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Insights into vitamin K-dependent carboxylation: home field advantage

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Vitamin K-dependent (VKD) proteins play critical roles in blood coagulation, bone metabolism, and other physiologic processes. These proteins undergo a specific post-translational modification called gamma (γ)-carboxylation which is critical to their biologic function.[1] The reaction, which occurs in the endoplasmic reticulum (ER) and requires reduced vitamin K, carbon dioxide and oxygen as co-factors, is catalyzed by γ-glutamyl carboxylase (GGCX). GGCX converts several glutamic acid residues (Glu) on its protein substrate [e.g. prothrombin, FVII, FIX, FX, PC, PS, PZ, and bone Gla protein (BGP)] to γ-carboxy-glutamic acid, otherwise known as Gla.[2] How does this enzyme pick its protein substrate and modify specific glutamic acid residues? In work spanning over 30 years, researchers identified a critical sequence called the propeptide region that is N-terminal to the mature protein (Figure 1). GGCX binds the propeptide and directs carboxylation of 9-13 Glu residues on the so-called Gla domain in a processive fashion.[2] The signal sequence and propeptide region are removed by peptidases prior to secretion of the mature VKD protein (Figure 1). For the VKD coagulation factors, the enhanced net negative charge following carboxylation in the Gla domain allows for high affinity divalent metal ion binding.[3] This changes the structural conformation of the Gla domain which facilitates binding to anionic phospholipids and localizes these proteins to the site of vascular injury.[3,4] Defects of VKD protein carboxylation cause bleeding disorders, and inhibition of this pathway is the basis of warfarin anticoagulation.[2]

Acquiring mechanistic information about GGCX and deciphering how the propeptide influences carboxylation has been challenging. Since GGCX is an integral membrane ER protein (Figure 1), extracting it in a functional state is difficult and requires artificial conditions to study it. Early work used crude microsomal extracts or detergent-solubilized liver microsomes following warfarin treatment or vitamin K-deficient animals which contained the enzyme and small amounts of endogenous protein substrate (e.g. prothrombin).[1] Advancements to this system incorporated artificial peptide substrates for GGCX such as FLEEL (residues 5-9 of rat prothrombin).[5] In the late 1980s, it was recognized that the propeptide sequence is critical for VKD protein carboxylation.[6] This insight led to the development of GGCX substrates that contained a propeptide sequence and portions of the Gla domain which are superior when compared to FLEEL alone.[7,8] These and other substrates have been used to demonstrate the importance of propeptide affinity in substrate recognition using either crude preparations or purified forms of GGCX and increased our understanding about the enzyme.[9] Further insights into the importance of the propeptide came from studies using mutant peptides and identification of naturally occurring mutations in the propeptide region of FIX.[10,11] However, this knowledge about the function of GGCX was obtained outside of its natural environment under artificial conditions. To better understand VKD carboxylation in its native milieu, Tie and Stafford developed a cell-based reporter assay to study γ-carboxylation and the entire VKD cycle.[12] In this system, a chimeric reporter-protein, FIXgla-PC is used, in which the PC backbone was replaced at the N-terminus with the FIX Gla domain.[12,13] This allowed for an ELISA-based quantification of carboxylated reporter protein using a capture antibody that recognizes only a fully carboxylated FIX Gla domain and an antibody against PC. The advantage of the system is that it allows for functional assessment of the VKD cycle enzymes, including GGCX, in an environment that requires the enzymes to interact with their physiologic substrates, a departure from systems previously employed.

In this issue of Haematologica, Hao *et al.* use this cell-based assay to study the role of the propeptide in directing carboxylation of VKD proteins.[14] Previous studies indicate that the propeptide region of VKD coagulation factors show considerable variation in their affinities for GGCX with FX, FIX and PC showing high (K_d ~1 nM), intermediate (K_d ~5 nM), and low affinity (K_d ~20 nM), respectively (Figure 1).[15] It is thought that these disparate affinities contribute to the heterogeneity in carboxylation in mammalian expression systems. Furthermore, it is thought that there is likely an optimal propeptide affinity that best directs carboxylation. To better understand how GGCX interacts with its protein substrates *via* propeptide binding in its natural environment, the authors created a series of chimeric

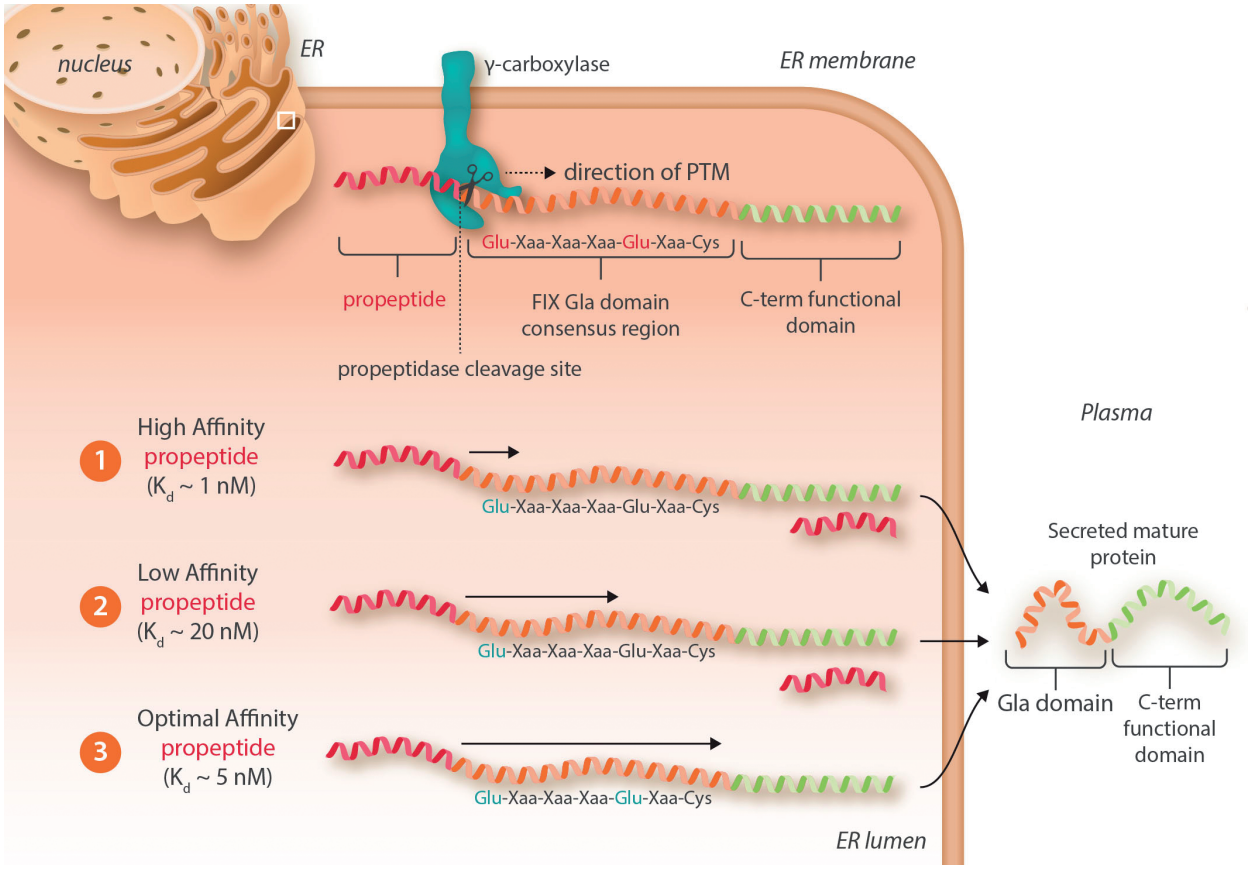

Figure 1. Carboxylation of vitamin K-dependent proteins by γ-carboxylase. The endoplasmic reticulum (ER) membrane-associated gamma-glutamyl carboxylase (GGCX) modifies glutamic acid (red) to gamma-carboxy-glutamatic acid (Gla, blue) within the Gla domain. GGCX recognizes and binds the substrate *via* the propeptide region (red helix) in a processive fashion. The affinity of the GGCX-propeptide complex determines relative efficiency of carboxylation as follows: 1) high affinity propeptides (K_d ~1 nM) result in significant uncarboxylated protein; 2) low affinity propeptides (K_d ~20 nM) are associated with moderate to normal carboxylated protein; and 3) optimal affinity propeptides (K_d ~5 nM) produce efficiently carboxylated protein. Glu: glutamic acid residues; FIX: factor IX; PTM: post-translational modification; C-term: C-terminus.

proteins in their cell-based assay. Propeptide sequences having a broad range of affinities for GGCX derived from FX, FIX, PC, and BPG were attached individually to the FIXgla-PC chimeric reporter. Hao *et al.* found that the FIX propeptide was the most efficient at directing carboxylation while the high affinity propeptide from FX and the low affinity propeptides from PC and BGP had reduced efficiency.[14] The data show that the FIX propeptide is optimal for both binding GGCX and releasing once the protein is carboxylated. These results differ when using synthetic propeptides, FLEEL and purified GGCX,[9] highlighting the importance of the cell-based system. Interestingly, the BGP propeptide, known to have a low affinity for GGCX, did not direct carboxylation of the reporter protein harboring the FIX Gla domain, but did direct carboxylation if the BGP Gla domain was used. This suggests that other determinants within BGP are needed for carboxylation of this protein. Enhancing the affinity of BGP propeptide for GGCX by mutating the -6 and -10 position rescued carboxylation of the chimeric reporter. The picture with the FX propeptide appeared to be different. This propeptide binds very tightly to GGCX and attempts to weaken the binding by mutation at the -6 and -10 position were unsuccessful. However, further changes to the propeptide revealed that the entire N-terminal portion of the propeptide determines carboxylation efficiency of VKD coagulation factors. Additional detailed investigation centered on known propeptide mutations. FIX mutations (-9 and -10 in the propeptide), for example, are known to cause warfarin hypersensitivity; a situation in which active FIX levels drop to <1% during anticoagulation therapy while the activity of other clotting factors is decreased to 30-40%.[10] The authors show that, in the cell-based system, these FIX mutant proteins were indeed hypersensitive to warfarin. Again, these data highlight the power of using the cell-based system to gain information about clinically relevant mutations.

The cell-based functional study presented by Hao *et al.*[14] provides further insights into GGCX function and the role of the propeptide during carboxylation in its natural environment. The findings are consistent with prior studies using purified GGCX and propeptide/FLEEL as a substrate. However, the work is nonetheless significant as it nicely shows that structure/function relationships about the propeptide and new insights about mutations in this region can be obtained. The finding that the FIX propeptide is optimal for efficient carboxylation should provide the framework to further understand the structural elements that mediate substrate recognition by GGCX and in the production of VKD coagulation factors. The work is also important as it highlights the power and utility of the cell-based

system to study GGCX and the entire vitamin K cycle. In fact, this group recently used this assay in a high-throughput capacity to screen small molecules that impact the vitamin K cycle, an exercise that would be not possible using prior approaches.[16] In summary, this elegant report confirms the critical role that the propeptide region plays in carboxylation of VKD proteins and highlights the utility of a novel cell-based assay that enables researchers to study membrane-associated enzymes in their natural, home environment.

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