Summary

Tissue Factor (TF) is a cofactor of Factor VIIa (F.VIIa) and is involved in the initiation of the extrinsic pathway of the blood coagulation cascade. TF is an integral membrane protein consisting of an extracellular domain (amino acids 1–219), a transmembrane domain (220–242), and a cytoplasmic domain (243–263). Based on structural and sequence similarities, TF belongs to the cytokine receptor superfamily.

Here, TF is studied using protein crystallography. For these experiments, purified protein solutions were available for TF–219, TF–243, TF–263, the murine anti–TF Fab fragment D3, the humanized anti–TF Fab fragment D3H44, and cell paste for the mutant TF–263 C245S.

The first part of this study addressed the crystallization of full–length TF, in order to obtain a model mimicing a full length cytokine receptor. So far, no structure of an integral cytokine receptor has been solved. Crystallization of the solubilized membrane proteins has proven difficult in the past, and also in this study the structure of full–length TF could not be solved. The crystals obtained were either too small for diffraction studies, or diffraction was limited to 7 Å only. However, the experiments described here, give valuable hints for the optimization of the crystallization conditions; this may ultimately lead to crystals well suited for X–ray diffraction studies.

In the second part of this study, the crystal structures of the anti–TF Fab fragments murine D3 and humanised D3H44 were solved and compared to study the structural aspects of the humanisation process. In addition, the antigen–antibody recognition process between TF and D3H44 was investigated. The crystal structure of the murine D3 Fab fragment was solved at a resolution of 2.4 Å. The structure of the humanised D3H44 antibody could be determined at a resolution of 1.85 Å, as well as the structure of the complex between the extracellular domain of TF and D3H44 at a resolution of 1.85 Å.

The comparison between the structures of the murine D3 and the humanised D3H44 revealed only small deviations between these structures despite different antibody fra-

meworks. The C_α atoms can be superimposed with a deviation of 1.27 Å (RMS, root-mean-square). In the main chain, most of the structural differences between D3 and D3H44 were located in those regions of the variable domain which are proximal to the constant domain.

During the humanisation process 7 amino acids from the antibody framework and 6 amino acids from the complementarity determining regions (CDR) have been mutated with the result of a 100–fold increased binding affinity compared to the D3 antibody. The most important mutation was the exchange Arg–H71–Ala in order to reinduce TF binding. The large side–chain of arginine sterically hinders the conformation of the CDR–H1 and –H2 loops which is necessary for TF binding. Further substitutions optimize either the hydrophobic contact between the light and the heavy chains or introduce new hydrogen bonds between the binding loops replacing water–mediated contacts.

The D3H44 structure shows numerous similarities to the structures of the previously published Fab fragments of anti–VEGF, anti–CD18, and anti–p185Her2. All these Fab fragments contain the same human framework.

The accessibility of the crystal structures of the D3H44 Fab fragment at 1.85 Å resolution, of the complex TF·D3H44 at 1.85 Å resolution and the previously published structure of the free TF at 1.7 Å resolution offers a unique opportunity to study the antigen–antibody recognition process. The conformational changes in D3H44 induced during complex formation are small and are mainly restricted to the reorientation of side–chains. The combining site in the TF·D3H44 complex contains a large number of polar interactions. In addition, 46 water molecules participate in the interface.

Independently from D3, another murine anti–TF antibody, 5G9, had been developed recognizing nearly the identical epitop on the C-terminal TF domain. The crystal structures of the free Fab fragment 5G9 and the complex between TF and 5G9 (TF·5G9) have been solved with a resolution of 2.5 Å and 3.0 Å, respectively. In a previously published report, the antigen–antibody recognition process has been studied between TF and the Fab fragment 5G9 and no major role for water molecules in the interface has been forseen. However, in the present study of TF and D3H44, water molecules play an important role in mediating contacts between both molecules.

In the Protein Data Bank, there are only two additional structures of antigen–antibody complexes for which the coordinates have been deposited together with the structures of the corresponding free antigen and free antibody. Structures at similar resolution are cytochrome c in complex with FabE8 and lysozyme in complex with HyHEL–63. Revisiting their antigen combining sites and applying the same criteria

as for the TF·D3H44 structure shows that these antigen–antibody complexes are quite similar with respect to the number of water molecules in the interface, the number of identical water molecules in the free components and the complex, and the number of water molecules, which are expelled by polar atoms of the binding partner.