellular posttranslational modification that antedates the divergence of molluscs, arthropods, and chordates. *Proc Natl Acad Sci U S A.* 2002;**99**(3):1264–9. doi: 10.1073/pnas.022637099. [PubMed: 11818531].
16. Czerwiec E, Begley GS, Bronstein M, Stenflo J, Taylor K, Furie BC, et al. Expression and characterization of recombinant vitamin K-dependent gamma-glutamyl carboxylase from an invertebrate, Conus textile. *Eur J Biochem.* 2002;**269**(24):6162–72. [PubMed: 12473112].
17. Kuo WL, Stafford DW, Cruces J, Gray J, Solera J. Chromosomal localization of the gamma-glutamyl carboxylase gene at 2p12. *Genomics.* 1995;**25**(3):746–8. [PubMed: 7759116].
18. Rishavy MA, Berkner KL. Vitamin K oxygenation, glutamate carboxylation, and processivity: defining the three critical facets of catalysis by the vitamin K-dependent carboxylase. *Adv Nutr.* 2012;**3**(2):135–48. doi: 10.3945/an.111.001719. [PubMed: 22516721].
19. Hallgren KW, Hommema EL, McNally BA, Berkner KL. Carboxylase overexpression effects full carboxylation but poor release and secretion of factor IX: implications for the release of vitamin K-dependent proteins. *Biochemistry.* 2002;**41**(50):15045–55. [PubMed: 12475254].
20. Rehemtulla A, Roth DA, Wasley LC, Kuliopulos A, Walsh CT, Furie B, et al. In vitro and in vivo functional characterization of bovine vitamin k-dependent gamma-carboxylase expressed in chinese hamster ovary cells. *Proc Natl Acad Sci.* 1993;**90**(10):4611–5. doi: 10.1073/pnas.90.10.4611.
21. Berkner KL. Expression of recombinant vitamin K-dependent proteins in mammalian cells: factors IX and VII. *Methods Enzymol.* 1993;**222**:450–77. [PubMed: 8412810].
22. Balland A, Faure T, Carvallo D, Cordier P, Ulrich P, Fournet B, et al. Characterisation of two differently processed forms of human recombinant factor IX synthesised in CHO cells transformed with a polycistronic vector. *Eur J Biochem.* 1988;**172**(3):565–72. [PubMed: 3280312].
23. Vatandoost J, Zomorodipour A, Sadeghizadeh M, Aliyari R, Bos MH, Ataei F. Expression of biologically active human clotting factor IX in Drosophila S2 cells: gamma-carboxylation of a human vitamin K-dependent protein by the insect enzyme. *Biotechnol Prog.* 2012;**28**(1):45–51. doi: 10.1002/btpr.723. [PubMed: 22012919].
24. Bandyopadhyay PK, Clark K, Stevenson BJ, Rivier JE, Olivera BM, Golic KG, et al. Biochemical characterization of Drosophila gamma-glutamyl carboxylase and its role in fly development. *Insect Mol Biol.* 2006;**15**(2):147–56. doi: 10.1111/j.1365-2583.2006.00619.x. [PubMed: 16640725].
25. Cheung A, Engelke JA, Sanders C, Suttie JW. Vitamin K-dependent carboxylase: influence of the "propeptide" region on enzyme activity. *Arch Biochem Biophys.* 1989;**274**(2):574–81. [PubMed: 2802629].
26. Galeffi P, Brownlee GG. The propeptide region of clotting factor IX is a signal for a vitamin K dependent carboxylase: evidence from protein engineering of amino acid -4. *Nucleic Acids Res.* 1987;**15**(22):9505–13. [PubMed: 3684602].
27. Handford PA, Winship PR, Brownlee GG. Protein engineering of the propeptide of human factor IX. *Protein Eng.* 1991;**4**(3):319–23. [PubMed: 1857716].
28. Hubbard BR, Ulrich MM, Jacobs M, Vermeer C, Walsh C, Furie B, et al. Vitamin K-dependent carboxylase: affinity purification from bovine liver by using a synthetic propeptide containing the gamma-carboxylation recognition site. *Proc Natl Acad Sci U S A.* 1989;**86**(18):6893–7. [PubMed: 2780546].
29. Furie B, Bouchard BA, Furie BC. Vitamin K-dependent biosynthesis of gamma-carboxyglutamic acid. *Blood.* 1999;**93**(6):1798–808. [PubMed: 10068650].
30. Huang M, Rigby AC, Morelli X, Grant MA, Huang G, Furie B, et al. Structural basis of membrane binding by Gla domains of vitamin K-dependent proteins. *Nat Struct Biol.* 2003;**10**(9):751–6. doi: 10.1038/nsb971. [PubMed: 12923575].
31. Vadivel K, Agah S, Messer AS, Cascio D, Bajaj MS, Krishnaswamy S, et al. Structural and functional studies of gamma-carboxyglutamic acid domains of factor VIIa and activated Protein C: role of magnesium at physiological calcium. *J Mol Biol.* 2013;**425**(11):1961–81. doi: 10.1016/j.jmb.2013.02.017. [PubMed: 23454357].
32. Stanley TB, Jin DY, Lin PJ, Stafford DW. The propeptides of the vitamin K-dependent proteins possess different affinities for the vitamin K-dependent carboxylase. *J Biol Chem.* 1999;**274**(24):16940–4. [PubMed: 10358041].
33. Camire RM, Larson PJ, Stafford DW, High KA. Enhanced gamma-carboxylation of recombinant factor X using a chimeric construct containing the prothrombin propeptide. *Biochemistry.* 2000;**39**(46):14322–9. [PubMed: 11087381].
34. Khorshidi S, Zomorodipour A, Behmanesh M, Vatandoost J, Bos MH. Functional expression of the human coagulation factor IX using heterologous signal peptide and propeptide sequences in mammalian cell line. *Biotechnol Lett.* 2015;**37**(9):1773–81. doi: 10.1007/s10529-015-1868-3. [PubMed: 26105559].
35. Vatandoost J, Abadi SH. Study of propeptide mutations effect on the human factor IX expression. *Cell Mol Res.* 2016;**in press**.
36. Suttie J, Vitamin K. Fat-soluble vitamins, their biochemistry and applications. Technomic Publishing Company; 1985. pp. 225–311.
37. Bush KA, Stenflo J, Roth DA, Czerwiec E, Harrist A, Begley GS, et al. Hydrophobic amino acids define the carboxylation recognition site in the precursor of the gamma-carboxyglutamic-acid-containing conotoxin epsilon-TxIX from the marine cone snail Conus textile. *Biochemistry.* 1999;**38**(44):14660–6. [PubMed: 10545191].
38. Berkner KL, Runge KW. The physiology of vitamin K nutriture and vitamin K-dependent protein function in atherosclerosis. *J Thromb Haemost.* 2004;**2**(12):2118–32. doi: 10.1111/j.1538-7836.2004.00968.x. [PubMed: 15613016].
39. Hallgren KW, Qian W, Yakubenko AV, Runge KW, Berkner KL. r-VKORC1 expression in factor IX BHK cells increases the extent of factor IX carboxylation but is limited by saturation of another carboxylation component or by a shift in the rate-limiting step. *Biochemistry.* 2006;**45**(17):5587–98. doi: 10.1021/bi051986y. [PubMed: 16634640].
40. Wallin R, Hutson SM, Cain D, Sweatt A, Sane DC. A molecular

mechanism for genetic warfarin resistance in the rat. *FASEB J.* 2001;**15**(13):2542–4. doi: 10.1096/fj.01-0337fje. [PubMed: 11641264].
41. Wajih N, Sane DC, Hutson SM, Wallin R. The inhibitory effect of calumenin on the vitamin K-dependent gamma-carboxylation system. Characterization of the system in normal and warfarin-resistant rats. *J Biol Chem.* 2004;**279**(24):25276–83. doi: 10.1074/jbc.M401645200. [PubMed: 15075329].
42. Honore B, Vorum H. The CREC family, a novel family of multiple EF-hand, low-affinity Ca(2+)-binding proteins localised to the secretory pathway of mammalian cells. *FEBS Lett.* 2000;**466**(1):11–8. [PubMed: 10648803].
43. Vorum H, Hager H, Christensen BM, Nielsen S, Honore B. Human calumenin localizes to the secretory pathway and is secreted to the medium. *Exp Cell Res.* 1999;**248**(2):473–81. doi: 10.1006/excr.1999.4431. [PubMed: 10222138].
44. Wajih N, Owen J, Wallin R. Enhanced functional recombinant factor VII production by HEK 293 cells stably transfected with VKORC1 where the gamma-carboxylase inhibitor calumenin is stably suppressed by shRNA transfection. *Thromb Res.* 2008;**122**(3):405–10. doi: 10.1016/j.thromres.2007.11.002. [PubMed: 18177690].
45. Thomas G. Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat Rev Mol Cell Biol.* 2002;**3**(10):753–66. doi: 10.1038/nrm934. [PubMed: 12360192].
46. Harrison S, Adamson S, Bonam D, Brodeur S, Charlebois T, Clancy B, et al. The manufacturing process for recombinant factor IX. *Semin Hematol.* 1998;**35**(2 Suppl 2):4–10. [PubMed: 9565160].
47. Suttie JW. Synthesis of vitamin K-dependent proteins. *FASEB J.* 1993;**7**(5):445–52. [PubMed: 8462786].
48. Gillis S, Furie BC, Furie B, Patel H, Huberty MC, Switzer M, et al. gamma-Carboxyglutamic acids 36 and 40 do not contribute to human factor IX function. *Protein Sci.* 1997;**6**(1):185–96. doi: 10.1002/pro.5560060121. [PubMed: 9007991].
49. Freedman SJ, Blostein MD, Baleja JD, Jacobs M, Furie BC, Furie B. Identification of the phospholipid binding site in the vitamin K-dependent blood coagulation protein factor IX. *J Biol Chem.* 1996;**271**(27):16227–36. [PubMed: 8663165].
50. Price PA, Fraser JD, Metz-Virca G. Molecular cloning of matrix Gla protein: implications for substrate recognition by the vitamin K-dependent gamma-carboxylase. *Proc Natl Acad Sci U S A.* 1987;**84**(23):8335–9. [PubMed: 3317405].
51. Pudota BN, Hommema EL, Hallgren KW, McNally BA, Lee S, Berkner KL. Identification of sequences within the gamma-carboxylase that represent a novel contact site with vitamin K-dependent proteins and that are required for activity. *J Biol Chem.* 2001;**276**(50):46878–86. doi: 10.1074/jbc.M108696200. [PubMed: 11591726].
52. Ratcliffe JV, Furie B, Furie BC. The importance of specific gamma-carboxyglutamic acid residues in prothrombin. Evaluation by site-specific mutagenesis. *J Biol Chem.* 1993;**268**(32):24339–45. [PubMed: 8226983].
53. Liu J, Jonebring A, Hagstrom J, Nystrom AC, Lovgren A. Improved expression of recombinant human factor IX by co-expression of GGCX, VKOR and furin. *Protein J.* 2014;**33**(2):174–83. doi: 10.1007/s10930-014-9550-5. [PubMed: 24567122].