

# Vitamin K-dependent proteins: Functions in blood coagulation and beyond

Omar Benzakour

Institut de Physiologie et Biologie Cellulaires, CNRS-UMR 6187, Université de Poitiers, Poitiers Cedex, France

Vitamin K or vitamin "Koagulation" (German spelling for coagulation) was discovered by the 1943 Nobel Prize winners Henrik Dam and Edward A. Doisy, as a fat soluble substance, the deficiency of which caused bleeding disorders. Vitamin K in its reduced form is required as a cofactor for the  $\gamma$ -glutamyl carboxylase enzyme that catalyses the  $\gamma$ -carboxylation of specific glutamic acid (Glu) residues of a subclass of proteins (1). This subclass of proteins was then termed vitamin K-dependent proteins (VKDP), or  $\gamma$ -carboxylated proteins or simply Gla-proteins. The enzymatic reaction generates  $\gamma$ -carboxyglutamate (Gla) and vitamin K 2,3,-epoxide which is then recycled back to the hydroquinone form by a reductase enzyme (1). Warfarin (3-[ $\alpha$ -acetylbenzyl-4-hydroxycoumarin]) inhibits the activity of the vitamin K epoxide reductase blocking the vitamin K cycle. This property of warfarin has led to its widespread use in anticoagulant therapy (2). In 2000, warfarin was ranked among the top-selling drugs with a turnover estimated at 500 million dollars. The  $\gamma$ -carboxylation process appears to be required for the activities of all Gla-proteins studied to date. Carboxylase substrates synthesised in the presence of warfarin are undercarboxylated and have impaired biological activities (3).

In the present theme issue, Shearer and Newman stress the importance of exploring vitamin K metabolism and catabolism pathways for a better understanding of pathologies linked to vitamin K deficiency (4). The occurrence of such pathologies is established in bone and arteries, two tissues that express many VKDP, whereby expression is hypothesised in others. The various molecular forms of vitamin K are different not only with regard to their co-factor activities but also with regard to their absorption, transport, cellular uptake, tissue distribution, turnover and catabolism. This paper provides a clear description of the nomenclature and chemical structure of K vitamins, namely phyloquinone (vitamin K<sub>1</sub>) and menaquinones (vitamins K<sub>2</sub>) as well as their dietary sources. Further research is certainly needed in this area to determine to what extent bacterially fermented food as well as intestinal flora contribute to the maintenance of vit-

amin K status. The effects of other putative non-cofactor functions of vitamin K, which potentially include the suppression of inflammation, prevention of brain oxidative damage and a role in sphingolipid synthesis as well as the possible existence of a receptor for vitamin K, are important issues that require long-term basic research to be clarified. The full consequences of dietary vitamin K deficiency on tissue distribution and metabolism of K vitamins as well as the consequences of oral anticoagulants on the metabolism of K vitamins remains to be assessed in the light of new findings on VKDP.

Only few proteins are known to contain Gla residues. These include: (i) certain proteins of the blood coagulation system: prothrombin, factors VII, IX and X, protein C, protein S and protein Z; (ii) the protein encoded by the growth arrest specific gene GAS6, which presents strong structural homology with protein S; (iii) Gla proteins expressed in mineralised tissues: osteocalcin and matrix Gla-protein (MGP); (iv) Gla-containing snake venom proteins and conotoxins; (v) four transmembrane Gla-proteins PRGP1, PRGP2, TMG3 and TMG4 whose functions are as yet unknown; (vi) some members of the connexin family of proteins (5). The precise role of the  $\gamma$ -carboxylation modification for connexin protein function is not as yet known.

Except for protein S and protein Z, VKDP of the blood-clotting cascade are serine protease zymogens and are activated by serine proteases. The role of protein Z in blood clotting is not fully clarified and neither cellular effects nor a specific pathology have been as yet associated with protein Z. The review by Vasse et al. (6) in the present issue covers this topic. Two further contributions in the present theme issue relate to the therapeutic functions of VKDP in haemostasis. In her concise review Ulla Hedner concentrates on the therapeutic use of factor VIIa in bleeding associated pathologies (7). The paper by Paul Monahan reviews the data obtained from factor IX knockout mice and other haemophilia B mouse models (8). Such mouse models have not only enabled a better understanding of the role of factor IX in haemostasis, thrombosis and wound healing but also pro-

## Correspondence to:

Omar Benzakour  
Institut de Physiologie et Biologie Cellulaires  
CNRS-UMR 6187, Université de Poitiers  
40 avenue du recteur Pineau, 86022 Poitiers Cedex, France  
Tel.: +33 5 49 45 3568, Fax: +33 5 49 45 4051  
E-mail: Omar.Benzakour@univ-poitiers.fr

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vided insights into new potential therapeutic approaches for haemophilia.

In addition to their role in the coagulation system, thrombin, protein S, activated protein C (APC), factor Xa, factor VII/VIII and Gas6 have all been shown to bind to cell-surface receptors and mediate cellular responses (9). Thrombin, but also factor Xa, factor VIIa and APC, activate members of G protein-coupled protease activated receptors (PARs). While PARs were originally extensively studied in relation to platelet aggregation, emerging evidence suggests that PAR1-PAR4 are involved in many other pathophysiological situations. Sokolova and Reiser (10) as well as Tofl et al. (11) illustrate two novel processes in which PARs seem to play a crucial role: neurodegeneration and sepsis. In addition to presenting the many facts arguing for a role of thrombin/PARs in the brain, the authors raise the important issue of the pattern of PAR expression in the brain and the interplay of receptors PAR-1, PAR-3 and PAR-4 in mediating thrombin's protective or deleterious effects (10). Elucidation of the roles of PARs in the brain may lead in the future to the use of thrombin inhibitors, PAR agonists and antagonists for treating stroke, Alzheimer's and Parkinson's disease. Sepsis represents another field in which PAR activation plays a crucial role. In this complex pathology that involves different vascular cells, both the interplay between PARs and endothelial protein C receptor modulates cellular responses to APC (11). While it is now established that recombinant APC is an effective treatment for severe sepsis, the mechanisms by which APC induces its pleiotropic and protective activities remain to be elucidated.

The paper by Schurgers et al. (12) is dedicated to a relatively new member of the VKDP family, MGP, which is the major known inhibitor of vascular calcification. Massive arterial calcifications are often associated with advanced atherosclerotic lesions. Vascular calcification and MGP activity are correlated with vitamin K2 intake. The authors provide convincing arguments in favor of using ELISA-based assays for undercarboxylated MGP as a potential biomarker for vascular calcification. If in the future, the relevance of undercarboxylated MGP monitoring is confirmed, it will certainly be a major breakthrough for cardiovascular disease screening. The molecular mechanisms involved in the inhibition of vascular calcification by MGP as well as the significance and the source of undercarboxylated MGP remain to be elucidated. If we assume that undercarboxylated MGP is released by apoptotic vascular cells, it is plausible that as atherosclerotic lesions evolve, cell death increases within the vessel wall allowing for undercarboxylated MGP to accumulate within the vascular tissue or in the circulation.

Among VKDP of the coagulation cascade, protein S and Gas6 present several unique features. They are produced at many extrahepatic sites and they activate a particular class of tyrosine kinase receptors referred to as TAM („Tyro3 Axl Mer“), whose members are Axl (also called Ufo and Ark), Tyro3 (also called Rse, Brt, Sky) and Mer (also called c-Eyk) (13, 14). The affinity of TAM receptors is much higher for Gas6 than for protein S (13). Nevertheless, given that the plasma concentration of protein S is much higher than that of Gas6 and that the distribution of expression of protein S and Gas6 differ, protein S would be an even more relevant physiological activator of TAM receptors than Gas6 (15). In the present theme issue, Fernández-Fernández

et al. (16) focus on the role of Gas6 and to some extent of protein S in haemostasis and inflammation. Gas6 knockout mice show a low incidence of thrombosis, which is partly due to a defect in platelet aggregation (16). These studies may lead in the future to the elaboration of new therapeutic strategies using Gas6 inhibitors with an aim to prevent or treat thrombosis.

TAM receptors were originally identified as tyrosine kinase receptors without known ligands and called „orphan receptors“. Later, Gas6 was discovered as a new member of the family of VKDP, as a homologue to protein S and as a ligand for TAM receptors. Since then, the study of cellular processes activated by TAM receptors and the phenotypes associated with the invalidation of the genes coding for Gas6 or for TAM receptors has opened a new research field with many potential therapeutic applications. Mice with knockouts of all three genes coding for TAM receptors (17) present a dysregulation of the immune system. Macrophages derived from Mer knockout mice are able to bind apoptotic cells but do not phagocytose them (18). Moreover, the process of spermatogenesis generates apoptotic cells and residual bodies that are normally phagocytosed by the Sertoli cells and this function is defective in TAM-/- mice (19). TAM-/- mice are infertile owing to the occlusion of the seminiferous tubules with apoptotic cells and residual bodies (19). Protein S, Gas6 and TAM receptors are expressed in the central nervous system (20). Furthermore, the  $\gamma$ -carboxylase enzyme is expressed in the central nervous system neuroepithelium during development and its expression persists in the brain during adulthood (21). This suggests that Gas6 and protein S produced locally can be  $\gamma$ -carboxylated and thus are able to activate TAM receptors in the brain in an autocrine or paracrine manner. TAM-/- mice present neurological abnormalities characterized by an increase in neuronal degeneration and the accumulation of apoptotic cells in the cerebellum, hippocampus and neocortex (19). The stacks of discs of the retina photoreceptor outer segments are daily renewed. Old discs are phagocytosed by the retinal epithelium. In TAM-/- mice, photoreceptor outer segment clearance is defective resulting in blindness (19). Studies *in vitro* confirmed that both Gas6 and protein S stimulate photoreceptor outer segment phagocytosis (22).

At least four VKDP are expressed in bone: osteocalcin, MGP, protein S and Gas6. While MGP is thought to control calcification, the precise function of osteocalcin in bone is still not well understood (4). Both Gas6 and protein S stimulate osteoclast functions (23). In humans, a congenital deficiency of protein S has been associated with low bone mineral density and osteonecrosis (24). During the remodelling process of the bone, small surface areas of bone matrix are removed by osteoclasts (resorption) and are subsequently replaced by the activity of osteoblasts. Osteoblasts can acquire a macrophage phenotype (CD68 expression) and possess phagocytic properties. Bone abnormalities were not studied in either Gas6-/- or TAM-/- mice and no laboratory has succeeded at present to generate protein S knock out mice. If in the future, such mice are produced they may contribute together with studies of bone abnormalities in Gas6-/- or TAM-/- mice to elucidate the roles of protein S and Gas6 in regulating phagocytosis in the bone.

Regulation of phagocytosis seems to be a common denominator by which Gas6 and protein S are major players in many

physiological processes. The location of oxidized phosphatidylserine residues at the outer surface of the plasma membrane is a signal of recognition of apoptotic cells by phagocytes also called an „eat me signal“. A specific receptor for oxidized phosphatidylserines was identified in macrophages (25). The major known opsonines for oxidized phosphatidylserines are MFG-E8 (EGF Globule Milk Fat Factor 8) and Gas6 (26, 27). Gas6 protein interacts with oxidized phosphatidylserines by its N-terminal Gla domain and with TAM receptors present on the surface of the phagocyte through its sex hormone-binding globulin carboxy-terminal domain (28). This enables the interaction between apoptotic cells and phagocytes. Furthermore, protein S is the main serum-derived factor responsible for binding to phosphatidylserines and stimulating lymphoid cell phagocytosis by mac-

rophages (29). The signaling cascade-Rho GTPase Rac1 regulates actin filament polymerisation resulting in changes to macrophage morphology and the internalisation of apoptotic cells. MER receptor activation by Gas6 led to the recruitment of phosphoinositol-3kinase and the phospholipase C  $\gamma$ , thereby activating the signalling cascade-Rho GTPase Rac1 which promotes apoptotic cell phagocytosis (28).

The present theme issue provides an update on most but not all VKDP. It highlights the importance of VKDP topic as an exciting field for basic research, which may lead in the future to the development of new therapeutic strategies aimed at targeting pathologies in which VKDP have established or newly discovered roles.

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