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Crystal structure of tissue factor in complex with antibody 10H10 reveals the signaling epitope

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ABSTRACT

Tissue factor (TF) initiates the extrinsic pathway of blood coagulation through sequential binding and activation of coagulation factors VII (FVII) and X (FX). In addition, through activation of G-protein-coupled protease activated receptors (PARs) TF induces cell signaling that is related to cancer, angiogenesis and inflammation. Monoclonal antibodies (mAbs) proved to be a useful tool for studying the interplay between TF signaling and coagulation. MAb 10H10 is unique in that it blocks the signaling pathway and thus inhibits angiogenesis and tumor growth without interfering with coagulation. It was also presumed that mAb 10H10 recognizes the cryptic pool of TF devoid of procoagulant activity. The crystal structure of the 10H10 Fab was determined in the absence and in the presence of the TF extracellular domain (ECD). The structures show that the antibody operates by the key-and-lock mechanism causing no conformational changes in either Fab or TF. The TF:10H10 interface is extensive and includes five segments of TF in both the N-terminal and C-terminal domains of the ECD. Neither the known epitope of FVII, nor the putative epitope of FX overlaps with the 10H10 binding site. The 10H10 epitope points to the likely location of the PAR2 exosite. It is also the hypothetical site of TF interaction with integrins that may play a major role in the encryption-decryption process.

1. Introduction

The coagulation cascade is initiated by the cell surface transmembrane receptor tissue factor (TF) which binds and activates the serine protease, factor VII (FVII). This complex then proteolytically activates the zymogen substrates, factor IX (FIX) and factor X (FX), and generates the two key intermediate enzymes for thrombin generation. Besides this, the TF:FVIIa complex induces cell signaling via activation of transmembrane G-protein-coupled protease activated receptors (PARs), primarily PAR2 [1,2]. This pathway has been implicated in preventing apoptosis, promoting migration and wound repair in non-cancerous epithelial cells, whereas in cancer cells TF-FVIIa-PAR2 signaling supports angiogenesis, tumor progression and metastasis [3]. The ternary TF:FVIIa:FXa complex is a more potent activator of PAR2 than the binary TF:FVIIa complex [4], however it is likely that both complexes play a role in vivo depending on the levels of TF and PAR2 expression and the availability of FX [5].

Activation of macromolecular substrates FX and FIX by TF:FVIIa involves interactions at the exosites in FVIIa and TF while the primary specificity residues at the catalytic cleft make a minor contribution [6]. Exosite-driven macromolecular substrate recognition is a common mechanism by which proteases of the coagulation system achieve their remarkable specificity [7]. Exosite engagement has also been demonstrated for PAR1 and PAR3 recognition by thrombin [8], however the presence of PAR2 exosites on TF:FVIIa is an open question [9].

MAbs have been used as specific probes for studying cellular processes and identifying functional regions involved in protein interactions [10]. Several anticoagulant mAbs have been characterized and their epitopes have been identified by TF mutagenesis. MAbs 7G11, 6B4 and HTF1 overlap with the FVIIa contact region, whereas more potent mAbs D3, 5G6 and 5G9 bind to the FX interaction region [11]. The crystal structure determination of the TF:5G9 and TF:D3 complexes [12,13] provided detailed information on the FX exosite.

Unlike other mAbs, 10H10 specifically blocks TF:FVIIa binary complex activation of PAR2 without inhibiting coagulation [14]. Treatment with 10H10 prevented tumor growth of aggressive breast

Abbreviations: CDR, complementarity determining region; CHES, *N*-cyclohexyl-2-aminoethanesulfonic acid; ECD, extracellular domain; EDTA, ethylenediaminetetraacetic acid; FVII, coagulation factor VII; FX, coagulation factor X; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mAb, monoclonal antibody; PAR, G-protein-coupled protease activated receptor; PDB, Protein Data Bank; PEG, polyethylene glycol; TF, tissue factor; VH, variable domain of the heavy chain; VL, variable domain of the light chain

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cancer cells and PAR2-dependent pregnancy complications in mouse models [15,16] and may present a therapeutic opportunity for cancer patients with impaired hemostasis. To provide insight into the mechanism of action of this antibody and to identify the epitope critically involved in TF signaling, we have determined the crystal structure of the 10H10 Fab alone and bound to the TF extracellular domain (ECD).

2. Materials and methods

2.1. Expression and purification of TF ECD and 10H10 Fab

The His-tagged TF ECD was expressed in *Escherichia coli* and purified using affinity and ion exchange chromatography. The cell lysate in 20 mM Na phosphate pH 7.5, 500 mM NaCl, 10% glycerol, 0.37 mg/mL lysozyme, 0.25 μL/mL benzonase, EDTA-free protease inhibitors was applied onto a 5-mL HisTrap HP column (GE Healthcare). TF ECD was eluted using a 100% to 40% gradient of 20 mM Tris, 500 mM NaCl, 500 mM imidazole, pH 7.0. Fractions containing TF ECD were pooled, dialyzed into 20 mM Tris, 50 mM NaCl, pH 7.0 and loaded onto a 16-mL Q HP column (GE Healthcare). The protein was eluted using a 50 mM to 1 M NaCl gradient in 20 mM Tris, pH 7.0. Fractions containing TF ECD were pooled and dialyzed against 20 mM Tris, pH 8.0, 50 mM NaCl.

10H10 mAb was isolated from hybridoma cell line TF9.10H10-3.2.1 (The Scripps Research Institute) and sequenced at Janssen R & D. The Fab fragment of 10H10 was constructed by fusing the mouse variable domains with human IgG1/κ constant domains that contained the 6xHis tag at the C-terminus of the heavy chain. Two Lonza-based vectors (Lonza Group, Switzerland) were used to construct expression plasmids for the heavy chain and the light chain, following the protocol described previously [17]. The plasmids were co-transfected into HEK 293 cells using Lipofectamine 2000 (Thermo Fisher). The leader sequences at the N-termini of the heavy and light chain directed the protein for secretion into cell culture medium consisting of DMEM (Dulbecco's Modified Eagle Medium), 10% FBS (fetal bovine serum) and 2 mM glutamine. The cell-culture supernatant was collected 5 days after transfection, centrifuged and loaded onto a 15-mL TALON column (GE Healthcare) in 50 mM Na phosphate, pH 7.1, 350 mM NaCl. The protein was eluted with a 0 to 80% gradient of 100 mM imidazole, 150 mM NaCl, 50 mM Na phosphate, pH 7.1. The fractions containing Fab were pooled and dialyzed into 20 mM Tris, pH 7.4, 50 mM NaCl. The Fab was further purified by size exclusion chromatography using a HiLoad 26/60 Superdex 200 column (GE Healthcare). The yield was 25 mg Fab per 1 L cell culture.

2.2. Complex preparation and crystallization

The TF:Fab complex was prepared by mixing 10H10 Fab with TF ECD at the molar ratio 1:1.2. The mixture was incubated for 20 min at room temperature and loaded on a Superdex 200 10/30 column (GE Healthcare) equilibrated with 20 mM HEPES, pH 7.5, 0.1 M NaCl. The shift in the elution profile indicated complex formation. Fractions corresponding to the main peak were pooled, concentrated to 11 mg/mL, and used for crystallization.

Crystallization of both the complex and free Fab was carried out by the vapor-diffusion method at 20 °C using a Hydra II robot (Thermo Scientific) in 96-well plates with the droplets containing protein and reservoir solution at 1:1 ratio. The screening was performed with the Hampton Research Crystal Screen HT and in-house screens [18]. Optimization of initial conditions yielded X-ray quality crystals that were obtained from 24% PEG 8000, 1.0 M sodium acetate, 5% PEG 400, 0.1 M cacodylate buffer, pH 6.5 (Fab) and from 18% PEG 8000, 0.1 M CHES, pH 9.5 (complex). Crystal data are given in Table 1.

Table 1
X-ray data and refinement statistics.

	10H10 Fab	10H10 Fab + TF
X-ray data		
Space group	$C222_1$	$I4_1$
Unit cell (Å)	81.77, 135.51, 88.25	116.86, 116.86, 106.86
Molecules/asymmetric unit	1	1
V_m (Å^3/Da)/solvent (%)	2.52/51	2.50/50
Resolution (Å)[a]	30–1.9 (1.95–1.90)	30–3.4 (3.5–3.4)
Number of measured reflections	367,469 (13,073)	71,313 (5081)
Number of unique reflections	38,664 (2712)	9573 (711)
Completeness (%)	99.4 (95.3)	96.7 (97.4)
Redundancy	9.5 (4.8)	7.4 (7.1)
R-merge (I)	0.060 (0.365)	0.105 (0.483)
Mean I/σ(I)	26.3 (4.2)	19.7 (3.4)
B factor from Wilson plot (Å^2)	30.8	74.9
Refinement		
Resolution (Å)	15–1.9	15–3.4
Number of all atoms	3756	4919
Number of water molecules	347	0
R-cryst	0.201	0.242
R-free (5% data)	0.243	0.289
RMSD bond lengths (Å)	0.010	0.011
RMSD bond angles (°)	1.3	1.5
Mean B-factor from model (Å^2)	32.7	30.6
Ramachandran plot, most favored (%)	90.7	83.1
Ramachandran plot, disallowed (%)	0.3	0.4

[a] Values for highest resolution shell are in parentheses.

2.3. X-ray structure determination

For X-ray data collection, one crystal of each the Fab and the complex was soaked for a few seconds in the mother liquor supplemented with 15% glycerol, and flash frozen in the stream of nitrogen at 100 K. Diffraction data were collected using a Rigaku MicroMax™-007HF microfocus X-ray generator equipped with an Osmic™ VariMax™ confocal optics, Saturn 944 CCD detector, and an X-stream™ 2000 cryocooling system (Rigaku). The X-ray data were processed with the program XDS [19].

The structures were determined by molecular replacement with the program Phaser [20] and refined using Refmac5 [21] and Coot [22]. The search model for 10H10 Fab was constructed from PDB entries 1MVU [23] for the light chain and 1D5I [24] for the heavy chain. The 10H10 Fab structure was refined at 1.9 Å resolution and was used, together with the TF structure from 1UJ3 [25], as a search model for the structure determination of the complex. The search using variable and constant domains of the Fab and the N- and C-terminal domains of TF ECD produced a structurally sensible solution that was refined at 3.4 Å resolution. Due to low resolution of the data, individual atomic temperature factors were not refined and water molecules were not added. X-ray data and refinement statistics are given in Table 1. Ramachandran statistics was calculated with PROCHECK [26]. The solvent accessible surface area was calculated with the program AREAIMOL from the CCP4 suite [27]. Figures were prepared with PyMol (Schrödinger, LLC).

3. Results

3.1. Structure of 10H10 Fab

The crystal structure of 10H10 Fab contains all 449 residues including the C-terminal His tag. The Fab has an elbow angle between the variable and constant domains of 135°, which is in the middle of the range observed for antibodies with the kappa light chain [28]. The conformations of the CDR loops L1, L2, L3, H1 and H2 can be classified