The third Trp-Lys-Ser (WKS) tripeptide motif in Tissue Factor is associated with a function site

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The tripeptide sequence Trp-Lys-Ser (WKS) is repeated three times in the extracellular ligand binding domain of human Tissue Factor (TF). Using site-directed mutagenesis, we replaced each of the WKS motifs in human TF by Arg-Lys-Gly (RKG), the least conserved replacement for the motif found in murine TF. This substitution in the first repeat W¹⁴KS, as well as a Trp¹⁴ → Arg substitution, resulted in a structurally altered protein, whereas a conservative hydrophobic Trp¹⁴ → Phe substitution resulted in a functionally normal protein. This suggests that Trp¹⁴ may contribute to a hydrophobic core rather than involvement of this motif in function. Replacement of the W⁴⁵KS and W¹⁵⁸KS motifs was associated with no detectable structural alterations; however, function was diminished with the RKG replacement of the third repeat. Mutant proteins with Lys¹⁵⁹ → Ala and Tyr¹⁵⁷ → Ala substitutions exhibited loss of function, whereas Tyr¹⁵⁶ → Ala and Ser¹⁶⁰ → Ala substitutions flanking the YWK sequence resulted in functional proteins. These data demonstrate that the W¹⁵⁸KS motif in human TF is associated with a functional site and identify Lys¹⁵⁹ in this motif as a functionally important residue.

INTRODUCTION

Cellular initiation of coagulation is mediated by Tissue Factor (TF), a cell-surface receptor and functional cofactor for the plasma serine proteinase VIIa [1,2]. There is no close sequence similarity between TF and other proteins, although structural similarity of the TF extracellular domain to the interferon γ -receptors and, more distantly, to the cytokine receptor family of proteins, has been suggested [3,4]. The sequence WKS was found three times in the mature human TF protein, a statistically improbable occurrence, and it was speculated that the tripeptide motif might have a functional significance [5]. Each of these motifs in human TF is encoded by a separate exon [6], and all WKS motifs are localized in the TF extracellular domain, which provides critical protein–protein interactions for binding of the ligand VIIa [7,8]. The present study uses site-directed mutagenesis to evaluate the functional significance of this motif at position 14–16 (encoded by exon 2), 45–47 (encoded by exon 3), and 158–160 (encoded by exon 4) [6]. This approach indicates a structural importance of Trp¹⁴ in the first repeat and provides evidence that the third repeat of the tripeptide motif is associated with a functional site in the TF extracellular domain.

MATERIALS AND METHODS

Site-directed mutagenesis and expression of TF in eukaryotic cells

The TF coding sequence inserted in the expression vector CDM8 [9,10] was modified using oligonucleotide-directed mutagenesis as described by Kunkel [11] with the modifications as previously reported [12]. Typically, oligonucleotides were designed which introduced the desired amino acid substitution as well as generated or destroyed restriction-endonuclease cleavage sites. The mutations were confirmed by dideoxynucleotide sequencing with Sequenase (United States Biochemical). Chinese-hamster ovary cells were transfected with mutated plasmid DNA

using the calcium phosphate method [13], followed by cell harvest after 72 h. Stable cell lines expressing certain TF mutants were generated and selected as described [12].

Functional characterization of the TF mutants

Transfected cells were harvested after detachment with 10 mM-EDTA and were resuspended in a small volume after repeated washes in Tris-buffered saline (TBS; 20 mM-Tris/150 mM-NaCl, pH 7.4). Portions containing a defined number of cells were sedimented, followed by removal of the supernatant for storage at -70°C . These cell pellets were either lysed with 2% Triton X-100 in TBS for antigen determination by e.l.i.s.a. [7] using two monoclonal antibodies to non-overlapping epitopes [14] or with 15 mM-octyl β -D-glucopyranoside for determination of functional activity in a one-stage clotting assay, as previously described [15]. Flow cytometry to evaluate monoclonal-antibody epitope expression and Western-blot analysis to reveal TF protein from transient transfection experiments were performed as previously described [12].

RESULTS AND DISCUSSION

Frequency of the WKS tripeptide sequence

The tripeptide motif WKS is found three times in TF. The motif was infrequent in the 1986 protein database [5]; however, random database search of 26 798 protein sequences found in the 1991 update of the Protein Identification Resource (PIR 27) from the National Biomedical Research Foundation suggests that the WKS sequence does not occur at a lower frequency than other possible combinations of the amino acids Trp, Lys and Ser. The number of proteins with the WKS motif is 362 (1.4%) in comparison to the other possible tripeptide permutations, which are SKW (235), KWS (248), WSK (329), SWK (365), or KSW (256). However, on the basis of this 1.4% frequency, random occurrence of WKS three times in a single protein has a probability of 2.5×10^{-6} .

Sequence comparison of human, rabbit and murine TF (Table 1) demonstrates a high degree of overall sequence conservation

Abbreviation used: TF, Tissue Factor.

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Table 1. Sequence conservation of the WKS motif in TF from human, mouse and rabbit

The three repeats of the WKS motif in human TF are found in three different exons. Substitutions for the three repeats found in the mouse and rabbit TF sequence are given. The WKS motif and substitutions in the other species are indicated in **bold** letters; adjacent residues are also shown. The one-letter code is used for the amino acid residues.

	Repeat I (exon 2)	Repeat II (exon 3)	Repeat III (exon 4)
Human	LTW K STN	GDW K SKC	YYW K SSS
Rabbit	LTW K STN	ENW K SKC	YYW K ASS
Mouse	LTW I STD	RNW K NKC	TYR K GSS

between the three species as well as partial conservation in the WKS motif [16]. The first repeat of the motif is identical between rabbit and human, which are functionally most comparable, whereas murine TF has a non-conservative Lys¹⁵ → Ile substitution. In the second repeat, only the murine TF sequence, which is functionally less active [17], shows a mismatch in the third position (Ser⁴⁷ → Asn). Multiple differences are found in the third repeat of the motif. The rabbit TF sequence has conservative Lys¹⁵⁹ → Arg and Ser¹⁶⁰ → Ala substitutions. The murine sequence has Trp¹⁵⁸ → Arg and Ser¹⁶⁰ → Gly substitutions, exhibiting the lowest degree of sequence conservation of

this motif. On the basis of this species comparison, a functional significance of a particular WKS motif cannot be inferred, and we addressed the question whether this tripeptide motif is functionally important by site-directed mutagenesis.

RKG substitutions for the WKS motif

We chose to replace each of the three WKS motifs in human TF with RKG, the least conserved substitution found in murine TF. Assuming comparable global folding of the TF protein of each of the three species, this substitution should not introduce alteration of overall protein structure in its location at the third position, W¹⁵⁸KS. However, if WKS has important residues for binding human Factor VIIa and inducing function [7], the RKG substitution may allow detection of functional requirement for the motif. Replacement of W¹⁴KS by RKG and transient expression resulted in marked decrease of biosynthesis of the mutant protein. It was not detectable by e.l.i.s.a. and only as a trace band by Western-blot analysis using a polyclonal monospecific antibody which was used to avoid false negative results due to selective loss of epitopes. This is consistent with severe alteration of the global fold of the mutant TF and lack of normal cellular processing.

The substitution of the W⁴⁵KS motif by RKG resulted in a level of expression of functional protein comparable to wild-type TF. However, no antigen was detectable by e.l.i.s.a, since the epitope of the capture antibody (TF9-6B4), which has recognition sites within residues 40–83 [14], was lost (Fig. 1). A stable cell line which expressed high levels of the mutant TF (TF_{R45G47}) was established, and the level of expression was estimated to be

Fig. 1. Expression of monoclonal-antibody epitopes on mutant TF

Cells expressing wild-type TF, TF_{R45G47}, TF_{R158G160} or TF_{A159} were allowed to react with the indicated monoclonal antibody (TF8-5G9, TF9-6B4, TF9-9C3) to non-overlapping epitopes, followed by detection with secondary, fluorescein-conjugated, antibody. Analysis by flow cytometry is depicted with the cell numbers plotted versus the relative fluorescence.

Table 2. Functional characterization of TF mutants

Cells expressing the indicated TF mutants were obtained from transient transfection experiments or represent stable cell lines (marked by *). The antigen content of the cell lysate determined by e.l.i.s.a. (concentration) is given as an indicator for levels of cellular expression. The specific functional activity was determined in a one-stage clotting assay, based on antigen levels determined by e.l.i.s.a. Normal *N*-linked glycosylation of the mutant protein is indicated by '+', based on mobility on SDS/PAGE and detection by Western-blot analysis. ND, not detectable; no reactivity indicates epitope loss for the capture antibody.

TF mutant	Specific activity		Concentration (ng/10 ⁶ cells)	<i>n</i>	Glycosylation
	(munits/ng)	(% of wild-type)			
TF	298 ± 87	100	50 ± 3	6	+
TF _{R14G16}	ND		ND	3	
TF _{R14}	ND		ND	3	
TF _{R14I15}	ND		ND	3	
TF _{F14}	265 ± 96	89	32 ± 2	4	+
TF _{A15}	178 ± 128	60	36 ± 2	4	+
TF _{R45G47} *		90	No reactivity	3	+
TF _{R158G160} *	41 ± 8	14	41 ± 3	3	+
TF _{A156} *	191 ± 53	64	37 ± 16	3	+
TF _{A157} *	39 ± 14	13	39 ± 15	3	+
TF _{F157}	372 ± 38	125	10 ± 1	4	+
TF _{R158}	64 ± 27	21	26 ± 24	4	+
TF _{A158}	152 ± 43	51	12 ± 4	4	+
TF _{A159}	24 ± 5	8	21 ± 6	3	+
TF _{I159}	109 ± 33	37	60 ± 20	3	+
TF _{A160}	283 ± 60	95	12 ± 2	5	+

88 ± 58 % of wild-type TF, *n* = 6, by flow cytometry using monoclonal antibodies to the other epitopes (Fig. 1). When viable cells expressing the mutant TF_{R45G47} or the wild-type TF were analysed for functional activity, similar results were obtained. The specific activity for TF_{R45G47} was 400 ± 63 munits/10⁵ cells and for wild-type TF was 507 ± 105 munits/10⁵ cells (mean ± S.D., *n* = 7). This demonstrates that TF_{R45G47} has normal specific activity and suggests that the W⁴⁵KS motif itself is not required for function of TF. Interestingly, W⁴⁵KS appears to be required for the epitope of TF9-6B4, a monoclonal antibody that completely inhibits Factor VII binding to TF [14], suggesting spatial proximity of the epitope to a Factor VII interactive site. The second WKS motif may therefore be near to, but not an intrinsic part of, this Factor VII-interactive site.

Substitution of the W¹⁵⁸KS motif by RKG resulted in an 86 % loss of functional activity (Table 2). The mutant protein: (i) was expressed at high levels, (ii) exhibited a normal pattern of TF band microheterogeneity by Western blot, which is characteristic of normal glycosylation of TF [10], and (iii) equivalently expressed all monoclonal-antibody epitopes (Fig. 1). This is consistent with proper global folding of the protein, and suggests a functional importance of the W¹⁵⁸KS motif in TF. We further addressed the functional defects of the first and third motifs by additional substitutions in the tripeptide motif as well as by replacing adjacent residues.

Structural importance of Trp¹⁴

The lack of expression of the mutant protein with the RKG substitution for the W¹⁴KS motif suggested that structural requirements for global folding of TF might have been lost. The Trp¹⁴ residue might contribute to the hydrophobic core of TF. Consistent with this speculation, Trp¹⁴ → Arg and the Trp¹⁴-Lys → Arg-Ile replacements in wild-type TF resulted in no detectable biosynthesis of protein (Table 2). The conservative hydrophobic substitution Trp¹⁴ → Phe resulted in normal levels of biosynthesis, *N*-linked glycosylation and functional activity of the mutant protein. In addition, a Lys¹⁵ → Ala substitution did not result in loss of function. This indicates that the W¹⁴KS motif

in TF is not a functional site, but that Trp¹⁴ may participate in folding of the nascent protein and may be critical for stabilizing the hydrophobic core of the TF extracellular domain.

Functional importance of the third WKS motif in TF

TF_{R158G160} demonstrated considerable loss of function without evidence of structural alterations. The Trp¹⁵⁸ → Arg substitution alone resulted in a functional defect (Table 2) comparable with the W¹⁵⁸KS → RKG substitution suggesting that the Trp¹⁵⁸ → Arg substitution causes the functional defect in the double mutant. In addition, the Ser¹⁶⁰ → Ala mutant exhibited normal function, indicating that the side chain of the serine residue is not requisite to interactions for assembly of the TF·Factor VIIa initiation complex. Replacement of Trp¹⁵⁸ by a charged Arg was not associated with apparent global structural changes. This implies that Trp¹⁵⁸ is not buried in the hydrophobic core but rather represents a surface Trp with the potential to interact with ligand or adjacent TF residues which mediate the interaction. However, in contrast with the Trp¹⁵⁸ → Arg mutant, the Trp¹⁵⁸ → Ala one appears to have almost normal cofactor function. This suggests that Trp¹⁵⁸ may not directly participate in functionally important protein-protein interactions and that the functional defect of the Trp¹⁵⁸ → Arg replacement may be due to localized alterations in conformation.

There is evidence that residues adjacent to Trp¹⁵⁸ are functionally important. The Lys¹⁵⁹ → Ala substitution leads to loss of functional activity comparable with the Trp¹⁵⁸ → Arg substitution. No structural alteration was detected for TF_{A159} based on monoclonal-antibody epitope expression (Fig. 1) and proper cellular processing. Whereas the Lys¹⁵⁹ → Ile substitution had relatively little effect on function, the Lys¹⁵⁹ → Ala substitution resulted in a significant loss of function. This suggests potential side-chain interactions or a space-filling role of Lys¹⁵⁹ that can be substituted adequately by the more bulky side chain of Ile, but not by the Ala, exchange. However, removal of the charged amino group of Lys¹⁵⁹ reduces the functional activity. Chemical modification of amino groups in TF with a homobifunctional cross-linking reagent had been shown to result in a loss of

function [18]. Since it is likely that the W¹⁵⁸KS motif is at the surface of TF rather than buried in a hydrophobic core (see above), the amino group of Lys¹⁵⁹ may be one of the functionally critical lysine residues susceptible to chemical modification.

The functional site also extends proximally from Trp¹⁵⁸, as demonstrated by the functional importance of Tyr¹⁵⁷. The Tyr¹⁵⁷ → Ala mutant resulted in an 87% loss of specific activity. There must be a critical role of the aromatic ring and only a minor contribution of the hydroxy group of Tyr¹⁵⁷, since the Tyr¹⁵⁷ → Phe replacement was fully functional. There could be structural importance for Tyr¹⁵⁷ to maintain local conformation by contribution to aromatic-aromatic interactions [19], or the aromatic ring could contribute to the interaction with ligand in addition to the charged group of Lys¹⁵⁹. The effect of the Trp¹⁵⁸ → Arg substitutions may be explained by slight alterations of the side-chain orientation of the adjacent residues Lys¹⁵⁹ and Tyr¹⁵⁷, which may be interactive with the ligand Factor VIIa. Similarly to Ser¹⁶⁰, the side chain of Tyr¹⁵⁶ can be deleted by replacement with Ala without loss of function. This analysis therefore provides evidence for a functionally important sequence span from Tyr¹⁵⁷ to Lys¹⁵⁹, with two flanking residues which appear to be unnecessary for specific receptor cofactor function. Tyr¹⁵⁷ and Lys¹⁵⁹ are also found in the mouse TF sequence. The reported lower functional activity of mouse TF in human plasma [17] can therefore not be accounted for by the lack of the two critical interactive residues in mouse TF. However, the spatial relationship of these two residues will differ depending on whether they are embedded in the Tyr-Tyr-Trp-Lys-Ser (YYWKS) or the Thr-Tyr-Arg-Lys-Gly (TYRKG) sequence (Table 1). The Trp¹⁵⁸ to Arg substitution in human TF appears to introduce a slight perturbation of the Tyr¹⁵⁷ and/or Lys¹⁵⁹ side chains resulting in loss of specific function. Similarly, these two residues may be oriented in the murine sequence less favourably for the interaction with human Factor VII. Although not proved by the present results, these differences in the local conformation at the third WKS position due to exchange of Trp by Arg and Ser by Gly in the mouse sequence could account for the variations in pro-coagulant activity of the two species.

Whereas Trp¹⁴ appears to be structurally important, no involvement of this residue, or of Lys¹⁵, in the formation of the functional TF·Factor VIIa complex could be demonstrated. Similarly, the non-conservative RKG substitution in the W⁴⁵KS motif did not significantly affect functional activity. However, substitutions in the W¹⁵⁸KS motif provided evidence for the participation of a WKS motif in a functional site in the C-terminus of the TF extracellular domain. Lys¹⁵⁹ of the WKS motif was identified as functionally important, and the functional significance of Tyr¹⁵⁷ indicates involvement of adjacent residues. There are likely to be other residues which contribute to this functional site, but are not in a linear proximity to the tripeptide

motif. The epitope of an inhibitory monoclonal antibody had been shown to have critical residues encoded by exon 4 and part of exon 5 [14]. In addition, covalent stabilization of carboxy disulphide loops in the TF extracellular domain is important for binding of Factor VII and function of TF [12]. These independent approaches therefore provide convergent evidence for the functional role of the C-terminal half of the TF extracellular domain. Single amino acid residues are identified which mediate functional interactions and provide localization of an interactive site in TF important to formation of the TF·Factor VIIa initiation complex on cells.

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