Tisssue factor and factor viia cross-species compatibility

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TABLE OF CONTENTS

1. ASTRACT

Knowledge about species compatibility is crucial for proper interpretation of data from *in vivo* experiments with human proteins in pharmacological models and of data from cross-species *in vitro* experiments. Information about the cross-species compatibility of tissue factor (TF) and coagulation factor (F) VII (FVII) has accumulated since the early history of coagulation research. Many observations were connected to the introduction and development of the prothrombin time (PT) assay where fibrin clot formation was observed when tissue extracts of different origins were added to recalcified human or non-human plasmas. Studies on cross-species TF-FVIIa compatibility entered into a new area with the cloning and recombinant expression of TF and FVII from a number of species as well as with the possibility of specific amino acid substitution. TF and/or FVIIa from cattle, dog, rabbit, mouse, rat and zebrafish have been purified and characterized in varying detail. In addition to adding knowledge about the species-specific TF-FVIIa interactions, cross-species studies often reveal information which adds to the general view of the structural and functional properties of the human TF-FVIIa complex. This review briefly outlines the features of human TF and FVIIa, their intermolecular interactions, and the biological effects of TF-FVIIa complex formation and compares this information to findings obtained in studies addressing TF or FVIIa of non-human origin. By examples we point to difficulties which may arise from the transcendence across species borders and how some cross-species data have advanced our understanding of the structure and function of the human TF-FVIIa complex.

2. INTRODUCTION

The early history of research on TF and coagulation has been described in several reviews (1-4). The classical theory on blood coagulation published by Morawitz in 1905 (5) suggested that tissue thromboplastin (also known as FIII or lipidated TF) activated prothrombin (FII) to thrombin (FIIa) which again converted fibrinogen (FI) to fibrin. An important further step forward was achieved by the introduction of a laboratory test in which a standardized thromboplastin preparation was added to re-calcified plasma and the time to clot formation, the prothrombin time (PT), was measured. The PT assay was at that time thought to measure the prothrombin concentration in plasma directly. The PT test was adapted in the clinic in the 1940'ies to monitor treatment with the newly introduced anticoagulant, dicumarol (reviewed in: 6).

Improved assay techniques soon revealed that additional factors were required for thrombin formation. FV (7) and FVII (8) were the first to be identified by testing of plasma from patients with specific hereditary deficiencies. A clear separation of the actions of FVIIa and FXa was, however, first possible with the specific identification of FX (9; 10).

Since the preparation of human tissue thromboplastin from brains was problematic for several obvious reasons it became common to use thromboplastins from various animal tissue sources for the general screening of human plasma samples. Pioneering studies on TF-FVIIa species compatibility with different animal thromboplastins and human and animal plasmas were reported by Stormorken (11) and Irsigler and colleagues (12).

With the discoveries of a number of new coagulation factors from VIII to XII it became a conceptual challenge to understand how the coagulation factors all worked together to transmit surface contact of blood into a response leading to thrombin generation and fibrin formation. The suggestion in 1964 by MacFarlane (13) and by Davie and Ratnoff (14) that coagulation should be understood as a cascade of consecutive and amplifying reactions in a chain of zymogen activation steps was a big step forward. In this theory surface exposure leads to activation of FXII to FXIIa, which activates FXI to FXIa, which again activates FIX to FIXa and so on. The role of FVIIa and a number of causes behind observed phenomena remained, however, unresolved with the single cascade model. The theories did for example not explain why FVII deficiency was sometimes associated with severe bleeding problems (15), when FXII or FXI deficiencies were predominantly asymptomatic (16-18).

A revision of coagulation with the TF-FVIIa complex in a central position was facilitated by progress in the purification of bovine TF (19) and FVII (20; 21). Important for the revision was also the observation that the TF-FVIIa complex could activate FIX (22). The current theory suggests that initiation of coagulation may proceed via either of two separate paths which merge at the FX activation level. This theory which operates with an intrinsic, FXII-dependent, and an extrinsic, TF-dependent, branch was subsequently advanced by Broze (23) and by Rapaport and Rao (2).

The new insight into the role of TF and the extrinsic pathway of coagulation made it possible to better perform and interpret studies on TF-FVIIa species compatibility with different animal thromboplastins and animal plasmas (24). The purification of the TF and FVII proteins in the seventies and eighties, the cloning of the human genes, and the subsequent cloning and recombinant expression of human and various animal TF and FVII proteins have provided additional knowledge about the species compatibility of these molecules.

However, information about TF and FVIIa interspecies compatibility is scattered in the literature on the many facets of TF and FVIIa biology. Furthermore, species compatibility is not always fully considered in studies with human proteins applied in *in vitro* as well as *in vivo* studies on TF and FVIIa. Finally, species compatibility may also pose a diagnostic challenge when TF of animal origin is used in testing (e.g. PT) of human samples, and vice versa when human TF or FVII is used for assaying samples of animal origin. Notably, studies on TF and FVIIa compatibility across species borders have provided useful information about important molecular interactions in the human TF-FVIIa complex. The aim of the present review is to gather and summarize a large body of the currently available information on these matters to support improved interpretation as well as planning of cross-species studies with TF and FVII.

3. TF AND FVIIA

FVIIa is a vitamin K-dependent serine protease. It is present in the circulation, primarily in its zymogen form, i.e. FVII, at a concentration of about 10 nM (25). Only approximately one percent of the total FVII antigen circulates in the activated enzyme FVIIa form while the remaining antigen circulates as the FVII zymogen (26; 27). The enzymatic activity of free circulating FVIIa is insufficient to initiate coagulation under physiological conditions. TF is an integral membrane protein which is expressed on a variety of cells located outside the vasculature (28) and normally not exposed to the circulation. Conversely, TF is exposed to the circulation at a site of vascular injury; and it is first when FVIIa binds to its natural high-affinity receptor, TF, that FVIIa attains its fully active, procoagulant state. When bound to TF, FVIIa activates FX to FXa which again activates more zymogen FVII to enzymatically active FVIIa. Thus, a reciprocal zymogen activation cycle is initiated and TF-bound FVII is rapidly converted to FVIIa by FXa mediated cleavage of the single scissile band at Arg152-Ile153. The activation of FVII to FVIIa by FXa leads to formation of additional TF-FVIIa complexes which trigger coagulation by additional FX activation (29).

The TF-FVIIa complex is also capable of inducing intracellular signal transduction mediated by

protease-activated receptors (PARs). This occurs either by cleavage of the PAR2 receptor by the TF-FVIIa complex or by cleavage of either of the PAR1 or PAR2 receptors by the ternary TF-FVIIa-FXa complex. PAR cleavage results in a variety of cellular responses (reviewed in: 30; 31).

Additional information about human TF and FVIIa structure and function is available in recent reviews (32; 33).

3.1. Purification, cloning and expression of TF

Historically, brain, placenta and lung tissue has been used as a source of crude tissue thromboplastin preparations. A simple way to prepare crude thromboplastins from mammals is the saline extraction method (34). Such crude preparations are useful for many purposes. However, the crude preparations are contaminated with varying quantities of pro-coagulant material of non-TF origin, e.g. endotoxin and coagulation factors and hence reagents with of a higher purity are often preferred.

Purification of TF was, however, hampered by its presence in relative low amount in tissues, and also by its lipoprotein nature which required detergent extraction and reconstitution of the biological activity in phospholipid vesicles. Thus, specific purification of the TF protein from crude tissue extracts was inefficient (35-37) until Bach and colleagues succeeded in purifying bovine TF to homogeneity by immunoaffinity chromatography (19). Bach and colleagues used bovine TF initially purified in µg scale by repeated preparative SDS-PAGE to raise the rabbit polyclonal antibody for the immunoaffinity procedure.

Affinity purification of human TF with the rabbit antibody against bovine TF failed. In this way species specificity played a historical role since the lack of cross-reactivity of the anti-bovine TF antibody to human TF prompted the development of an alternative affinity purification procedure using human FVII immobilized to a solid support (19; 38). In analogy, human TF was also purified by human FVII affinity chromatography using an immobilized FVII antibody which allowed specific binding of TF (39). These different methods all yielded homogenous TF with a high specific activity after relipidation and could still be used as inspiration for purification of TF from additional species. The mature and fully glycosylated human TF protein was shown, irrespective of purification method, to migrate with an apparent molecular weight of approximately 47 kDa on SDS-PAGE (38-40).

Purification of human TF was followed by its cloning by several groups (39; 41-43). The TF protein is encoded by the *F3* gene. In humans, *F3* (Gene ID: 2152) is located at chromosome 1; specifically: 1p22-p21 (44; 45). Human *F3* constitutes six exons separated by five introns and spans a total of 12.6 kilobase (kb) pairs (43). The mature human TF mRNA transcript comprises 2.4 kb (44).

TF cDNA sequences from a variety of animal species, including bovine (46), rabbit (47), guinea pig (48), rat (49), and mouse (47; 50) are now available. In addition, TF sequences from chimpanzee (Gene ID: 457038), sumatran orangutan (Gene ID: 100173124), pig (Gene ID: 396677), dog (Gene ID: 490153), hen (Gene ID: 429084) and zebrafish (Gene ID: 567257) have been predicted.

Advances in recombinant technology have provided tools for expression of TF in bacteria (51), yeast (52; 53), insect cells (54) and mammalian cells (51). Indeed, with the successful cloning of human TF followed recombinant expression of the soluble TF ectodomain (i.e. TF truncated N-terminally of the transmembrane domain), often referred to as soluble TF or merely sTF and solution of its crystal structure (55; 56). TF produced in *E.coli* does not contain any carbohydrate and therefore the soluble and full length human proteins migrate with apparent molecular weights of 25 and 36 kDa, respectively, on SDS-PAGE. Althoug lacking its glycans, TF produced in bacteria is still functional in activity assays (57).

In general, soluble TF can be purified to homogeneity by anion exchange chromatography without the need for affinity matrixes. With minor modifications to the protocol used for production of human soluble TF, our group has produced recombinant soluble murine (58) as well as canine TF (59), and soluble porcine, rabbit, rat and zebrafish TF in *E.coli* (H.R.Stennicke, unpublished).

3.2. TF structure and function

Mapping of the sequence identified TF as an integral membrane glycoprotein receptor with homology to the cytokine-receptor superfamily (60). This is a unique class of signal transducing receptor proteins specific for a diverse group of hematopoietic factors and growth hormones. The discovery of this kinship initiated a search for a possible involvement in signal transduction. Homology did not extend to the short intracellular TF domain and it was obvious that a putative TF mediated signaling proceeded by a path different from that used by other members of the family.

Cellular responses induced in both human and canine cells by TF-FVIIa complex formation were first reported by the group headed by Hans Prydz (61). This group also noted that the proteolytic activity of FVIIa was mandatory for the signaling. It is now recognized that in addition to its well known triggering of the extrinsic coagulation cascade, binding of FVIIa to cell surface TF triggers intracellular signal transduction via protease activated receptors (PARs) (30; 31).

Human TF consists of a 219-amino acid residue extracellular domain, a 23-residue hydrophobic transmembrane region, and a 21-residue intracellular domain (Figure 1). The extracellular domain which comprises two fibronectin modules is characterized by a high sequence homology between species including residues interacting with FVII and sequences coding for glycosylation. Notably, an extra stretch has been adapted in mouse and rat TF at the interface between the two fibronectin modules (Figure 1 B). In all species TF contains a typical trans-membrane domain and also a short

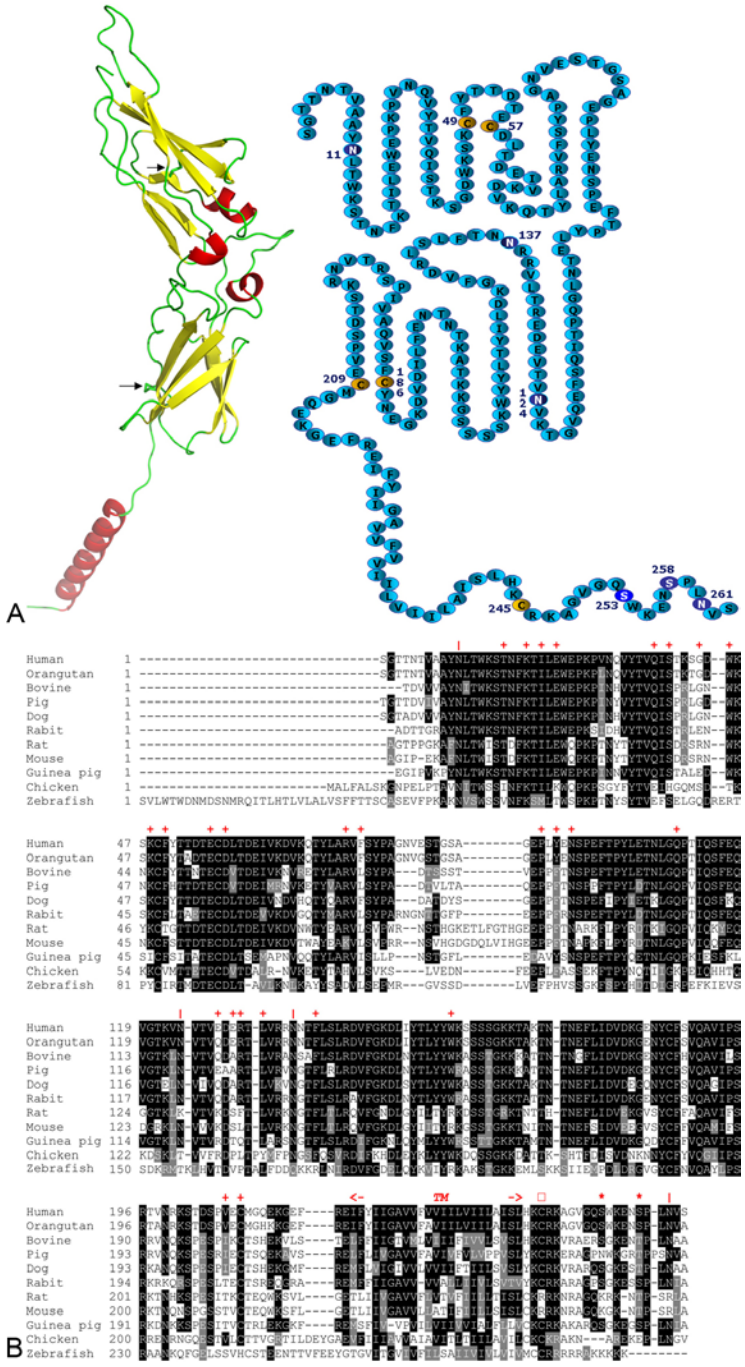

Figure 1. A. Human tissue factor (TF) structure and sequence. Left: TF structure in a cartoon representation. Helix turns are shown in red, strands in yellow and loops in green. The two disulfides are shown in green using stick representation. Right: Sequence of human TF. The disulphides are shown in amber. Potential N-glycosylation, O-glycosylation, phosphylation, and palmitoylation are shown in blue. See the main text for a detailed description. B. Sequence alignment of human and animal TF. The sequences of the mature proteins were taken from the UniProt data base when available. Alternatively they were determined using SignalP (http://www.cbs.dtu.dk/services/SignalP/). The transmembrane region, i.e. residues corresponding 220 through 242 in human TF, is indicated by arrows. Crosses in red denote residues in human TF which are in close contact with human FVIIa as determined from the X-ray structure of the human sTF-FVIIai complex (pdb entry code 1dan) and vertical lines in red show potential N-glycosylation sites in human TF. The alignment was performed in ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html) was used for graphics display.

intracellular domain. Although not essential for normal development, it is striking that the intracellular domains of all species TF are equipped with potential Cys-SH sites for palmitoylation, and, except for hen and zebrafish, also with Ser/Thr sites for phosphorylation (Figure 1 B).

TF is subject to several post tranlational modifications reported to regulate its pro-coagulant activity (see: 32). The extracellular domain of human TF contains four cysteines arranged in two disulfide bonds (Figure 1). The Cys49–Cys57 disulfide bond serves as a firm link between two β-sheets in the fibronectin-like domain. The Cys186-Cys209 bridge may undergo redox-dependent opening catalyzed by protein disulfide isomerase (PDI) resulting in structural rearrangement of TF (62). Three potential N-glycosylation sites, Asn11, Asn124, and Asn137, are distributed on the TF extracellular domain. Recently, a report showed that recombinant TF was less active than placenta derived TF with respect to TF-FVIIa mediated activation of FX and that this difference was due to different glycosylation patterns (57). Notably, FX activation with placenta derived TF was decreased after de-glycosylation of the TF protein. Thus, suggesting that the presence of fucosylated and/or sialylated sugars on TF slightly enhanced FX activation by the TF-FVIIa complex. In agreement with earlier studies, binding of FVIIa to TF was apparently unaffected by the glycosylation of TF (57). The intracellular domain of TF contains potential sites for post tranlational modifications; a free cysteine, Cys-SH 245, which is susceptible to palmitoylation (63), and Ser 253/258 phosphorylation sites (64; 65). Palmitoylation may target TF to lipid rafts on the cell surface and possibly quench its pro-coagulant potency and enhance angiogenesis (66). This effect appears to be antagonized by phosphorylation of Ser 253/258 (67; 68). Clearly, the effect of TF-FVIIa-induced signaling and resultant posttranslational modification of TF is an area which awaits further elucidation.

3.3. Purification, cloning and expression of FVII

The trace concentration of FVII and its sensitivity to proteolytic activation were challenges in initial attempts to isolate the protein from plasma. Bovine FVII was the first to be purified to homogeneity (20). The original purification procedure involved a multitude of steps: barium sulphate adsorption, DEAE-Sephadex adsorption, chromatography on benzamidine-agarose, heparin-agarose, and finally preparative electrophoresis. Application of a similar purification procedure with 19.5 L human plasma resulted in a yield of 2.6 mg human FVII (69). Obviously, it may not be feasible to purify FVII from plasma in all species as such an approach requires large plasma volumes. Thus, studies with FVII from smaller animal species will in practice be limited to using recombinant proteins.

Fortunately, it is now possible to isolate recombinant FVII from culture media in a two step procedure comprising anion exchange followed by immuno-affinity chromatography (70). This efficient approach to purification of human FVII relies on the existence of a specific calcium-dependent antibody. An antibody with these properties may not be initially available for the purification of FVII of non-human origin. Hence, much valuable knowledge can be gathered from early sources on the purification of bovine and human FVII.

The human FVII protein is encoded by the *F7* gene (Gene ID: 2155) located on the long arm of chromosome 13, specifically 13q34 (71). Human F7 constitutes 8 exons separated by 8 introns and spans a total of 12.8 kb pairs (72; 73). The mature human FVII mRNA transcript is encoded by exons 1 through 8 and comprises 3.1 kb (minor differences between two transcript variants); exons la and lb and part of exon 2 encode a prepro leader sequence that is removed during processing. Thus, the remainder of exon 2 and exons 3 through 8 encode the mature protein (73).

Recombinant production of FVII from various species has become possible with the increasing availability of the FVII cDNA's. The amino acid sequences of FVII from these species are aligned with the sequence of human FVII (Figure 3). Because FVII undergoes advanced posttranslational processing, (e.g. γ-carboxylation), mammalian cell systems, including the human 293 embryonic kidney (HEK 293), baby hamster kidney (BHK) and Chinese hamster ovary (CHO) cell lines, have predominantly been applied for expression of functional FVII. Additionally, functional FVII can be expressed in insect cells co-transfected with human γ –carboxylase (74). To date, cDNAs and recombinant proteins of human (72; 75), rabbit (76; 77), zebrafish (78), rat (79), murine (58; 80), and canine (59; 81) origin have been reported.

3.4. FVIIa structure and function

FVIIa belongs to the family of vitamin K-dependent coagulation proteases. Structurally, FVIIa closely resembles FIXa, and FXa. During activation the single chain FVII zymogen is activated to the two chain active serine protease, FVIIa, by proteolysis of the Arg152-Ile153 scissile bond (72). This limited proteolysis results in an active enzyme with an N-terminal light chain of 152 residues and a heavy chain of 254 residues, linked by a disulphide bridge. The N-terminal of the light chain contains a γ-carboxyglutamic acid (Gla) domain (residues 1–45) followed by two repeating copies of a 36 amino acid epidermal growth factor (EGF) like domains (residues 46–88 and 89–139 for EGF 1 and EGF 2, respectively) (73). The EGF domains are followed by a so-called connecting region that contains the activation Arg152-Ile153 cleavage site. The final 254 amino acids of the protein, i.e. residues 153–406, comprise the serine protease domain identical to the heavy chain. Notably, FVIIa remains in a zymogen-like state after activation and only becomes an efficient catalyst when associated with its protein cofactor, TF (82; 83).

3.5. Interaction of FVIIa with TF

Residues of importance for FVIIa recognition of TF have been identified in both proteins by amino acid substitution techniques (84; 85) and species comparison (see section 4). This combined with the X-ray crystallographic structure of the complex of soluble human TF and active site inhibited FVIIa (FVIIai) (86) (Figure 2)

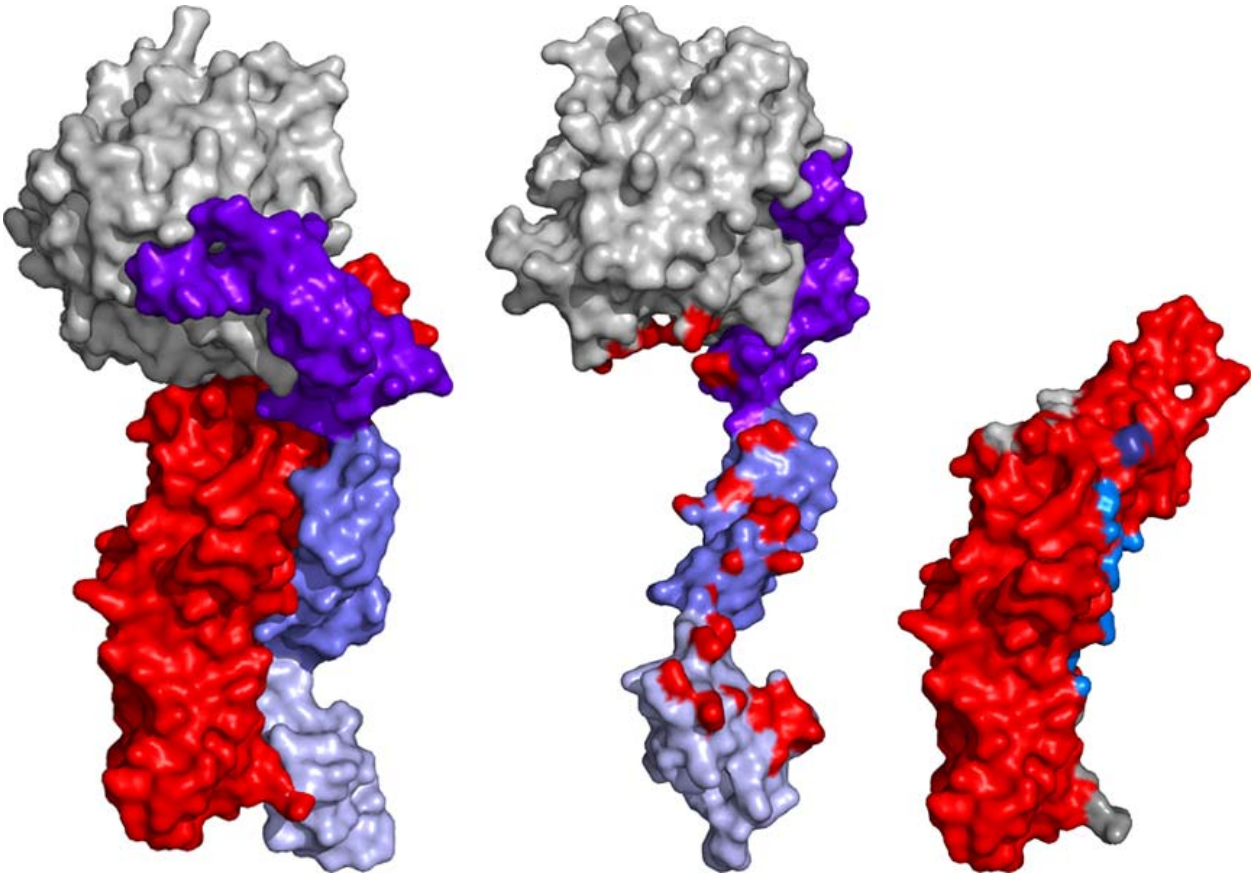

Figure 2. Model of the human TF-FVIIa complex. The model is based on the X-ray structure (PDB entry 1dan), thus it represents soluble human TF in complex with active site inhibited human FVIIa, and is shown in surface rendered representation. Loops missing in the original structure have been modelled. Left: entire TF-FVIIa complex; red, TF (residues 3-219); grey, FVIIa protease domain; dark blue, FVIIa EGF2; blue, FVIIa EGF1; light blue, FVIIa Gla-domain. Center: FVIIa alone, rotated relative to the orientation in the left figure; TF is removed from the complex and the TF-interacting surface of FVIIa is exposed. Atoms in FVIIa which are less than 3.5Å from TF are shown in red. Right: TF alone; FVIIa is removed and the FVIIa-interacting surface on TF is exposed. Atoms in TF which are less than 3.5Å from FVIIa are coloured according to their closest partner.

provide a starting-point for modeling of cross-species TF-FVIIa complexes and for the interpretation of data on TF-FVIIa interactions across species borders (Figure 3). Membrane interaction is mediated by the C-terminal portion of TF and by the FVIIa Gla domain. The orientation of the structure is likely roughly vertical to a putative membrane. Thus, in addition to assisting in facilitating TF binding, the FVIIa light chain aids in the proper positioning of the protease domain and its active site above the membrane surface (87).

3.6. Binding of human FVIIa to sTF, lipidated TF and cell surface TF

3.6.1. FVIIa binding to sTF

Binding of TF to FVIIa induces a marked enhancement of the amidolytic activity of FVIIa (83). This property was used to determine a binding equilibrium constant (K_D) of 13 nM for binding of bovine FVIIa to recombinant bovine $sTF_{(1-213)}$ (53). By competition experiments the authors also showed that the affinity for TF was increased when the active site of FVIIa was blocked as was also suggested in an earlier report by Bach *et al.* (88). K_D values in the 3 - 10 nM range were identified for the binding of human FVIIa to human $sTF_{(1-219)}$ by surface plasmon resonance (SPR) (89-92). In addition, SPR studies showed that the K_D's for binding of zymogen FVII and activated FVIIa to human sTF were comparable (93) and also confirmed that occupancy of the active site increased the affinity of FVIIa for sTF (91). The strong binding to sTF was obtained especially due to a slow dissociation of the complexes with half-lives of 16 ± 1 min for human FVIIa and 50 ± 4 min for human FFR-FVIIai, respectively (91). We have reported SPR data for murine-human and canine-human sTF-FVIIa cross-species interactions. Notably, we found that the binding of human FVIIa to immobilized mouse sTF failed to induce a detectable signal (58) whereas human FVIIa bound efficiently (K_D = 3 nM) to immobilized canine sTF (59).

3.6.2. FVIIa binding to lipidated TF and to cell surface TF

Procoagulant activity of TF-FVIIa on cells saturates at picomolar concentrations of FVIIa (94)

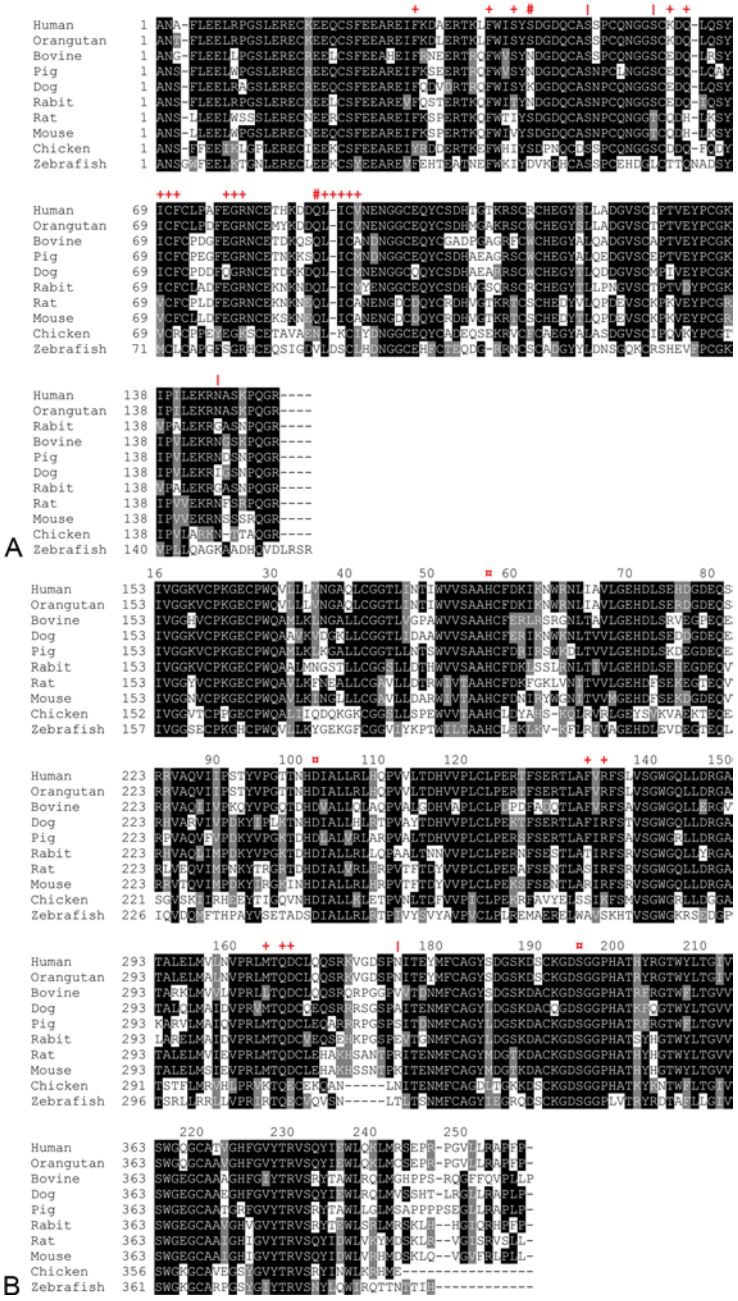

Figure 3. A. Sequence alignment of the light chain of human and animal FVIIa. The light chain consists of a Gla doman (residues 1-45) and two EGF domains (EGF1: residues 46-88; EGF2: residues 89-134), position 45 and 88 are indicated by a hash mark. Note that the major portion of the glutamic acid residues in the Gla domain have been post-translationally gamma-carboxylated. Crosses in red denote residues which are in close contact with TF as determined from the X-ray structure of the human TF-FVIIa complex (PDB entry 1dan). Vertical lines in red show potential O-glycosylation and N-glycosylation sites in human FVIIa. The alignment was performed in ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html) was used for graphics display. B. Sequence alignment of the heavy chain of human and animal FVIIa. In addition to the sequence numbering, which is based on that of human FVIIa, the chymotrypsin sequence numbering is included above the sequences. Active site residues are marked by ¤, crosses in red denote residues which are in close contact with TF as determined from the X-ray structure of the human TF-FVIIa complex (PDB entry 1dan). The vertical line in red show potential N-glycosylation site in human FVIIa. The alignment was performed in ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html) was used for graphics display.

whereas the TF-FVIIa binary complex signals efficiently at approximately 10 nM FVIIa (62; 95).

Studies with binding of ^{125}I-labled human FVIIa to human carcinoma J82 (96) and OC-2008 (94) cells reported K_D values between 0.3 nM and 12 nM with various degrees of apparent cooperatively. Recent studies with J82 (91), MDA-MB 231 (95), and WI38 (59) cells yielded K_D values close to those found for binding of human FVIIa to human sTF. Notably TF-dependent FVIIa/PAR2 signaling followed a saturation pattern similar to that obtained in ^{125}I-FVIIa binding studies. FVIIa EC_{50} values in the range 3-8 nM were obtained for TF-FVIIa-induced expression of the cytokines, IL-8, CXC1, and GM-CSF by MDA-MB-231 cells indicating that this FVIIa activity reflects its binding to the same TF cell surface pool as that determined by binding experiments (95).

Although reasonable consensus exists about binding of FVIIa to sTF and TF exposed on cells this is not the case with binding of FVIIa to lipidated TF as indicated by the wide range of dissociation equilibrium constants reported in the literature. Early binding studies on full length relipidated TF applied bovine TF and FVIIa. TF was incorporated in large lipid vesicles of various PC/PS compositions and binding was measured by equilibrium sedimentation, FX activation activity and fluorescence anisotropy (88; 97). An apparent K_D value ~ 7 pM was obtained by fluorescence anisotropy. K_D values determined by TF stimulation of FVIIa amidolytic activity ranged from ~ 40 to 300 pM for TF in PS/PC and 3 nM for TF in PC vesicles (98; 99). A recent reinvestigation of the binding to relipidated TF using SPR with TF embedded in a PS/PC phospholipid bilayer coated onto a lipophilic chip (100). In this study, the K_D for the binding of PS/PC-embedded TF was approximately 50 pM. This high affinity was primarily the result of a slow dissociation of FVIIa from PS/PC-embedded TF. This makes it difficult to establish true equilibrium condition and the authors suggested that this complication together with differences in lipid composition and lipidation techniques may account for the wide variation among apparent K_D values reported in earlier studies. Thus, results may vary quite significantly not only between species, but also within the same species. It is therefore crucial to conduct species comparisons under as identical assay conditions as possible.

The lower K_D determined for lipidated TF on PS/PC bilayer coincide with estimates of FVIIa EC_{50} values based on TF-FVIIa mediated activation of FX or FIX whereas they disagree with estimates of K_D's for FVIIa binding to cell surface TF and SPR data. The reason for this is not clear. The dilemma was further illuminated by studies which compared the time courses for establishing maximal FX activation activity and ^{125}I-FVIIa binding (94). ^{125}I-FVIIa binding was saturated very slowly in contrast to a rapid (half maximal saturation reached within one min) saturation of another pool of TF responsible for FX activation.

The question remains whether the picomolar EC_{50} of FVIIa for TF-stimulated FX activation reflects binding to a small TF pool with high affinity for FVIIa which support FX activation in coexistence with a larger "cryptic" TF pool of lower affinity without the capacity to mediate FX activation. The EC_{50} of FVIIa for the FX activation on cells varies with the cell line but is generally in the picomolar range (~ 40 pM) about two orders of magnitude lover than the K_D values determined in binding experiments (95). In addition to species compatibility, cross-species studies have to include this divergence in the assessment of experimental data, and as discussed below also when evaluating PT data.

3.7. Effect of FFR-FVIIai on PT in human plasma and plasmas from other species

The prothrombin clotting time (PT) obtained by mixing of calcium, thromboplastin and plasmas from various species served as a useful first indicator of TF-FVII species compatibility. The study by Janson *et al.* (24) is a frequently cited source of data on inter-species PT. However, certain reservations are in place before jumping to conclusions about species compatibility from such data. Firstly, TF-dependent initiation of clotting should predominate over contact activation. Secondly, the crucial reaction with regard to species specificity might be activation of FVII by FXa as well as activation of FX by FVIIa. Finally, species thromboplastins might contain FVII/FVIIa trace impurities especially when prepared from lungs or placentas. These impurities may have adverse effects on e.g. assay sensitivity to plasma FVII (101).

With these reservations in mind we have applied the PT assay to investigate the species compatibility between human FVIIa and TF from a number of species. A variation of the PT assay with species thromboplastin applied in plasma from the same species was developed for this purpose. To estimate binding of human FVIIa to TF from various species we measured the competition in the PT assay of various concentrations of active site blocked FVIIa (FFR-FVIIai) and plotted the data as reciprocal PT (i.e. 1/PT) versus the logarithmic FFR-FVIIai concentration (log [FFR-FVIIai]). This presentation has the advantage that 1/PT has the dimension, s^{-1}, which alludes to a "clotting rate". Hence, it is possible to calculate an IC_{50} from the resulting S-shaped curve (Table 1).

Incubation and mixing procedures are crucial in this PT assay as illustrated in the example in with human lipidated TF (Innovin®) in human plasma (Figure 4). In procedure "A", TF, Ca^{2+}, and FFR-FVIIai are incubated together before mixing with plasma. Inhibition by FFR-FVIIai is very efficient (IC_{50} = 15 pM) with this procedure compared to procedures "B" (IC_{50} = 22.5 nM) and "C" (IC_{50} >> 1000 nM).

These diverging FFR-FVIIai inhibitor profiles are accounted for by the character of TF-FVIIa complex formation. A very slow dissociation of FFR-FVIIai or FVIIa from their respective complexes with TF provides a plausible explanation for the results in procedures "A" and "C". Dissociation of TF complexes with half-lives close to one hour (91) or slower (92) are much too slow to occur in the time frame of the PT assay. Clotting activity with

Table 1. Inhibition of the prothrombin time (PT) by human FFR-FVIIai

Species	IC_{50}, nM *	PT, sec #
Man	16.2	13.9
Mouse	116.3	107.9
Rat	26.5	54.1
Rabbit	15.2	15.2
Pig	23.6	29.8

Homologous plasma and thromboplastin was used in each combination, e.g. human plasma + human thromboplastin. *Bregengaard and Petersen, previously unpublished results. # Data from Janson et al., 1984 (24). Refer to the main text and the cited reference for a detailed description of the PT methods.

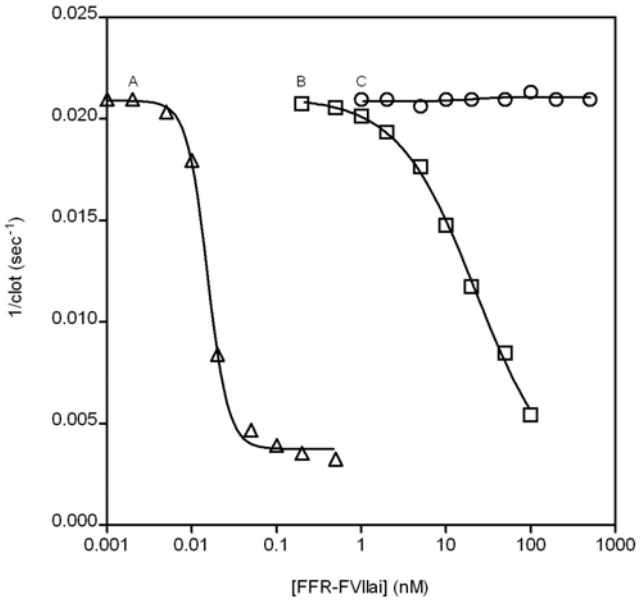

Figure 4. Prothrombin time (PT) measured in human plasma with three different mixing procedures. "A" 23 pM lipidated human TF (Innovin®) in 20 mM Hepes, 140 mM NaCl, 1 mg/ml BSA, pH 7.4 with 20 mM $CaCl_2$ was incubated with various concentrations (0.002 nM – 0.2 nM) of FFR-FVIIai for 15 min at 25°C before 75 µl of this blend was mixed with 75 µl normal citrated plasma two-fold diluted in 20 mM Hepes, 140 mM NaCl, 1 mg/ml BSA, pH 7.4. "B" 23 pM lipidated TF (Innovin®) in 20 mM Hepes, 140 mM NaCl, 1 mg/ml BSA, pH 7.4 with 25 mM $CaCl_2$ was incubated for 15 min at 25°C before 75 µl of this blend was mixed with 75 µl two fold diluted plasma containing various concentrations (0.5 nM -50 nM) of FFR-FVIIai. "C" 23 pM lipidated human TF (Innovin®) in 50 mM Tris, 100 mM NaCl, 1% BSA, pH 7.4 containing 25 mM $CaCl_2$ and 10 nM FVIIa was incubated for 15 min at 25°C before 50 µl of this blend was mixed with 100 µl two fold diluted FVII-depleted plasma containing various concentrations (2.0 – 500 nM) of FFR-FVIIai. The time to fibrin clot formation was measured on an ACL 300 Coagulation Analyzer (ILS Laboratories, Italy).

procedure "A" is therefore likely to reflect an established equilibrium between FFR-FVIIai and TF in the pre-incubation mixture. The results with procedure "C" simply reflect that dissociation of FVIIa from the preformed TF-FVIIa complex is too slow for any significant replacement of FVIIa with FFR-FVIIai in the time frame of the PT assay. Finally with procedure "B", in the absence of pre-formed TF-FVIIa complex, the results are expected to reflect relative on rates for FVIIa and FFR-FVIIai when they compete for TF upon mixing. FFR-FVIIai reacts approximately 1.5 fold faster with TF than FVIIa (91). Thus, with a plasma concentration of 5 nM FVII in the final mixture one would expect an IC_{50} of 3.3 nM. The actual IC_{50} found (Table 1) is somewhat higher.

The data (Table 1) summarizes the IC_{50} values obtained when homologous systems of thromboplastin and plasma from different species are mixed according to procedure "B" in the presence of various concentrations of the active site inhibited human FVIIa competitor. The IC_{50} value gives an estimate of how efficient human FFR-FVIIai competes for the TF from a certain species. As illustrated by the experiments in Figure 3, the on rates for binding of FFR-FVIIai to species TF are likely to impact the IC_{50} values obtained by the procedure applied. The IC_{50} values complement "classical" PT values (24) and are assumed to correlate inversely with the procoagulant effect of human FVIIa in species plasma.

3.8. Species compatibility with TF-FVIIa substrates, FX, FIX, and PARs

Measurements of the inhibitory effect of FFR-FVIIai in animal plasma stimulated with homologous thromboplastin provide a ranking of the affinity of human FFR-FVIIai to TF from the animal species. Presumably, such data also gives clues to the relative affinity of human

FVIIa for the species specific TF. Obviously, it is, however, not possible from such experiments to draw firm conclusions about the capability of the animal-human TF-FVIIa complex to trigger coagulation in animal plasma or to activate FX and FIX from the animal species. Limited information is available from PT experiments which employed mixing of homologous thromboplastins and plasmas in the presence of various concentrations of human FVIIa (Bregengaard, unpublished results). These data may throw some light on this question. It was observed that increasing concentrations of human FVIIa induced similar shortenings of PT in human, baboon, pig, dog, rat, and goat plasmas. Compared to the effect in human plasma, human FVIIa was less efficient in mouse plasma and more efficient in rabbit plasma.

The interaction of TF-FVIIa with PAR2 appears to be largely independent on exosite interactions and depend strongly on primary interactions between the FVIIa active site and the PAR2 cleavage site (31). This may suggest a low cross species compatibility for the TF-FVIIa interaction with PAR2 as also confirmed by a very low efficiency of murine FVIIa-induced PAR2 signaling in human carcinoma MDA-MB 231 cells in spite of an efficient binding of murine FVIIa to human TF (102).

4. SELECTED HUMAN-ANIMAL CROSS-SPECIES TF AND FVII COMPATIBILITIES

4.1. Bovine TF and FVIIa

Bovine TF and FVII served as important model molecules in the development of methods for purification of human TF and FVII because large amounts of tissue (e.g. brain) and plasma could be readily obtained.

Bovine-bovine, human-bovine, bovine-rabbit, and bovine-canine TF-FVIIa interactions have been partially explored for different purposes. For example, bovine TF has been used in *in vitro* assays together with bovine (103) or human FVII/FVIIa (e.g. 96; 104).

Notably, Wildgoose and covorkers showed with competition binding experiments that human TF binds bovine FVII with decreased affinity indicating structural incompatibility in regions of TF- FVIIa interaction (96). Based on sequence differences between bovine and human FVII, three peptides were rationally designed to further explore the interaction of human FVII with human TF. One of the three peptides designed affected the interaction of FVIIa with cell-surface human TF as well as FVIIa mediated FX activation (96). However, our inspection of the X-ray crystallographic structure of the human TF-FVIIa complex (86) showed that this peptide corresponds to the so-called 60-loop which borders one part of FVIIa's active site cleft. Hence, its influence on FX activation may be due to competition with the substrate by binding to FX's activation peptide. Other parts of the interface may be responsible for the reduced binding of human TF to bovine FVIIa. In particular, side chains in the binding region between EGF1 of FVIIa and TF seem important. In human TF, Glu-130 is likely in close proximity to Glu-62 in bovine FVII which during complex formation may result in electrostatic repulsion and explain a low affinity. Notably, the combination of residue Ala-130 in bovine TF and Lys-62 in human FVII will not cause repulsion as suggested for the human-bovine TF-FVIIa complex.

Bovine TF has also been applied *in vivo* to address the possibility of using TF as a FVIII bypassing agent in hemophilia A (105). Specifically, bovine TF was dosed to rabbits with antibody induced hemophilia A and to dogs with congenital hemophilia A. Although the TF infusions seemed to improve the bleeding phenotype of the hemophilic animals, correct interpretation of data from such models was, and is still, a difficult task. The purity of the TF preparation was relatively low, interactions between bovine TF and rabbit or canine FVII are poorly characterized and no control experiments including e.g. an inhibitory anti-bovine TF antibody were conducted.

4.2. Canine TF and FVIIa

Canine TF binds human and canine FVIIa with comparable affinities suggesting that human TF-FVIIa interactions can be reliably recapitulated when human recombinant FVIIa is applied in canine models (59). *In vivo* support hereof was provided by a study showing that human recombinant FVIIa worked as an efficient replacement therapy in congenitally FVII deficient Beagle dogs (106). Contrasting the favorable compatibility of canine TF with human FVIIa, the binding of canine FVIIa to human TF was found to be decreased and to result in a decreased pro-coagulant activity of the complex even with canine FX as the substrate (59). Compared to the relatively straight forward compatibility aspects in non-clinical research with canine models, the asymmetry across the canine-human species border gives rise to serious challenges in veterinary medicine, especially when human TF is used in diagnostic assays (107-109).

The asymmetry of the heterologous human-canine/canine-human complexes may be accounted for by our modeling based on the human TF-FVIIa complex (86). This suggest that unfavorable interactions may exist between Glu-130 in human TF and Glu-62 in canine FVII resulting in a similar repulsion as suggested above to be responsible for the low affinity of the human-bovine TF-FVII complex. Other interactions may be of importance as well, such as putative unfavorable contacts formed at the interface between the C-terminal region of human TF and the heavy chain of canine FVIIa. Here, Glu-95 in human TF may exert an electrostatic repulsion on Glu-170 in canine FVIIa. This potential clash is absent in the heterologous canine-human TF-FVIIa and in the autologous canine TF-FVIIa complexes because of the presence of the uncharged Thr-130 in canine TF.

Madin Derby canine kidney (MDCK) epithelial cells constitutively express canine TF. The MDCK cell line has been used by us and others to explore TF sorting in cells (110; 111), TF-FVIIa mediated cell signaling (61; 112) as well as human-canine cross-species interactions of TF and FVIIa (59).

Hemophilic dogs are important pre-clinical animal models and served a pivotal role in the discovery of

recombinant human FVIIa as a bypassing therapy for inhibitor complicated hemophilia A and B (113). Hemophilic dogs are uniquely "human-like" in their disease phenotype with spontaneous bleeding episodes and in their response to human recombinant FVIIa in clinically relevant doses (114). Studies with FVII deficient Beagles (81; 115; 116) have provided valuable information, e.g. about the pharmacokinetics (117) and pharmacodynamics (106) of FVII and FVIIa. The dog studies proved to closely recapitulate the human setting (118; 119). Such information together with knowledge on cross-species TF-FVIIa interactions is important for optimal interpretation of data obtained in studies with administration of human FVIIa to dogs (106; 113; 120; 121) and also for interpretation and potential translation of gene therapy-based approaches with FVIIa expression (122).

4.3. Rabbit TF and FVIIa

The rabbit is an important experimental animal model. Accordingly, cross-species rabbit TF-FVIIa interactions, e.g. human-rabbit (24) and bovine-rabbit (105), have been explored. Owing to relative ease of access to rabbit brains as a source of lipidated TF, rabbit TF has been used for decades as an initiator of coagulation in assays measuring the pro-coagulant activity of human FVII(a). Generally, rabbit TF is considered an excellent match for human FVIIa. Notably, similarity between human and rabbit TF allowed partial purification of rabbit TF employing an antihuman TF antibody in an immunoaffinity process (123).

Characterization of recombinant rabbit FVII showed that rabbit- and human TF interacted favorably with rabbit FVIIa (77). In addition, human TF had previously been observed to work as a more potent inducer of clot formation in rabbit plasma than in human plasma (24). These observations led Williamson and colleagues (124) to hypothsize that the observed discrepancy in FVII clotting activities might reside in the five amino acid residue differences between the rabbit and human FVII EGF1 domains. To address this hypothesis they 'rabbitized' the human FVII EGF1 domain either by exchanging the entire EGF1 domain creating a human-rabbit FVII chimera or by S53N, K62E, P74A, A75D or T83K single amino acid substitutions. Indeed, 'rabbitization' of human FVII did result in human FVII variants with increased TF binding and increased procoagulant activity.

The increased activity of 'rabbitized' human FVII is in apparent conflict with the hypothesis that we offered in section 4.1 and section 4.2 to explain the unfavorable interactions in heterologous complexes of human TF with bovine and canine FVIIa. This applies especially to the K62E mutation in FVII which, according to our simplified hypothesis, should result in a repulsive interaction with Glu-130 in human TF. It is possible that the Lys to Glu mutation at position 62 in human FVIIa changes the local interface. This may result in new TF-FVIIa interactions which are not immediately predictable from available X-ray structures. The study by Williamson and colleagues illustrates the importance of in depth explorations of species-differences and how such data might leverage the understanding of human TF-FVIIa interactions. Clearly, the current *in silico* models of TF-FVIIa complexes needs further sophistication to account for all available empirical data on species compatibility.

4.4. Murine TF and FVIIa

Early observations indicated that mouse thromboplastin was inefficient in inducing clotting of human plasma (24). The human-mouse incompatibility was further elucidated in a later study where the stimulation by human-mouse chimeras of the extra-cellular TF domain was studied with human recombinant FVIIa and partly purified mouse FVII (125). It was concluded from this study that the region (residues 40-105) in human TF coded by exon 3 was essential for the interaction with human FVIIa and could not be replaced with the corresponding sequence from mouse TF. These data were corroborated and extended by results with recombinantly produced mouse TF and FVIIa (58). This study indicated that mouse FVIIa interacts efficiently with human TF and FX. Data also showed that the heterologous complex between human sTF and mouse FVIIa was more efficient in activating human FX than the corresponding autologous complex with human FVIIa. The most efficient activation of human FX was obtained with the autologous mouse-mouse sTF-FVIIa complex, whereas the heterologous mouse-human sTF-FVIIa complex was essentially inactive. The observed stimulation of mouse FVIIa with human as well as mouse TF, and stimulation of human FVIIa only with human TF, and not with mouse TF again substantiated the notion of a unidirectional human-mouse asymmetry and incompatibility.

The most notable sequence difference between murine and human TF is a large loop (residues 83 to 94) inserted in murine TF (Figure 1 B). According to our modeling, this loop is in close vicinity to the protease interaction region. It contains 6 extra residues that might prevent a close contact with the protease domain of human FVIIa and most likely accounts for the lack of affinity for human FVIIa.

Murine models are attractive for detailed *in vivo* studies on TF and FVIIa for obvious reasons. The mice are small, they are easily bred and can be genetically engineered to create knock-out and transgene animals. However, the physiological characteristics of mouse TF made it difficult to apply a direct approach along these lines in studies of TF biology and TF-FVII interactions using human FVII or FVIIa in mice. Thus, human-mouse incompatibility poses an obstacle in studies of TF biology and TF-FVII interactions in mice (24; 58; 125). Furthermore, it was discovered that targeted disruption of the murine TF gene was incompatible with normal embryonic development such that TF-null fetuses died between gestation day 9.5 and 10.5 due to impaired vitelline vessel formation and fatal embryonic bleeding events (126-128). This suggested that TF played a pivotal role in angiogenesis but obviously prevented studies in adult TF knock-out mice.

Due to its incompatibility with mouse TF, human FVIIa can not be used to address general questions about TF-FVIIa interactions in murine models unless the mice are

manipulated to express human TF. Consequently, mice that express human TF in place of murine TF have been developed. To date, three such strains, i.e. the low-TF strain (129), the human chromosome vector (HCV) strain (130), and the TF knock-in (TFKI) strain (131), have successfully been created. All three strains have provided valuable insights into TF-related processes (132). Although the low-TF and HCV mice developed to term, they had a significantly shorter life span and exhibited hemorrhage and fibrosis in the heart. This was probably due to the fact that TF expression was subnormal at approximately one and twenty percent of normal in the low-TF and HCV mice, respectively. Conversely, TFKI mice exhibit normal hemostasis and 'normal' levels of human TF in all tissues, i.e. TF levels that are similar to those of murine TF in wild-type mice. The TFKI mice develop normally and do not show signs of cardiac hemorrhage and fibrosis, unless challenged with an inhibitory antihuman TF antibody (131). Thus, TFKI mice represent true "TF-humanized" mice and can be used to screen the effects of human TF interacting drugs in mice (133).

Mice lacking FVII would, in analogy to TF deficient mice, be valuable in studies exploring biological and pharmaceutical effects of FVII and FVIIa. However, mice completely deficient in FVII suffer fatal perinatal bleeding after having developed normally *in utero* (134). Thus, completely FVII deficient mice are unavailable for pharmacological studies. As an alternative, mice with severe but incomplete FVII deficiency have been created (135). In addition to being FVII deficient these mice did, however, also exhibit relative deficiencies in coagulation factors VIII, IX, XI, and XII. It may therefore be difficult to completely dissect the role of FVII in these mice and improved models are desirable.

Subcutaneous xenograft mouse models that use human or murine cultured tumor cells injected into the subcutaneous tissue of immunodeficient mice exist. Such models are popular in studies on the role of TF in tumor biology (130). They possess a number of potential pitfalls and possibilities in relation to species compatibility. Mouse fibrosarcoma cells overexpressing TF were applied in the first study xenograft mouse model to examine the effect of TF expression on tumor development (136). Although mouse FVIIa binds with high affinity to human TF it is e.g. a very inefficient inducer of human PAR2 signaling (102). Of special interest in relation to species compatibility is the application of xenograph models in studies on the role of tumor-derived versus host-derived TF in tumor progression (130). In this kind of studies it is possible to exploit the species differences between human and murine TF with various combinations of mouse strains, human/murine tumor cell lines, specific antibodies as well as with human and murine FVIIa, and FFR-FVIIai.

Accumulating evidence that TF is expressed on endothelial cells on blood vessels associated with solid tumors but not on normal vessels has spurred an interest in TF as a target for cancer treatment (137; 138). By conjugating potent drugs to FVII, this targeted drug delivery system has the potential to enhance therapeutic efficacy, while reducing toxic side effects. This principle was tested in a study where a synthetic curcumin analogue 'EF24' was coupled to the active site of human FVIIa. Compared with un-targeted EF24, the TF-targeted molecule induced apoptosis in tumor cells and significantly reduced tumor size in human breast cancer xenografts in athymic nude mice (139). A recent study described the application of a murine FVII variant (K341A) covalently coupled to a photosensitive drug (verteporfin) for this purpose. Mouse breast cancer EMT6 cells were injected to establish the subcutaneous xenograft model, and targeting tumor cells with the mouse specific FVII conjugate was shown to improve the specificity and efficiency of the photodynamic therapy in this model (140).

Pharmacological intervention with recombinant human FVIIa in murine hemophilia models (141-144) showed that surprisingly high dose of FVIIa was required for efficient hemostasis (145; 146). In the murine tail bleeding model the dosage of human FVIIa required to obtain hemostasis in hemophilia A mice was approximately one hundred fold higher compared with the dose that has been proven clinically efficacious in human hemophilia patients. The reason for this difference in dosing requirements across the human-murine species border is a matter of controversy. It is, on one hand, possible to argue that the decreased compatibility of murine TF with human FVIIa may explain the high dose requirement. This is in accordance with in vitro studies which suggest i) an absolute requirement for TF for the by-passing action of FVIIa in hemophilia, and ii) that pharmacological concentrations of FVIIa is needed to compete with endogenous zymogen FVII for binding to TF (147-149). The by-passing function of FVIIa was on the other hand found by others to be TF-independent, just as the need for supra physiological concentrations of FVIIa was suggested to be caused by its low affinity for surface exposed phospholipids (29; 150). The FVIIa variant NN1731 (146; 151; 152) exhibits markedly increased activity in absence of TF (153). When bound to TF NN1731 exhibits an activity comparable to that of wild type FVIIa; and compared with FVIIa it also binds with similar affinities to TF and phospholipids. The fact that NN1731 is a more potent bypassing agent than FVIIa seems to argue in favor of the TF-independent mechanism. So far, the mechanism for the by-passing activity of recombinant human FVIIa remains unresolved, and so do the related mouse-human TF-FVIIa cross-species phenomena.

4.5. Rat TF and FVIIa

Rat TF as well as human and rat FVIIa have been applied in rats to address various aspects of TF-FVIIa interactions *in vitro* (154) and *in vivo* (155; 156). Recombinant rat FVII was successfully produced employing the HEK293 cell line (79). In addition soluble rat TF was produced in *E.coli* and SPR data on rat-human TF-FVIIa cross-species compatibility were reported (157). The SPR experiments were, however, performed at very high concentrations of proteins which make the results difficult to interpret. Hence, further exploration of rat-human cross-species TF-FVIIa interactions is warranted.

4.6. Zebrafish TF and FVIIa

Both TF and FVII have been identified in tissues from zebrafish (78; 158). This narrows the evolutionary window for development of the vertebrate coagulation cascade (> 430 million years). Molecular evolution of the vertebrate blood coagulation system was recently further enlightened by a study comprising cloning of five K vitamin-dependent proteases (including FVII) from puffer fish and chicken (159). The interspecies interactions of human-zebrafish, and rabbit-zebrafish TF-FVIIa have been compared to intraspecies zebrafish TF-FVIIa interactions (78). Notably, zebrafish TF-FVIIa interactions showed a significant degree of species specificity as demonstrated by the lack of response in zebrafish plasma to human or rabbit TF in the form of crude tissue thromboplastin (160).

5. PERSPECTIVES

Complex formation between TF and FVIIa plays a crucial role in a number of biological activities including coagulation, inflammation, tissue repair, and angiogenesis. These are activities that are implicated in the pathogenesis of serious diseases, e.g. hemophilia, intravascular coagulation, and cancer. Optimal use of animal models for studying the pathology of these diseases requires a detailed knowledge about species TF-FVIIa interactions in the specific model applied.

The present review references a subpopulation of the large body of literature in which species borders are crossed in relation to TF and FVIIa. Due to lack of species specific reagents, many studies have historically used a variety of human and non-human reagents; many of the studies have only indirectly – if at all – assessed the question about TF-FVIIa compatibility across the species borders passed. The result is a diffuse picture with information scattered on the many faces of TF and FVIIa biology. Recent advances in recombinant technology offer means to rationally probe TF and FVIIa interactions across species borders (58; 59; 124). A starting point could be to 'revisit' historically valuable studies e.g. that of Janson and colleagues (24), using new recombinant reagents such as TF, FVIIa, and active site inhibited FVIIa. Hopefully, additional reports specifically targeting cross-species interactions of TF with FVIIa will become available.

Computational modeling of the TF-FVIIa complex can be used to inspire new studies and also to interpret results obtained in the laboratory. At present, *in silico* exploration of TF and FVIIa interactions is based primarily on the crystal structure of active site inhibited human FVIIa in complex with soluble human TF (86). This potentially limits the extrapolative power of the currently available modeling approaches. Notably, based on inspection of the modeled human-bovine and human-canine TF-FVIIa complexes, we proposed that the decrease in affinity of human TF for bovine and canine relative to human FVIIa may partially be explained by electrostatic repulsion of human TF by Glu-62 in the FVIIa molecule from these two species. However, this hypothesis seems in apparent conflict with the results obtained with a 'rabbitized' human FVIIa variant in which residue Lys-62 in human FVII was replaced by a Glu residue (124). Rather than reducing the affinity, the substitution resulted in a three fold increased affinity of the human FVIIa K62E variant for human TF. It is clear that the present conception of the TF-FVIIa complex is far from complete and that a future resolution of the crystal structures of non-human or human-animal TF-FVIIa complexes may advance our insights and sophisticate *in silico* modeling of species compatibility.

Species differences can also be utilized in diagnostic medicine. For example, the human FVII variant "Padua", i.e. FVII R304Q, which causes a qualitative – but not quantitative – FVII deficiency in the patient, can be diagnosed by the use of TF from ox and man or rabbit. Specifically, the procoagulant activity of FVII Padua, as assessed in the PT assay, is normal when the TF source is bovine (100 % relative to a normal pooled human plasma [NHP]), slightly decreased with porcine TF (65 % relative to NHP), and significantly decreased with human (35 % relative to NHP) or rabbit (10 % relative to NHP) TF (161).

As the present review illustrates, knowledge about intra and inter-species interactions between TF and FVIIa has improved significantly over the past three decades. However, it also clearly illustrates and emphasizes that there is potentially much still to be learned by a more stringent exploration of TF-FVIIa interactions with proteins from non-human sources and from cross-species studies.

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