REVIEW ARTICLE

Familial multiple coagulation factor deficiencies: new biologic insight from rare genetic bleeding disorders

B. ZHANG and D. GINSBURG

Departments of Internal Medicine and Human Genetics and the Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI, USA

Summary. Combined deficiency of factor (F)V and FVIII (F5F8D) and combined deficiency of vitamin K-dependent clotting factors (VKCFD) comprise the vast majority of reported cases of familial multiple coagulation factor deficiencies. Recently, significant progress has been made in understanding the molecular mechanisms underlying these disorders. F5F8D is caused by mutations in two different genes (*LMAN1 and MCFD2*) that encode components of a stable protein complex. This complex is localized to the secretory pathway of the cell and likely functions in transporting newly synthesized FV and FVIII, and perhaps other proteins, from the ER to the Golgi. VKCFD is either caused by mutations in the γ-carboxylase gene or in a recently identified gene encoding the vitamin K epoxide reductase. These two proteins are essential components of the vitamin K dependent carboxylation reaction. Deficiency in either protein leads to under-carboxylation and reduced activities of all the vitamin K-dependent coagulation factors, as well as several other proteins. The multiple coagulation factor deficiencies provide a notable example of important basic biological insight gained through the study of rare human diseases.

Keywords: γ-carboxylation, ER, Golgi, factor V, factor VIII, Vitamin K.

Introduction

Familial multiple coagulation factor deficiencies are a group of rare inherited disorders characterized by the simultaneous decrease in the levels of two or more coagulation factors. Recent progress has led to a better understanding of the molecular mechanisms underlining combined deficiency of factor (F)V and FVIII (F5F8D) and combined deficiency of vitamin K-dependent clotting factors (VKCFD). These studies have also yielded significant insights into ER to Golgi protein

Correspondence: Professor D. Ginsburg, Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI 48109–0650, USA. Tel.: 734-647-4808; fax: 734-936-2888; e-mail: ginsburg@umich.edu

transport and vitamin K metabolism, respectively. Little is known about other types of multiple coagulation factor deficiencies, as only isolated case reports are available.

Combined deficiency of FV and FVIII (F5F8D)

Biosynthesis of FV and FVIII

Hemostasis is mediated by the regulated and sequential activation of serine proteases in the coagulation cascade. FV and FVIII are two large plasma glycoproteins that function as essential cofactors for the proteolytic activation of prothrombin and FX, respectively. FV is synthesized primarily in hepatocytes and megakaryocytes and is found in the plasma and α granules of platelets as a 330-kDa single chain polypeptide [1]. In contrast, there has been long-standing uncertainty as to the primary tissue source for the biosynthesis of FVIII. Though earlier results suggested the hepatocyte [2–5], a recent study identified liver sinusoidal endothelial cell as a significant source of circulating FVIII [6]. FVIII is synthesized at low levels and is processed upon secretion to a heterodimer consisting of an 80-kDa light chain in association with a 200-kDa heavy chain fragment [7]. The light chain is bound through non-covalent interactions to a primary binding site at the amino-terminus of von Willebrand factor (VWF). The VWF interaction is required to stabilize FVIII in plasma. FV and FVIII circulate as inactive precursors that are activated through limited proteolysis by thrombin. FV and FVIII share similar domain structure (A1-A2-B-A3-C1-C2), and undergo similar extensive post-translational modifications, including signal peptide cleavage, formation of conserved disulfide bonds, addition of multiple oligosaccharide structures, and sulfation of specific tyrosine residues [8]. Both protein cofactors are also inactivated through proteolysis by activated protein C (APC). Despite these similarities, the plasma concentration of FVIII is ~40-fold lower than that of FV, and expression of FVIII in heterologous systems is similarly inefficient. The latter observation appears to be due to lower expression of FVIII mRNA, as well as inefficient secretion of the primary translation product.

Genetic deficiency of FVIII results in classic hemophilia (hemophilia A) whereas inherited FV deficiency leads to

parahemophilia, a rare autosomal recessive condition exhibiting a similar hemorrhagic phenotype. Combined deficiency of FV and FVIII is an autosomal recessive disease that was first described in 1954 by Oeri *et al.* [9]. Patients with this disorder exhibit plasma FV and FVIII antigen and activity levels in the range of 5–30% of normal. Bleeding symptoms are similar to those observed in patients with single deficiencies of FV or FVIII [10–12]. The most commonly noted are epistaxis, menorrhagia and excessive bleeding during or after trauma, surgery, or labor. Inheritance of F5F8D is autosomal recessive and distinct from the coinheritance of both FV deficiency and FVIII deficiency. To our knowledge, at least 140 patients in 81 families have been diagnosed with F5F8D to date, with over half of the families from the Mediterranean region. However, it is likely that F5F8D is under-diagnosed, in part due to its often mild bleeding manifestations. Some of the F5F8D patients may be misdiagnosed as having mild hemophilia or parahemophilia. This disorder appears to be particularly prevalent among Middle Eastern Jews and non-Jewish Iranians, estimated at ~1 : 100 000 [12]. This high frequency is probably due, at least in part, to the high incidence of consanguineous marriages in these populations [13].

Molecular basis of F5F8D

Using a homozygosity mapping approach, the gene for F5F8D was localized to the long arm of chromosome 18 in nine unrelated Jewish families of Sephardic and Middle Eastern origin [14], and in 19 families from Iran, Pakistan, and Algeria [15]. Subsequent positional cloning analysis of the same nine Israeli families identified two founder mutations in *LMAN1* (also known as *ERGIC-53*), a gene of previously unknown function [16]. To date, 17 different mutations have been identified [16–18]. All but one of the mutations are either nonsense or frameshift alleles whose truncated protein products would be predicted to lack normal LMAN1 function (Fig. 1). The only missense mutation, a substitution of threonine for the initiator methionine, is also predicted to result in the absence of a protein product. The diverse nature of the mutations indicates multiple independent genetic origins. However, founder mutations may account for all or the majority of F5F8D in isolated populations. For example, one of the originally reported mutations was found to be prevalent in Jews originating from the island of Djerba in Tunisia, while this mutation was not found in North African Jews [13].

Though mutations in *LMAN1* account for F5F8D in the majority of patients with this disease, no *LMAN1* mutations could be identified in ~30% of affected families [17,18]. Homozygosity mapping using 10 of the latter pedigrees localized the disease gene to the short arm of chromosome 2. Positional cloning identified a novel gene, now termed *MCFD2* (**m**ultiple **c**oagulation **f**actor **d**eficiency gene 2), as the second cause for F5F8D [19]. Seven different mutations were identified, including two missense mutations (Fig. 1). *MCFD2* encodes an EF-hand domain protein that colocalizes with LMAN1 in the ER-Golgi intermediate compartment (ERGIC). MCFD2 does not contain an ER retention motif. The intracellular localization of MCFD2 relies on its interaction with LMAN1, as intracellular MCFD2 is markedly reduced in lymphocytes derived from LMAN1 deficient patients. MCFD2 interacts with LMAN1 in a calcium-dependent manner. Missense mutations within the second MCFD2 EF-hand domain (D129E and I136T) disrupt this interaction and result in F5F8D [19].

Biological insights from studies of F5F8D

A central problem in cell biology is how newly synthesized proteins are sorted for transport to their final destinations. Sorting involves both selection of cargo proteins intended for different destinations, and efficient separation from the components of the ER and Golgi. The Golgi body is generally regarded as the major sorting machinery and is the site of extensive post-translational modification of transported proteins. However, it has become increasingly clear that ER exit is also an important point for protein sorting. Correctly folded proteins destined for secretion by anterograde transport towards the Golgi are packaged in the ER into COPII-coated vesicles [20]. In yeast, the COPII coat consists of the small GTPase Sar1p and the heterodimeric protein complexes Sec23/24p and Sec13/31p [21], in which Sec24p appears to be involved in the recognition of sorting signals [22,23]. These vesicles then uncoat and fuse with each other to form the ERGIC, also referred to as vesicular tubular clusters (VTCs). Resident proteins recycle from the ERGIC back to the ER in COPI-coated vesicles. The COPI coat consists of the small GTPase ARF and a coatomer of seven-subunit complexes [21]. Two distinct models have been proposed to explain how ER resident proteins are segregated away from soluble cargo proteins during vesicle formation [24,25]. Secretion of certain abundant proteins is consistent with a bulk flow model in which cargo moves by default and requires no export signals [26]. In contrast, the receptor-mediated export model envisions selective packaging of secreted proteins into budding COPII vesicles with the help of cargo receptors (Fig. 2), and is supported by recent observations of sorting in ER-derived transport vesicles [27–29].

Identification of cargo receptors has proven difficult. Although LMAN1 was first identified in 1987 [30,31], its function had remained an enigma in cell biology. LMAN1 is a 53- kDa homo-hexameric transmembrane protein, and along with VIP36 [32], VIPL [33,34] and ERGL [35], belongs to a recently defined class of animal lectins [36,37]. Due to its intracellular localization and its calcium-dependent mannose binding [38,39], LMAN1 was postulated to function as a cargo receptor for glycoproteins. The unexpected finding of *LMAN1* gene mutations as a cause of F5F8D provided the first direct evidence for a mammalian ER cargo receptor, suggesting that LMAN1 specifically mediates export of FV and FVIII from ER to Golgi [16]. Two potential cargo receptors were recently described in yeast [40,41].
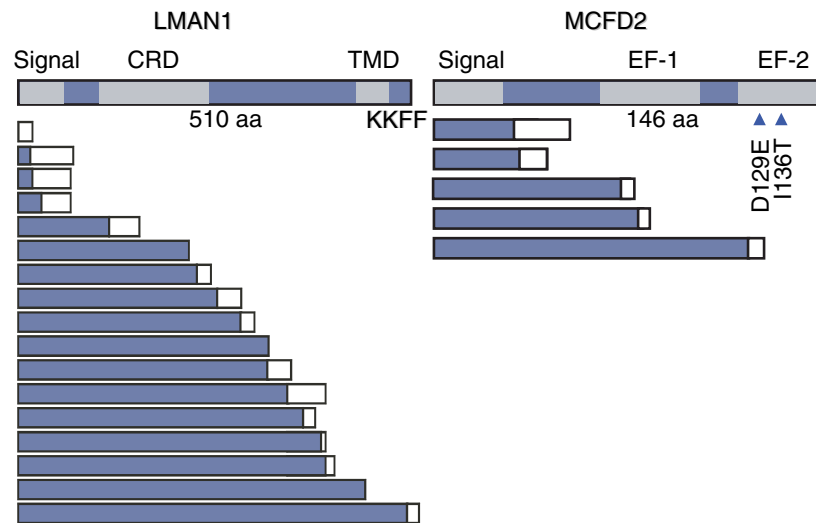
Fig. 1. Hypothetical protein products from the LMAN1 and MCFD2 genes carrying patient mutations. (a) All mutations in LMAN1 identified to date are predicted to result in complete loss of protein function. CRD: carbohydrate recognition domain; TMD: transmembrane domain. The C-terminal ER exit/retrieval signal KKFF is noted. (b) Most mutations in MCFD2 are predicted to result in frameshift ahead of the second EF hand domain. Two missense mutations also occur in the second EF hand and were shown to abolish binding to LMAN1 (19). White rectangles at the C-terminal end of the truncated LMAN1 and MCFD2 proteins indicate additional amino acid sequences resulting from frameshifts.
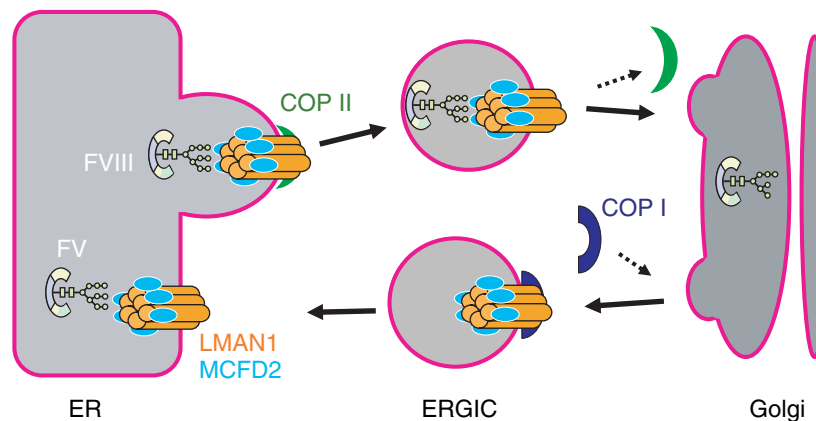
Fig. 2. Model of receptor-mediate ER to Golgi transport of FV and FVIII. Correctly folded FV/FVIII molecules are envisioned to be recruited to the COPII-coated vesicles budding from the ER by binding to the LMAN1-MCFD2 complex. Release of FV/FVIII from LMAN1-MCFD2 occurs in the ERGIC, where COPII coats are replaced by the COPI coats. The LMAN1-MCFD2 complex is recycled back to the ER in COPI-coated retrograde vesicles as FV and FVIII are transported to the Golgi.

The luminal segment of LMAN1 contains a carbohydrate recognition domain (CRD) that shares homology to lectins from leguminous plants (Fig. 1). Point mutations of conserved amino acids in the CRD abolish mannose binding [38,39]. The crystal structure of the CRD domain of rat LMAN1 was recently solved, and shown to consist of primarily β-sandwich folds that are most similar to leguminous lectins, and also resemble the CRD of the ER folding chaperone calnexin that also interacts with FVIII [42]. LMAN1 contains a C-terminal diphenylalanine motif that interacts with COPII coat proteins, potentially directing LMAN1 to vesicles budding from the ER [43]. This motif, together with a dilysine ER retrieval signal [44], results in constitutive recycling of LMAN1 between the ER and Golgi apparatus [45,46]. Correct trafficking of LMAN1 also requires oligomerization (hexamer) of LMAN1, which is mediated by cooperation of the ER luminal and transmembrane domains [47]. MCFD2 lacks the C-terminal KDEL retrieval signal that mediates recycling of many other soluble ER resident proteins [48], via binding to the KDEL receptor [49,50]. MCFD2 is retained in the ER and the ERGIC through an alternative mechanism that is dependent on its direct interaction with LMAN1 [19]. The crystal structure of the LMAN1-CRD [42] also identified a surface patch of conserved residues on the opposite side of the mannose-binding site that could serve as a binding site for MCFD2 or an additional ligand.

MCFD2-deficiency does not alter the subcellular localization of LMAN1, suggesting that it is not required for the ER exit and recycling of LMAN1 [19]. MCFD2 could function at the point of either cargo loading or cargo unloading during the ER to Golgi transport of FV and FVIII (Fig. 2). $Ca^2 \pm$ MCFD2 could form a receptor complex with the luminal domain of LMAN1 in which MCFD2 may serve as the cofactor that specifically captures correctly folded FV and FVIII in the ER lumen. This complex could then be stabilized by interactions between LMAN1 and the oligosaccharides and/or amino acid side chains of FV or FVIII. This cargo-containing complex is then packaged into COPII-coated vesicles for budding. An intriguing implication of this model is that the primary cause of F5F8D is defective MCFD2, and failure to retain MCFD2 in the ER is the mechanism by which LMAN1 mutations cause F5F8D. Alternatively, MCFD2 may be required for the dissociation of FV and FVIII from LMAN1 in the ERGIC, perhaps in response to the dissociation of COPII coats from transport vesicles, or a decrease in pH and Ca + concentration in the ERGIC [51]. Dissociation of cargo from LMAN1 seems to depend on the lower pH in the ERGIC, which may affect calcium binding [51,52]. All of these models imply a direct interaction between MCFD2 and/or LMAN1 and their cargo. Indeed, FVIII was detected in a coimmunoprecipitation complex with overexpressed LMAN1 [53].

Clear MCFD2 orthologs with > 60% amino acid identity are evident in a number of vertebrate species, including mouse, rat and zebrafish. Possible homologs of MCFD2 may also exist in invertebrate species, with the sequence conservation most striking in the C-terminal EF-hand domain [19]. Though there are no other clear human homologs with sequence similarity extending along the full length of *MCFD2*, a subset of other EF-hand-containing proteins could potentially define a class of adaptors/chaperones that might serve similar functions for other secreted proteins. Close homologs of LMAN1 can also be identified in multiple vertebrate and invertebrate species including *C. elegans* and *D. melanogaster*. Of note, invertebrates separated from vertebrates prior to the evolution of the blood clotting system, suggesting a more general role for the *MCFD2/LMAN1* pathway in the biosynthesis of a broad class of secretory proteins, in addition to FV and FVIII. Consistent with this notion, mutations in the *Drosophila LMAN1* homolog, *rhea*, cause a late embryonic recessive lethal phenotype, resulting from somatic wing and muscle developmental defects [54,55]. However, inactivation of the *C. elegans* homolog of *LMAN1* by an RNAi approach resulted in no discernable phenotype (B. Zhang, R. Ellis, and D. Ginsburg, unpubl. data).

Though no other abnormalities have yet been identified in F5F8D patients, it is likely that the ER to Golgi transport of other proteins is also altered, though at a level insufficient to produce a clinical phenotype. A selective delay in secretion of procathepsin C was observed in HeLa cells that overexpress a dominant-negative form of LMAN1 [56]. A cathepsin-Z-related protein (catZr) has also been shown to crosslink to LMAN1 [57], though catZr secretion has not

been directly examined in LMAN1 or MCFD2-deficient cells. Interestingly, a recent report presented *in vitro* evidence that the rat ortholog of MCFD2 may function as a stem-cell derived neuronal survival factor [58]. Although F5F8D patients with MCFD2 deficiency do not present obvious neurological symptoms, a subtle defect in humans or a species-specific neurological phenotype in *MCFD2* null mice cannot be ruled out.

Both FV and FVIII are heavily glycosylated, particularly in the B domains. In cells overexpressing a dominant negative LMAN1 mutant, secretion of wild-type FV and FVIII was impaired, while secretion of B domain-deleted FV and FVIII was unaffected. These results suggest that the B domains may be important in mediating LMAN1-dependent secretion of FV and FVIII [59]. A small amount of B domain sequence with six consensus N-linked glycosylation sites is sufficient to mediate efficient FVIII secretion [60]. In addition to sugar residues, protein–protein interactions appear also to contribute to the interaction between FVIII and LMAN1 [53].

Combined deficiency of vitamin K-dependent clotting factors (VKCFD)

Vitamin K and γ-carboxylation

γ-glutamyl carboxylase (GGCX) catalyzes a post-translational modification of glutamate (Glu) residues into γ-carboxyglutamate (Gla) residues [61,62]. This carboxylation is required for the activities of coagulation FII, FVII, FX and FX, as well as the anticoagulant factors protein C, protein S and protein Z [63,64]. Carboxylation occurs at Glu residues located in a homologous ∼45 a.a. 'Gla domain'. The presence of Gla residues enables these proteins to adapt to a calcium-dependent conformation that allows binding to phospholipids. Other proteins known to undergo γ-carboxylation include osteocalcin [65], matrix Gla protein [65], Gas6 [66], nephrocalcin A-D [67], as well as several putative carboxylated proteins identified through nucleotide database screening (PRGP1, PRGP2, TMG3 and TMG4) [68,69]. Vitamin K is an essential cofactor for γ-carboxylase [61,62]. As carbon dioxide is added to Glu to form Gla, the reduced form of vitamin K (vitamin K hydroquinone) is oxygenated to form vitamin K 2,3 epoxide. Another enzyme, vitamin K epoxide reductase (VKOR), is required to regenerate the vitamin K hydroquinone, completing the so-called vitamin K cycle (Fig. 3).

γ-carboxylation occurs in the ER. GGCX appears to bind to the propeptide at the amino terminus of its substrates [70,71]. Several point mutations in the propeptide substantially reduce or eliminate carboxylation of FIX [71,72]. The carboxylated proteins are then transported to the Golgi for secretion, where the propeptide sequence is removed. VKOR activity was identified 30 years ago [73]; however, little is known about the biochemistry of this enzyme, as it is extremely refractory to purification. The oral anticoagulant warfarin functions by inhibiting VKOR and thereby blocking the regeneration of active vitamin K hydroquinone [74]. As a consequence, the
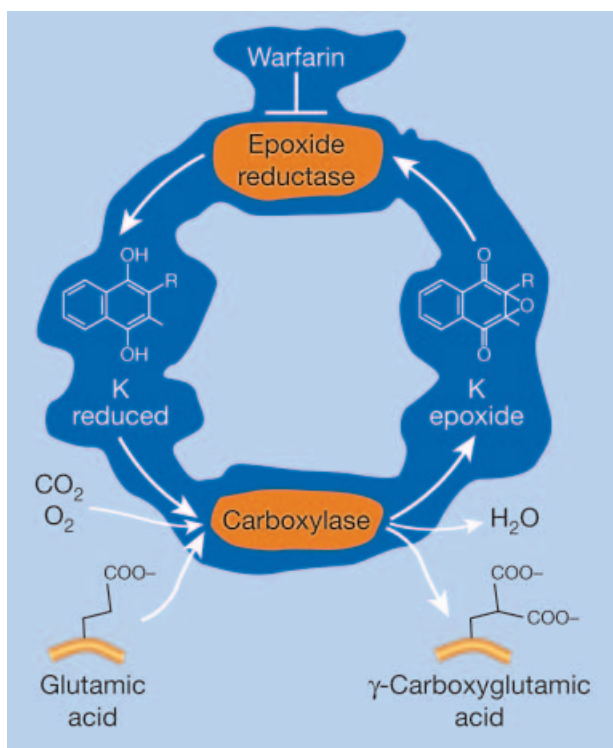
Fig. 3. Vitamin K cycle. Vitamin K is an essential cofactor for γ-carboxylase (GGCX). GGCX adds a carbon dioxide to glutamic acid to form γ-carboxyglutamic acid, at the same time that the reduced form of vitamin K (vitamin K hydroquinone) is oxygenated to form vitamin K 2,3 epoxide. Vitamin K epoxide reductase (VKOR) is required to regenerate the vitamin K hydroquinone, completing the vitamin K cycle. Warfarin inhibits VKOR to limit the carboxylation of vitamin K-dependent coagulation factors. Reprinted by permission from Nature (115), copyright (2003) Macmillan Publishers Ltd.

vitamin K-dependent coagulation factors are under-carboxylated, leading to a reduction in functional activity.

Clinical symptoms of VKCFD

VKCFD is an autosomal recessive disorder characterized by deficiency of all vitamin K-dependent clotting factors. The first case of VKCFD was described in 1966 in an infant girl who had bleeding from the first week of life [75]. The proband was found to have low or undetectable levels of FII, FVII, FIX and FX, with no evidence of hepatic damage or malabsorption. High doses of vitamin K partially restored the clotting factors (FII, FVII, FIX, and FX) in this patient toward normal and prevented bleeding [76]. Only a few additional cases of VKCFD2 have been reported since [77–88]. Clinical symptoms of VKCFD vary in accordance with procoagulant protein levels [87]. Large amounts of vitamin K can partially correct symptoms in most, but not all [86,89] of the cases. Bleeding can include intracranial hemorrhage in the first weeks of life, sometimes leading to a fatal outcome. Hemarthrosis and mucocutaneous bleedings may follow antibiotic therapy, due to decreased vitamin K production by gut bacteria. Skeletal

defects have been reported in some probands, presumably resulting from undercarboxylation of bone Gla-proteins [77,82]. Diagnosis of familial VKCFD requires differentiation from acquired forms of the disorder that can be caused by accidental ingestion warfarin products, intestinal malabsorption of vitamin K, and liver dysfunctions.

Molecular basis of VKCFD and perspective on vitamin K-dependent γ-carboxylation

Studies of VKCFD have provided important insights into the mechanisms of the vitamin K cycle and protein carboxylation. VKCFD is a heterogeneous disorder with at least two subtypes. VKCFD1 is defined by defective γ-carboxylase (GGCX) activity, first reported in Devon Rex cats [90]. CCGX was purified to homogeneity in 1991 [91] and its gene cloned the same year [92]. Recently, two different homozygous point mutations in the *GGCX* gene have been reported in three consanguineous human families [79,81,84]. The first mutation results in the substitution of arginine for leucine at residue 394 (L394R) in four members of an Arab kindred. The second mutation that changes a tryptophan at residue 501 to serine (W501S) was reported in two Lebanese families. In addition, another family was found to have an 8 nucleotide deletion in the first intron of *GGCX*, which may disrupt a *cis* transcriptional element [85]. GGCX carrying the L394R mutation demonstrated a threefold reduced activity compared with the wild type protein [79]. L394 is in a 25 amino acid stretch of the enzyme that is highly conserved among all species with a known *GGCX* gene, including invertebrates such as the fruit fly and mosquito [62]. The primary defect associated with L394R appears to be reduced glutamate-substrate binding [93,94]. L394 may be directly involved in glutamate binding or may stabilize the binding site. The effect of the W501S mutation is still under investigation. It was noted, however, that W501 is in proximity to one of the regions involved in propeptide binding [81].

VKCFD2 results from functional deficiency of vitamin K 2,3-epoxide reductase (VKOR) [82,87]. Purification and cloning of VKOR turned out to be a challenging task. A genome wide linkage analysis in 2 pedigrees localized the disease gene to a 20-Mb segment of chromosome 16p [95], a rather large interval for positional cloning. It was noted, however, that a 4.0-Mb subinterval in this region corresponds to the warfarin resistance loci in rats and mice [96], suggesting that the warfarin resistance loci in rodents and VKCFD2 locus in humans may be caused by allelic mutations in the same gene [95]. Indeed, systematic screening of candidate genes in this region led to the identification of the vitamin K epoxide reductase complex subunit 1 gene (*VKORC1*) [97]. This gene was so named because of evidence suggesting that VKOR is a multisubunit complex [98,99]. A homozygous mutation that replaces arginine 98 by tryptophan was identified in two families. In addition, four heterozygous mutations were detected in warfarin resistant individuals. Li *et al.* identified the same gene independently using an alternative approach that involved knocking down

expression of individual candidate genes in the VKCFD2 disease interval with short interference RNAs and testing for reduced VKOR activity [100].

VKORC1 is a small protein of 163 amino acids (∼18 kDa) with up to three potential transmembrane domains. Like γ-carboxylase, it is localized to the ER via the C-terminal dilysine ER retention motif [97]. Expression of VKORC1 alone in insect cells confers warfarin-sensitive VKOR activity, raising the possibility that VKOR may be encoded by a single gene [100]. The identification of a point mutation that decreases VKOR activity and mutations that increase the warfarin resistance of VKOR provide critical information about the residues important for enzymatic activity.

γ-Carboxylation is absolutely required for the function of the vitamin K-dependent blood coagulation factors. Though its importance for the function of other carboxylated proteins seems likely, this is less clearly established. The function of the γ-carboxylated protein Gas6 is not well defined. However, *Gas6*-deleted mice exhibit a defect in platelet function [101] and appear to be protected from glomerular injury in a nephrotoxic nephritis model [102]. A mutation in the matrix Gla protein (MGP) gene causes Keutel syndrome [103], a human disorder characterized by abnormal cartilage calcification, peripheral pulmonary stenosis and midfacial hypoplasia. MGP deficient mice develop soft tissue calcifications, and uniformly succumb to aortic rupture within 2 months of birth [104]. Osteocalcin is an inhibitor of osteoblast function. Mice lacking osteocalcin exhibit increased bone formation without impairing bone resorption [105]. Administration of warfarin during pregnancy results in warfarin embryopathy, characterized by a spectrum of birth defects including abnormal mid-facial development, stippling of the epiphyses, and mental retardation [82,106–108]. Patients with VKORC1 mutations do not closely resemble those of warfarin embryopathy, though bone deformation has been noted [82]. The phenotypic differences may be explained by maternal complementation in VKCFD2 patients of the fully γ-carboxylated form of the protein responsible for the teratogenic effect.

The rarity of VKCFD and the fact that only missense mutations in GGCX and VKORC1 have been identified suggest that complete deficiency in either of these enzymes has severe consequences. Indeed, deletion of the *Ggcx* gene in mice is partially embryonic lethal [109]. Null mice surviving to term died uniformly at birth of massive intra-abdominal hemorrhage [109]. These results exclude the existence of a redundant carboxylase pathway and indicate a role for γ-carboxylated proteins in early mammalian development.

Other inherited diseases that exhibit blood coagulation defects

Sporadic cases of combined deficiencies for other coagulation factors have been reported [110–113]. Many of these patients may represent the chance inheritance of two different disease genes. In addition, several inherited diseases may exhibit coagulation deficiencies as a consequence of a more general metabolic defect. For example, congenital disorders of glycosylation (CDG) are a rapidly growing family of inherited diseases affecting any of the steps involved in glycosylation of proteins in the ER and Golgi. CDG types 1a and 1b have been associated with abnormal coagulation factor levels [114].

Concluding remarks

The powerful tools provided by the human and mouse genome projects have made it possible to identify the causes for several previously mysterious combined coagulation factor deficiencies. The identification of VKORC1 should significantly expedite the biochemical study of the vitamin K epoxide reductase. For example, we are now in a much better position to tackle long-standing questions such as whether VKOR is a multicomponent enzyme, and where the electrons come from that reduce vitamin K epoxide. Warfarin is currently the only highly active oral anticoagulant drug and is widely used in the treatment of thromboembolic diseases. Problems associated with the use of warfarin include difficulty in determining the right dosage and controlling the risk of bleeding. Better understanding of VKOR should provide information that may guide the design of safer vitamin K antagonists. Further elucidation of the LMAN1-MCFD2 secretory pathway will enhance our understanding of FV and FVIII biosynthesis, and provide fundamental insight into the basic biological process of ER-Golgi transport. To date, LMAN1-MCFD2 remains the only cargo receptor identified in higher eukaryotes. The requirement of a soluble protein (MCFD2) as an essential subunit of the cargo receptor distinguishes it from the known cargo receptors in yeast. Identification of other proteins relying on LMAN1-MCFD2 for efficient secretion should provide further insight into this important biologic pathway. FV and FVIII share little homology to two other putative ligands of LMAN1, cathepsin C and catZr. With the identification of additional ligands, it may be possible to define the common structural features that mediate these interactions. Finally, future studies may exploit the LMAN1-MCFD2 pathway as a novel drug target for the treatment of thrombophilia, and for the more efficient production of recombinant proteins, including FVIII.

References

1 Chiu HC, Schick PK, Colman RW. Biosynthesis of factor V in isolated guinea pig megakaryocytes. *J Clin Invest* 1985; **75**: 339–46.

2 Bontempo FA, Lewis JH, Gorenc TJ, Spero JA, Ragni MV, Scott JP, Starzl TE. Liver-transplantation in hemophilia-A. *Blood* 1987; **69**: 1721–4.

3 Kelly DA, Summerfield JA, Tuddenham EGD. Localization of Factor-VIIIC antigen in guinea-pig tissues and isolated liver-cell fractions. *Br J Haematol* 1984; **56**: 535–43.

4 Wion KL, Kelly D, Summerfield JA, Tuddenham EGD, Lawn RM. Distribution of factor-VIII messenger-RNA and antigen in human-liver and other tissues. *Nature* 1985; **317**: 726–9.

5 Zelechowska MG, Vanmourik JA, Brodniewiczproba T. Ultrastructural-localization of Factor-VIII procoagulant antigen in human-liver hepatocytes. *Nature* 1985; **317**: 729–30.

6 Do H, Healey JF, Waller EK, Lollar P. Expression of factor VIII by murine liver sinusoidal endothelial cells. *J Biol Chem* 1999; **274**: 19587–92.

7 Kaufman RJ, Wasley LC, Dorner AJ. Synthesis, processing, and secretion of recombinant human factor VIII expressed in mammalian cells. *J Biol Chem* 1988; **263**: 6352–62.

8 Kaufman RJ. Post-translational modifications required for coagulation factor secretion and function. *Thromb Haemost* 1998; **79**: 1068–79.

9 Oeri J, Matter M, Isenschmid H, Hauser F, Koller F. Angeborener mangel an faktor V (parahaemophilie) verbunden mit echter haemophilie A bein zwei brudern. *Med Probl Paediatr* 1954; **1**: 575–88.

10 Peyvandi F, Tuddenham EG, Akhtari AM, Lak M, Mannucci PM. Bleeding symptoms in 27 Iranian patients with the combined deficiency of factor V and factor VIII. *Br J Haematol* 1998; **100**: 773–6.

11 Ginsburg D. Hemophilia and Other Inherited Disorders of Hemostasis and Thrombosis. In: *Emery and Rimoin's Principles and Practice of Medical Genetics*, Vol. II. Rimoin DL, Connor, JM, Pyeritz, RE, eds. Churchill Livingstone, New York, 1996 :1651–76.

12 Seligsohn U, Zivelin A, Zwang E. Combined factor V and factor VIII deficiency among non-Ashkenazi Jews. *N Engl J Med* 1982; **307**: 1191–5.

13 Segal A, Zivelin A, Rosenberg N, Ginsburg D, Shpilberg O, Seligsohn U. A mutation in LMAN1 (ERGIC-53) causing combined factors V and VIII deficiency is prevalent in Jews originating from the island of Djerba in Tunisia. *Blood Coagulation and Fibriolysis*, 2004; **15**: 99–102.

14 Nichols WC, Seligsohn U, Zivelin A, Terry VH, Arnold ND, Siemieniak DR, Kaufman RJ, Ginsburg D. Linkage of combined factors V and VIII deficiency to chromosome 18q by homozygosity mapping. *J Clin Invest* 1997; **99**: 596–601.

15 Neerman-Arbez M, Antonarakis SE, Blouin J-L, Akhtari M, Afshar Y, Tuddenham EGD. The locus for combined factor V-factor VIII deficiency (F5F8D) maps to 18q21, between D18S849 and D18S1103. *AJHG* 1997; **61**: 143–50.

16 Nichols WC, Seligsohn U, Zivelin A, Terry VH, Hertel CE, Wheatley MA, Moussalli MJ, Hauri H-P, Ciavarella N, Kaufman RJ, Ginsburg D. Mutations in the ER-Golgi intermediate compartment protein ERGIC-53 cause combined deficiency of coagulation factors V and VIII. *Cell* 1998; **93**: 61–70.

17 Neerman-Arbez M, Johnson KM, Morris MA, Mcvey JH, Peyvandi F, Nichols WC, Ginsburg D, Rossier C, Antonarakis SE, Tuddenham EGD. Molecular analysis of the ERGIC-53 gene in 35 families with combined factor V-factor VIII deficiency. *Blood* 1999; **93**: 2253–60.

18 Nichols WC, Terry VH, Wheatley MA, Yang A, Zivelin A, Ciavarella N, Stefanile C, Matsushita T, Saito H, de Bosch NB, Ruiz-Saez A, Torres A, Thompson AR, Feinstein DI, White GC, Negrier C, Vinciguerra C, Aktan M, Kaufman RJ, Ginsburg D, Seligsohn U. .ERGIC-53 gene structure and mutation analysis in 19 combined factors V and VIII deficiency families. *Blood* 1999; **93**: 2261–6.

19 Zhang B, Cunningham MA, Nichols WC, Bernat JA, Seligsohn U, Pipe SW, McVey JH, Schulte-Overberg U, de Bosch NB, Ruiz-Saez A, White GC, Tuddenham EG, Kaufman RJ, Ginsburg D. Bleeding due to disruption of a cargo-specific ER-to-Golgi transport complex. *Nature Genet* 2003; **34**: 220–5.

20 Schekman R, Orci L. Coat proteins and vesicle budding. *Science* 1996; **271**: 1526–33.

21 Springer S, Spang A, Schekman R. A primer on vesicle budding. *Cell* 1999; **97**: 145–8.

22 Miller EA, Beilharz TH, Malkus PN, Lee MCS, Hamamoto S, Orci L, Schekman R. Multiple cargo binding sites on the COPII subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles. *Cell* 2003; **114**: 497–509.

23 Mossessova E, Bickford LC, Goldberg J. SNARE selectivity of the COPII coat. *Cell* 2003; **114**: 483–95.

24 Wieland FT, Gleason ML, Serafini TA, Rothman JE. The rate of bulk flow from the endoplasmic reticulum to the cell surface. *Cell* 1987; **50**: 289–300.

25 Warren G, Mellman I. Bulk flow redux? *Cell* 1999; **98**: 125–7.

26 Martinez-Menarguez JA, Geuze HJ, Slot JW, Klumperman J. Vesicular tubular clusters between the ER and Golgi mediate concentration of soluble secretory proteins by exclusion from COPI-coated vesicles. *Cell* 1999; **98**: 81–90.

27 Kuehn MJ, Herrmann JM, Schekman R. COPII–cargo interactions direct protein sorting into ER-derived transport vesicles. *Nature* 1998; **391**: 187–90.

28 Muniz M, Morsomme P, Riezman H. Protein sorting upon exit from the endoplasmic reticulum. *Cell* 2001; **104**: 313–20.

29 Nehls S, Snapp EL, Cole NB, Zaal KJ, Kenworthy AK, Roberts TH, Ellenberg J, Presley JF, Siggia E, Lippincott-Schwartz J. Dynamics and retention of misfolded proteins in native ER membranes. *Nat Cell Biol* 2000; **2**: 288–95.

30 Saraste J, Palade GE, Farquhar MG. Antibodies to rat pancreas Golgi subfractions: identification of a 58-kD cis-Golgi protein. *J Cell Biol* 1987; **105**: 2021–9.

31 Schweizer A, Fransen JAM, Bachi T, Ginsel L, Hauri HP. Identification, by a monoclonal-antibody, of a 53-Kd protein associated with a tubulo-vesicular compartment at the cis-side of the Golgi-apparatus. *J Cell Biol* 1988; **107**: 1643–53.

32 Fiedler K, Simons K. Characterization of VIP36, an animal lectin homologous to leguminous lectins. *J Cell Sci* 1996; **109**: 271–6.

33 Neve EPA, Svensson K, Fuxe J, Pettersson RF. VIPL, a VIP36-like membrane protein with a putative function in the export of glycoproteins from the endoplasmic reticulum. *Exp Cell Res* 2003; **288**: 70–83.

34 Nufer O, Mitrovic S, Hauri HP. Profile-based data base scanning for animal L-type lectins and characterization of VIPL, a novel VIP36-like endoplasmic reticulum protein. *J Biol Chem* 2003; **278**: 15886–96.

35 Yerushalmi N, Keppler-Hafkemeyer A, Vasmatzis G, Liu XF, Olsson P, Bera TK, Duray P, Lee B, Pastan I. ERGL, a novel gene related to ERGIC-53 that is highly expressed in normal and neoplastic prostate and several other tissues. *Gene* 2001; **265**: 55–60.

36 Hauri H, Appenzeller C, Kuhn F, Nufer O. Lectins and traffic in the secretory pathway. *FEBS Lett* 2000; **476**: 32–7.

37 Fiedler K, Simons K. A putative novel class of animal lectins in the secretory pathway homologous to leguminous lectins. *Cell* 1994; **77**: 625–6.

38 Arar C, Carpentier V, Le Caer JP, Monsigny M, Legrand A, Roche A. C. ERGIC-53, a membrane protein of the endoplasmic reticulum-Golgi intermediate compartment, is identical to MR60, an intracellular mannose-specific lectin of myelomonocytic cells. *J Biol Chem* 1995; **270**: 3551–3.

39 Itin C, Roche AC, Monsigny M, Hauri HP. ERGIC-53 is a functional mannose-selective and calcium-dependent human homologue of leguminous lectins. *Mol Biol Cell* 1996; **7**: 483–93.

40 Belden WJ, Barlowe C. Role of Erv29p in collecting soluble secretory proteins into ER-derived transport vesicles. *Science* 2001; **294**: 1528–31.

41 Muniz M, Nuoffer C, Hauri HP, Riezman H. The Emp24 complex recruits a specific cargo molecule into endoplasmic reticulum-derived vesicles. *J Cell Biol* 2000; **148**: 925–30.

42 Velloso LM, Svensson K, Schneider G, Pettersson RF, Lindqvist Y. Crystal structure of the carbohydrate recognition domain of p58/ERGIC- 53, a protein involved in glycoprotein export from the endoplasmic reticulum. *J Biol Chem* 2002; **277**: 15979–84.

43 Kappeler F, Klopfenstein DR, Foguet M, Paccaud JP, Hauri HP. The recycling of ERGIC-53 in the early secretory pathway. ERGIC-53 carries a cytosolic endoplasmic reticulum-exit determinant interacting with COPII. *J Biol Chem* 1997; **272**: 31801–8.

44 Nufer O, Guldbrandsen S, Degen M, Kappeler F, Paccaud JP, Tani K, Hauri HP. Role of cytoplasmic C-terminal amino acids of membrane proteins in ER export. *J Cell Sci* 2002; **115**: 619–28.

45 Klumperman J, Schweizer A, Clausen H, Tang BL, Hong W, Oorschot V, Hauri HP. The recycling pathway of protein ERGIC-53 and dynamics of the ER-Golgi intermediate compartment. *J Cell Sci* 1998; **111**: 3411–25.

46 Tisdale EJ, Plutner H, Matteson J, Balch WE. p53/58 binds COPI and is required for selective transport through the early secretory pathway. *J Cell Biol* 1997; **137**: 581–93.

47 Nufer O, Kappeler F, Guldbrandsen S, Hauri HP. ER export of ERGIC-53 is controlled by cooperation of targeting determinants in all three of its domains. *J Cell Sci* 2003; **116**: 4429–40.

48 Munro S., Pelham HR. A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 1987; **48**: 899–907.

49 Lewis MJ, Sweet DJ, Pelham HR. The ERD2 gene determines the specificity of the luminal ER protein retention system. *Cell* 1990; **61**: 1359–63.

50 Semenza JC, Hardwick KG, Dean N, Pelham HR. ERD2, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell* 1990; **61**: 1349–57.

51 Appenzeller-Herzog C, Roche AC, Nufer O, Hauri HP. pH-induced conversion of the transport lectin ERGIC-53 triggers glycoprotein release. *J Biol Chem*, 2004; **279**: 12943–50.

52 Hauri HP, Kappeler F, Andersson H, Appenzeller C. ERGIC-53 and traffic in the secretory pathway. *J Cell Sci*, 2000; **113**: 587–96.

53 Cunningham MA, Pipe SW, Zhang B, Hauri HP, Ginsburg D, Kaufman RJ. LMAN1 is a molecular chaperone for the secretion of coagulation factor VIII. *J Thromb Haemost* 2003; **1**: 2360–7.

54 Martin-Bermudo MD, Brown NH. The localized assembly of extracellular matrix integrin ligands requires cell-cell contact. *J Cell Sci* 2000; **113 Part 21**: 3715–23.

55 Prout M, Damania Z, Soong J, Fristrom D, Fristrom JW. Autosomal mutations affecting adhesion between wing surfaces in *Drosophila melanogaster*. *Genetics* 1997; **146**: 275–85.

56 Vollenweider F, Kappeler F, Itin C, Hauri HP. Mistargeting of the lectin ERGIC-53 to the endoplasmic reticulum of HeLa cells impairs the secretion of a lysosomal enzyme. *J Cell Biol* 1998; **142**: 377–89.

57 Appenzeller C, Andersson H, Kappeler F, Hauri HP. The lectin ERGIC-53 is a cargo transport receptor for glycoproteins. *Nat Cell Biol* 1999; **1**: 330–4.

58 Toda H, Tsuji M, Nakano I, Kobuke K, Hayashi T, Kasahara H, Takahashi J, Mizoguchi A, Houtani T, Sugimoto T, Hashimoto N, Palmer TD, Honjo T, Tashiro K. Stem cell-derived neural stem/progenitor cell supporting factor is an autocrine/paracrine survival factor for adult neural stem/progenitor cells. *J Biol Chem* 2003; **278**: 35491–500.

59 Moussalli M, Pipe SW, Hauri HP, Nichols WC, Ginsburg D, Kaufman RJ. Mannose-dependent endoplasmic reticulum (ER)-Golgi intermediate compartment-53-mediated ER to Golgi trafficking of coagulation factors V and VIII. *J Biol Chem* 1999; **274**: 32539–42.

60 Miao HZ, Sirachainan N, Palmer L, Kucab P, Cunningham MA, Kaufman RJ, Pipe SW. Bioengineering of coagulation factor VIII for improved secretion. *Blood* 2004; **103**: 3421–9.

61 Furie B, Bouchard BA, Furie BC. Vitamin K-dependent biosynthesis of γ-carboxyglutamic acid. *Blood* 1999; **93**: 1798–808.

62 Presnell SR, Stafford DW. The Vitamin K-dependent carboxylase. *Thomb Haemost* 2002; **87**: 937–46.

63 Furie B, Furie BC. Molecular basis of vitamin K-dependent gamma-Carboxylation. *Blood* 1990; **75**: 1753–62.

64 Han X, Fiehler R, Broze GJ. Isolation of a protein Z-dependent plasma protease inhibitor. *Proc Natl Acad Sci USA* 1998; **95**: 9250–5.

65 Hauschka PV, Lian JB, Cole DEC, Gundberg CM. Osteocalcin and Matrix Gla Protein – Vitamin K-Dependent Proteins in Bone. *Physiol Rev* 1989; **69**: 990–1047.

66 Stitt TN, Conn G, Gore M, Lai C, Bruno J, Radziejewski C, Mattsson K, Fisher J, Gies DR, Jones PF *et al*. The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. *Cell* 1995; **80**: 661–70.

67 Mustafi D, Nakagawa Y. Characterization of calcium-binding sites in the kidney-stone inhibitor glycoprotein nephrocalcin with vanadyl ions – electron-paramagnetic-resonance and electron-nuclear double-resonance spectroscopy. *Proc Natl Acad Sci USA* 1994; **91**: 11323–7.

68 Kulman JD, Harris JE, Haldeman BA, Davie EW. Primary structure and tissue distribution of two novel proline-rich gamma-carboxyglutamic acid proteins. *Proc Natl Acad Sci USA* 1997; **94**: 9058–62.

69 Kulman JD, Harris JE, Xie L., Davie EW. Identification of two novel transmembrane gamma-carboxyglutamic acid proteins expressed broadly in fetal and adult tissues. *Proc Natl Acad Sci USA* 2001; **98**: 1370–5.

70 Diuguid DL, Rabiet MJ, Furie BC, Liebman HA, Furie B. Molecular-basis of hemophilia-b – a defective enzyme due to an unprocessed propeptide is caused by a point mutation in the factor-IX precursor. *Proc Natl Acad Sci USA* 1986; **83**: 5803–7.

71 Jorgensen MJ, Cantor AB, Furie BC, Brown CL, Shoemaker CB, Furie B. Recognition site directing vitamin-k-dependent gamma-carboxylation resides on the propeptide of factor-IX. *Cell* 1987; **48**: 185–91.

72 Huber P, Schmitz T, Griffin J, Jacobs M, Walsh C, Furie B, Furie BC. Identification of Amino-Acids in the Gamma-Carboxylation Recognition Site on the Propeptide of Prothrombin. *J Biol Chem* 1990; **265**: 12467–73.

73 Zimmerma.A, Matschin.JT. Biochemical basis of hereditary resistance to warfarin in rat. *Biochem Pharmacol* 1974; **23**: 1033–40.

74 Wallin R, Martin LF. Vitamin-K-dependent carboxylation and vitamin-k metabolism in liver – effects of warfarin. *J Clin Invest* 1985; **76**: 1879–84.

75 Mcmillan CW, Roberts HR. Congenital combined deficiency of coagulation factors 2 7 9 and X – report of a case. *N Engl J Med* 1966; **274**: 1313–&.

76 Chung KS, Bezeaud A, Goldsmith JC, Mcmillan CW, Menache D, Roberts HR. Congenital deficiency of blood-clotting Factor-II, Factor-VII, Factor-IX, and Factor-X. *Blood* 1979; **53**: 776–87.

77 Boneh A, BarZiv J. Hereditary deficiency of vitamin K-dependent coagulation factors with skeletal abnormalities. *Am J Med Genet* 1996; **65**: 241–3.

78 Brenner B, Tavori S, Zivelin A, Keller CB, Suttie JW, Tatarsky I, Seligsohn U. Hereditary deficiency of all vitamin K-dependent procoagulants and anticoagulants. *Br J Haematol* 1990; **75**: 537–42.

79 Brenner B, Sánchez-Vega B, Wu S-M, Lanir N, Stafford DW, Solera J. A missense mutation in γ-glutamyl carboxylase gene causes combined deficiency of all vitamin K-dependent blood coagulation factors. *Blood* 1998; **92**: 4554–9.

80 Goldsmith GH. Jr, Pence RE, Ratnoff OD, Adelsteinn DJ, Furie B. Studies on a family with combined functional deficiencies of vitamin K-dependent coagulation factors. *J Clin Invest* 1982; **69**: 1253–60.

81 Mousallem M, Spronk HM, Sacy R, Hakime N, Soute BA. Congenital combined deficiencies of all vitamin K-dependent coagulation factors. *Thromb Haemost* 2001; **86**: 1334–6.

82 Pauli RM, Lian JB, Mosher DF, Suttie JW. Association of congenital deficiency of multiple vitamin K-dependent coagulation factors and the phenotype of the warfarin embryopathy: clues to the mechanism of teratogenicity of coumarin derivatives. *Am J Hum Genet* 1987; **41**: 566–83.

83 Pechlaner C, Vogel W, Erhart R, Pumpel E, Kunz F. A new case of combined deficiency of vitamin-k dependent coagulation-factors. *Thromb Haemost* 1992; **68**: 617.

84 Spronk HM, Farah RA, Buchanan GR, Vermeer C, Soute B. A. Novel mutation in the gamma-glutamyl carboxylase gene resulting in congenital combined deficiency of all vitamin K-dependent blood coagulation factors. *Blood* 2000; **96**: 3650–2.

85 Thomas A, Stirling D. Four factor deficiency. *Blood Coagul Fibrinol* 2003; **14** (Suppl. 1): S55–S57.

86 Vicente V, Maia R, Alberca I, Tamagnini GP, Lopez BA. Congenital deficiency of vitamin K-dependent coagulation factors and protein C. *Thromb Haemost*, 1984; **51**: 343–6.

87 Oldenburg J, von Brederlow B, Fregin A, Rost S, Wolz W, Eberl W, Eber S, Lenz E, Schwaab R, Brackmann HH, Effenberger W, Harbrecht U, Schurgers LJ, Vermeer C, Muller CR. Congenital deficiency of vitamin K dependent coagulation factors in two families presents as a genetic defect of the vitamin K-epoxide-reductase-complex. *Thromb Haemost* 2000; **84**: 937–41.

88 Ekelund H, Lindeberg L, Wranne L. Combined deficiency of coagulation Factor-II, Factor-VII, Factor-IX, and Factor-X – a case of probable congenital origin. *Pediatric Hematol Oncol* 1986; **3**: 187–93.

89 Johnson CA, Chung KS, McGrath KM, Bean PE, Roberts HR. Characterization of a variant prothrombin in a patient congenitally deficient in Factor-II, Factor-VII, Factor-IX and Factor-X. *Br J Haematol* 1980; **44**: 461–9.

90 Soute BAM, Ulrich MMW, Watson ADJ, Maddison JE, Ebberink RHM, Vermeer C. Congenital deficiency of all vitamin K-dependent blood coagulation factors due to a defective vitamin K-dependent carboxylase in Devon Rex cats. *Thromb Haemost* 1992; **68**: 521–5.

91 Wu SM, Morris DP, Stafford DW. Identification and purification to near homogeneity of the vitamin K-dependent carboxylase. *Proc Natl Acad Sci USA* 1991; **88**: 2236–40.

92 Wu S-M, Cheung W-F, Frazier D, Stafford DW. Cloning and expression of the cDNA for human gamma-glutamyl carboxylase. *Science* 1991; **254**: 1634–6.

93 Mutucumarana VP, Stafford DW, Stanley TB, Jin DY, Solera J, Brenner B, Azerad R, Wu SM. Expression and characterization of the naturally occurring mutation L394R in human gamma-glutamyl carboxylase. *J Biol Chem* 2000; **275**: 32572–7.

94 Mutucumarana VP, Acher F, Straight DL, Jin DY, Stafford DW. A conserved region of human vitamin K-dependent carboxylase between residues 393 and 404 is important for its interaction with the glutamate substrate. *J Biol Chem* 2003; **278**: 46488–93.

95 Fregin A, Rost S, Wolz W, Krebsova A, Muller CR, Oldenburg J. Homozygosity mapping of a second gene locus for hereditary combined deficiency of vitamin K-dependent clotting factors to the centromeric region of chromosome 16. *Blood* 2002; **100**: 3229–32.

96 Kohn MH, Pelz HJ. A gene-anchored map position of the rat warfarin-resistance locus, Rw, and its orthologs in mice and humans. *Blood* 2000; **96**: 1996–8.

97 Rost S, Fregin A, Ivaskevicius V, Conzelmann E, Hortnagel K, Pelz HJ, Lappegard K, Seifried E, Scharrer I, Tuddenham EG, Muller CR, Strom TM, Oldenburg J. Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature* 2004; **427**: 537–41.

98 Cain D, Hutson SM, Wallin R. Assembly of the warfarin-sensitive vitamin K 2,3-epoxide reductase enzyme complex in the endoplasmic reticulum membrane. *J Biol Chem* 1997; **272**: 29068–75.

99 Begent LA, Hill AP, Steventon GB, Hutt AJ, Pallister CJ, Cowell DC. Characterization and purification of the vitamin K1 2,3 epoxide reductases system from rat liver. *J Pharm Pharmacol* 2001; **53**: 481–6.

100 Li T, Chang CY, Jin DY, Lin PJ, Khvorova A, Stafford DW. Identification of the gene for vitamin K epoxide reductase. *Nature* 2004; **427**: 541–4.

101 Angelillo-Scherrer A, de Frutos PG, Aparicio C, Melis E, Savi P, Lupu F, Arnout J, Dewerchin M, Hoylaerts MF, Herbert M, Colen D, Dahlback B, Carmeliet P. Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. *Nature Med* 2001; **7**: 215–21.

102 Yanagita M, Ishimoto Y, Arai H, Nagai K, Ito T, Nakano T, Salant DJ, Fukatsu A, Doi T, Kita T. Essential role of Gas6 for glomerular injury in nephrotoxic nephritis. *J Clin Invest* 2002; **110**: 239–46.

103 Munroe PB, Olgunturk RO, Fryns JP, Van Maldergem L, Ziereisen F, Yuksel B, Gardiner RM, Chung E. Mutations in the gene encoding the human matrix Gla protein cause Keutel syndrome. *Nat Genet* 1999; **21**: 142–4.

104 Luo G, Ducy P, Mckee MD, Pinero GJ, Loyer E, Behringer RR, Karsenty G. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature* 1997; **386**: 78–81.

105 Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A, Karsenty G. Increased bone formation in osteocalcin-deficient mice. *Nature* 1996; **382**: 448–52.

106 Hall JG, Pauli RM, Wilson KM. Maternal and fetal sequelae of anticoagulation during pregnancy. *Am J Med* 1980; **68**: 122–40.

107 Howe AM, Lipson AH, desilva M, Ouvrier R, Webster WS. Severe cervical dysplasia and nasal cartilage calcification following prenatal warfarin exposure. *Am J Med Genet* 1997; **71**: 391–6.

108 Menger H, Lin AE, Toriello HV, Bernert G, Spranger JW. Vitamin K deficiency embryopathy. A phenocopy of the warfarin embryopathy due to a disorder of embryonic vitamin K metabolism. *Am J Med Genet* 1997; **72**: 129–34.

109 Zhu A, Raymond R, Zheng X, Westrick RJ, Furie BC, Furie B, Kaufman RJ, Ginsburg D. Abnormalities of development and hemostasis in γ-carboxylase deficient mice. *Blood* 1998; **92**: 152a (Abstract).

110 Machin SJ, Miller BR. Congenital combined factor VII and factor VIII deficiency. *Acta Haematol* 1980; **63**: 167–9.

111 Soff GA, Levin J. Familial Multiple Coagulation-Factor Deficiencies.1. Review of the Literature – Differentiation of Single Hereditary Disorders Associated with Multiple Factor Deficiencies from Coincidental Concurrence of Single Factor Deficiency States. *Semin Thromb Hemost* 1981; **7**: 112–48.

112 Soff GA, Levin J, Bell WR. Familial Multiple Coagulation-Factor Deficiencies.2. Combined Factor-VIII, Factor-IX, and Factor-XI Deficiency and Combined Factor-IX and Factor-XI Deficiency – 2 Previously Uncharacterized Familial Multiple Factor Deficiency Syndromes. *Semin Thromb Hemost* 1981; **7**: 149–69.

113 Sano M, Saito H, Shimamoto Y, Sugiura I, Ohtsubo H, Kohda H, Yamaguchi M. Combined hereditary factor XI (plasma thromboplastin antecedent) deficiency, von Willebrand's disease, and xeroderma pigmentosum in a Japanese family. *Am J Hematol* 1993; **44**: 129–33.

114 Marquardt T. Denecke J. Congenital disorders of glycosylation: review of their molecular bases, clinical presentations and specific therapies. *Eur J Pediatr* 2003; **162**: 359–79.

115 Sadler JE. Medicine: K is for koagulation. *Nature* 2004; **427**: 493–4.