RESEARCH HIGHLIGHT

Osteocalcin has many tricks to get γ-carboxylated

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Osteocalcin is a bone-derived hormone whose bioactivity is regulated by vitamin K-dependent γ-carboxylation. Using cryo-electron microscopy, Cao et al. elucidate the structure of the osteocalcin precursor bound to the vitamin K-dependent γ-carboxylase, revealing unique and complex interactions between the enzyme and its substrate.

γ-carboxylation of glutamic acid (Glu) residues is a post-translational modification catalyzed by the vitamin K-dependent γ-carboxylase (VKGC or GGCX). This enzyme is localized within the endoplasmic reticulum (ER) membrane and uses vitamin K hydroquinone (KH_2) as a co-factor. KH_2 is epoxidated by the γ-carboxylase, a process that generates a superbase that deprotonates the γ-carbon of a Glu residue, allowing the addition of the carboxyl group.[1] γ-carboxylated residues (Gla), which increase the calcium ion (Ca^{2+}) affinity of proteins, are present in several clotting factors (e.g., prothrombin, factor IX, factor X, etc.), explaining the essential role of vitamin K in blood coagulation. This modification is also found in matrix Gla protein (MGP), an extracellular matrix (ECM) protein that prevents calcification of arteries and cartilage,[2] and in GAS6, a signaling molecule implicated in immunity, cancer and diabetes.[3] Osteocalcin, also called bone Gla protein (BGP), is another small vitamin K-dependent protein produced specifically by osteoblasts and containing three Glu residues that can be γ-carboxylated.[4] The γ-carboxylated form of osteocalcin (cOCN) accumulates in the bone ECM due to its high affinity for hydroxyapatite, the mineral component of bone. As the most abundant non-collagenous protein of bone ECM, cOCN may play a role in the proper maturation of bone mineral.[5] When uncarboxylated or undercarboxylated, osteocalcin is released in the circulation and functions as a hormone to promote insulin sensitivity, enhance male fertility, and influence brain functions.[4]

Vitamin K-dependent coagulation factors such as factor IX are recognized by the γ-carboxylase through a conserved sequence (i.e., **F**X_5**A**X_3**L**) contained in their propeptide region. The tight interaction with this propeptide sequence is also essential to stimulate γ-carboxylase and epoxidase enzymatic activity, allowing the sequential carboxylation of multiple Glu residues within a single substrate.[6] In contrast to the other Gla proteins, osteocalcin propeptide (i.e., **F**X_5GX_3V), which does not match the conserved recognition sequence, binds weakly to the γ-carboxylase and does not activate γ-carboxylase.[7] Moreover, it was found that decarboxylated osteocalcin can be γ-carboxylated in vitro by the γ-carboxylase in the absence of its propeptide,[8] suggesting that osteocalcin is recognized through different and unknown molecular interface(s).

A study by Cao et al.[9] published in *Cell Research* provides important insights into the mechanism by which the osteocalcin precursor interacts with and activates γ-carboxylase. Using an ingenious approach, they expressed in mammalian cells pro-osteocalcin in fusion with the γ-carboxylase, allowing equimolar co-purification of the enzyme and its substrate. They then determined by cryo-electron microscopy (cryo-EM) the structure of osteocalcin bound to the γ-carboxylase in the presence of its native low-affinity propeptide. The obtained high-quality cryo-EM maps revealed that osteocalcin binds to the γ-carboxylase in a partially folded conformation through multiple interaction interfaces. One surprising finding is that the native osteocalcin propeptide, which has very poor affinity for γ-carboxylase when tested alone,[7] binds stably to the enzyme when attached to mature osteocalcin. Another important observation emanating from the structure analysis is that the mature region of osteocalcin interacts extensively with γ-carboxylase, and that these additional interactions occur even in the presence of the low-affinity native osteocalcin propeptide. This latest result is consistent with the previous observation that the enzyme can bind and γ-carboxylate mature osteocalcin lacking the propeptide in vitro.[8]

Further analyses revealed that, when bound to γ-carboxylase, the C-terminal region of mature osteocalcin adopts a structure characterized by two helices forming an "L" shape, that fits in a similarly shaped groove in the enzyme. The interactions between osteocalcin helices and γ-carboxylase occur primarily through hydrophobic contacts. Disruption of these hydrophobic interactions through mutations in osteocalcin C-terminal region or the γ-carboxylase groove significantly reduces the capacity of osteocalcin to stimulate enzymatic conversion of VKH_2 to VK epoxide, underscoring their importance for enzymatic activation.

In a previously published structure of γ-carboxylase bound to factor IX, the C-terminal region of the Glu-rich region of factor IX, which lacks the hydrophobic residues present in osteocalcin C-terminus, was not found to form any interaction with the enzyme.[6] These results highlight an important difference in the recognition of osteocalcin and coagulation factors by the γ-carboxylase. In vitamin K-dependent coagulation factors, high-affinity binding of the propeptide is sufficient to activate the γ-carboxylase (Fig. 1a), while osteocalcin needs to bind the enzyme through its C-terminal domain to allow a conformational change that in turn stabilizes the low-affinity binding of its propeptide. Therefore, stable binding of pro-osteocalcin to the γ-carboxylase and its subsequent carboxylation, relies on an avidity-based mechanism whereby multiple interactions between the enzyme and its substrate compensate for the relatively weak affinity of each individual region (Fig. 1b).

The understanding of the unique mechanism underlying osteocalcin recognition by the γ-carboxylase has important biological

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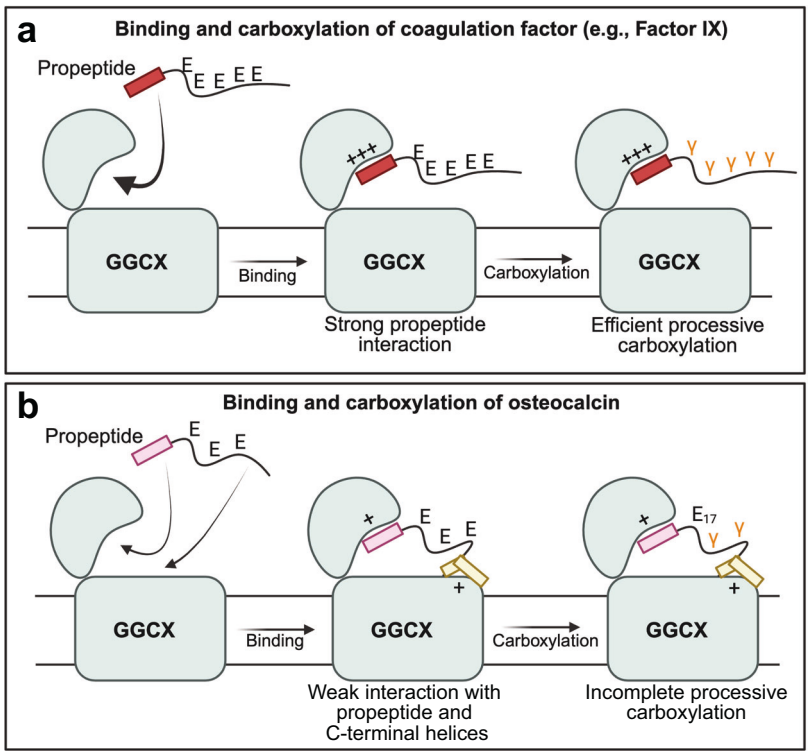

Fig. 1 Comparison of the binding and carboxylation mechanism of factor IX and osteocalcin. a Coagulation factors, such as factor IX, are recognized by the γ-carboxylase (GGCX) through strong binding of their propeptide (+++). This strong interaction facilitates efficient processivity of the enzyme, resulting in complete γ-carboxylation of the Glu residues (E). **b** Osteocalcin is recognized by GGCX through multiple weak interactions (+) involving its propeptide and two C-terminal helices. This may result in incomplete processing and undercarboxylation of osteocalcin on Glu_{17} (E_{17}), allowing the production of bioactive osteocalcin. Created in BioRender.com.

implications. Vitamin K-dependent coagulation factors are very efficiently carboxylated on each of their multiple Glu residues due to the high affinity of the enzyme for their propeptide (Fig. 1a). The lower affinity of the osteocalcin propeptide probably results in reduced processivity of the γ-carboxylase, thereby allowing the production of fully carboxylated and undercarboxylated osteocalcin (Fig. 1b). Indeed, Cao et al. found that a significant proportion of osteocalcin is undercarboxylated on Glu_{17} following interaction with γ-carboxylase in the presence of VKH_2. This is of particular interest, since Glu_{17} osteocalcin (Glu_{13} in mice) was proposed to be the bioactive form of the hormone in the circulation.[10,11] Overall, the different mechanisms by which osteocalcin is recognized by γ-carboxylase may allow the osteoblast to adapt its production of bioactive undercarboxylated osteocalcin in response to specific physiological cues, such as insulin, exercise or acute stress, which were previously shown to modulate serum levels of undercarboxylated osteocalcin.[4,12] The discovery also suggests that it could be possible to develop small molecules targeting the interactions between γ-carboxylase and osteocalcin C-terminal helices to increase the production of bioactive osteocalcin without impacting coagulation factor activity. This may represent a novel therapeutic approach for the treatment of diabetes or cognitive decline.

Recently, ER Gla protein (ERGP) was identified as a novel transmembrane γ-carboxylated protein expressed by pancreatic β cells and hepatocytes.[13] This protein lacks the sequence found in the propeptide region of the other Gla proteins, including osteocalcin, but is nevertheless carboxylated by the γ-carboxylase in a vitamin K-dependent manner on multiple Glu residues located in its luminal domain. Based on the work of Cao et al., it would be interesting to test whether ERGP is recognized by γ-carboxylase through multiple weak binding sites, as it is the case for osteocalcin.

In summary, the work of Cao et al. shows that mammalian γ-carboxylase has more than one way to recognize vitamin K-dependent proteins. The elucidation of the structural basis for the γ-carboxylation of osteocalcin also set the stage for developing novel therapies for bone and metabolic disorders.

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ADDITIONAL INFORMATION

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