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Crystallization of an anti-factor IX antibody and its complex

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Initiation, propagation and regulatory processes of blood coagulation occur at the cell surface. During blood coagulation, many coagulation factors are anchored onto cell-surface membranes through their N-terminal carboxyglutamic acid-rich (Gla) domains found on such vitamin K-dependent blood coagulation factors as factors VII, IX, X, prothrombin, factors C and S. 10C12 is a conformation-specific calcium-dependent anti-factor IX antibody, which is directed at the calcium-stabilized Gla domain and interferes with factor IX–membrane interaction. In a variety of animal models, 10C12 has been demonstrated to be effective anticoagulant in attenuating thrombosis without severe bleeding. The crystallization of the Fab fragment of 10C12 and its complex with the Gla domain of human factor IX was not trivial. Here, the crystallization conditions and unusual aspects of this crystallization process are reported.

1. Introduction

Blood coagulation plays an important role in the human body, preventing excessive loss of blood after vascular damage. Abnormal blood coagulation leads to thrombosis. Factor IX plays an important role in the propagation stage of the blood coagulation cascade (Furie & Furie, 1988). Factor IX can be activated to factor IXa in the presence of calcium ions either by factor XIa (intrinsic pathway, Fig. 1) or by the complex of factor VIIa–tissue factor bound to the membrane surface (extrinsic pathway). Factor IXa and its cofactor, factor VIIIa, assemble into a membrane surface-bound enzyme complex (tenase) that can efficiently activate factor X to factor Xa in the presence of calcium ions. Factor Xa, in the presence of factor Va, can activate prothrombin to thrombin, which will finally lead to fibrin clot formation. While the tissue factor–factor VIIa complex is the major trigger of the blood coagulation process, the factor IXa–factor VIIIa complex (tenase) sustains the coagulation process.

The activity of factor IXa is dependent on its specific binding on physiological cell surfaces, such as on activated platelets and endothelial cells, through its N-terminal γ-carboxyglutamic acid-rich (Gla) domains. The Gla domain is a membrane-binding motif also found in other vitamin K-dependent blood-coagulation factors such as factors VII, IX, X, prothrombin, factors C and S. These proteins require vitamin K for the post-translational synthesis of γ-carboxyglutamic

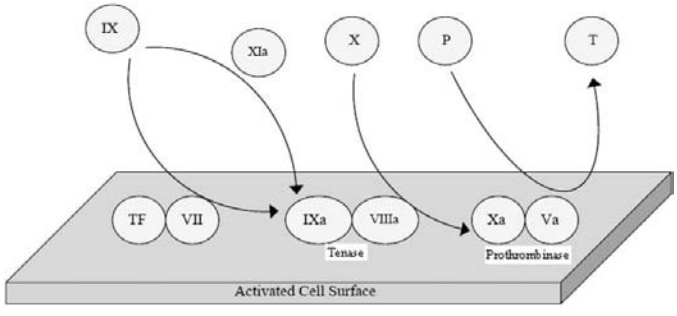

Figure 1
Factor IX plays an important role in sustaining blood coagulation process (P, prothrombin; T, thrombin).

acid, an amino acid clustered in the Gla domain. In the presence of calcium ions, the Gla domain folds properly into a globular domain and supports interaction of these vitamin K-dependent blood coagulation factors with cell-surface membranes containing phosphatidylserine.

Anti-factor IX antibody 10C12 was one of a panel of antibodies identified from a phage display single-chain Fv (scFv) human antibody library screened against synthetic factor IX Gla domain in the presence of calcium ions (Suggett *et al.*, 2000). 10C12 was then reformatted into Fab′2 form with two Fab fragments connected by a leucine zipper. 10C12 is a calcium-dependent and calcium-specific antibody of factor IX; there was no binding in the presence of magnesium ions. The binding to factor IX Gla domain has high affinity with a dissociation constant (K_d) of 1.6 n*M*. Interestingly, 10C12 binding to Gla domain is highly specific to factor IX Gla domain, despite the high sequence homology among all Gla domains. 10C12 was found to strongly inhibit tenase function and factor IX binding on endothelial cells and has been demonstrated as an effective anticoagulant in several animal models with minimal bleeding compared with heparin (Refino *et al.*, 1999, 2002).

Here, we present the crystallization of 10C12 Fab fragment and its complex with human factor IX Gla domain. The crystal structure of the complex between 10C12 and factor IX may shed light on the potential factor IX-induced conformational change upon 10C12 antibody binding.

2. Experimental

10C12 Fab′2 was the kind gift of Dr Daniel Kirchhofer (Genentech). Protein concentration was assayed using the Advanced Protein Assay (Cytoskeleton, Denver, CO, USA). Papain and cysteine were purchased from Sigma–Aldrich. Gla domain peptide (amino acids 1–47) was synthesized according to the literature (Jacobs *et al.*, 1994).

2.1. Preparation of 10C12 Fab fragment

Papain (27.8 mg ml^{-1}) was activated at 310 K for 15 min with 10 m*M* cysteine in 0.1 *M* sodium acetate pH 5.5, 12.5 m*M* EDTA. The final concentration of activated papain was 13.9 mg ml^{-1}. 75 µl of activated papain was added to 500 µl of 10C12 Fab′2 (1.6 mg ml^{-1}). The digestion mixture was incubated at 310 K for at least 2 h and was then stored at 193 K. For purification, the reaction mixture was thawed to room temperature and immediately applied to a Superdex 75 gel-filtration column. The protein was then eluted with 0.1 *M* NaCl, 0.2 *M* sodium acetate pH 5.5. The purified protein was concentrated in a Millipore Ultrafree concentrator with 5 kDa molecular-weight cutoff to a concentration of 15 mg ml^{-1} for crystallization trials.

To make 10C12 Fab–factor IX Gla domain complex, 4.0 ml of 10C12 Fab fragment (0.18 mg ml^{-1}) eluted from a Superdex 75 column was mixed with 1.0 ml of factor IX Gla domain at 0.50 mg ml^{-1}. The complex was purified on a HiLoad 16/60 Superdex 75 gel-filtration column with a column volume of 120 ml. The complex eluted at 52.5 ml with a buffer of 0.2 *M* NaCl, 2 m*M* $CaCl_2$, and 0.1 *M* HEPES pH 7.5. The purified complex was concentrated in a Millipore Ultrafree concentrator with 5 kDa molecular-weight cutoff at 6000*g* to a concentration of 4.0 mg ml^{-1} for crystallization.

2.2. Crystallization of 10C12 Fab and its complex with factor IX Gla domain

The crystals of 10C12 Fab and 10C12 Fab–factor IX Gla domain complex were both formed using the vapour-diffusion method. 10C12 Fab was crystallized with 3.0 *M* ammonium sulfate, 0.1 *M* HEPES, 2% MPD pH 7.5. Crystals of 10C12 Fab–factor IX Gla domain complex were formed at a precipitant condition of 2.3 *M* ammonium sulfate, 0.1 *M* HEPES pH 7.5, 5 m*M* calcium chloride and 2% MPD. The contents of both crystals were confirmed by SDS–PAGE.

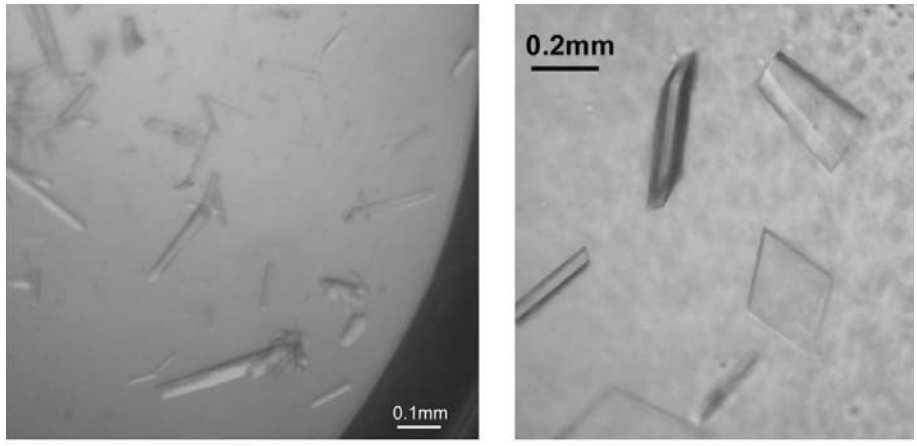

Figure 2
Crystals of 10C12 Fab (*a*) and its complex with factor IX Gla domain (*b*).

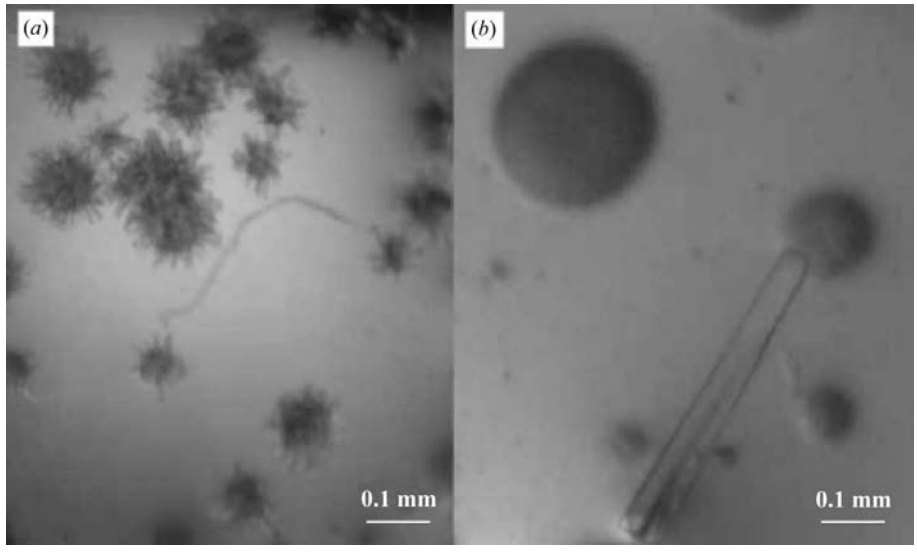

Figure 3
Large yellowish oily droplets appeared in the crystallization setup of 10C12 Fab, and served as nucleation surface (*a*). Single crystal grown out of the surface of the droplets (*b*)

2.3. X-ray data collection

X-ray diffraction data were collected either with in-house X-ray systems or synchrotron sources (BNL NSLS beamlines). The in-house X-ray system used was a Bruker Proteum R system equipped with an FR591 9 kW Cu $K\alpha$ rotating anode, a Montel 200 focusing multilayer optics and a Smart 6000 CCD detector. The crystal was quickly (<1 s) dipped into a cryoprotectant solution (mother liquor containing 25% glycerol) and immediately frozen at 103 K in a liquid-nitrogen stream prior to data collection.

3. Results and discussion

10C12 is an engineered anti-human factor IX antibody directed against the membrane-anchoring domain of human factor IX Gla domain. As a conformational specific and calcium-dependent antibody, 10C12 shows no binding to factor IX in the absence of calcium ion. It is a potent and highly specific antibody (K_d = 1.6 nM), recognizing only factor IX and not other coagulation factors, despite high sequence identity among all the Gla domains of vitamin K-dependent blood-coagulation proteins. 10C12 was found to strongly inhibit tenase function and factor IX binding on endothelial cells and has been demonstrated in several animal models as an effective anticoagulant without bleeding (Refino *et al.*, 1999, 2002). The crystal structure of 10C12 and its complex with antigen will reveal the structure basis of these characteristics.

10C12 Fab crystals were formed from vapour diffusion against 3.0 M ammonium sulfate, 2% MPD, 0.1 M HEPES pH 7.5 (Fig. 2). This crystallization requires unusually high precipitant concentration (3.0 M ammonium sulfate). Increasing the concentration of the protein solution did not lower the precipitant concentration required. During the crystallization setup, the addition of precipitant into protein solution caused immediate protein precipitation. The precipitant, however, dissolved overnight to yield a clarified solution and crystals appeared in a period of 3–4 weeks. This phenomenon was similar to that in the crystallization of sar1-GDP protein (Huang *et al.*, 2002), where a high concentration of precipitant (25% PEG 4000) caused protein precipitation that took several days to dissolve.

The long crystallization time of 10C12 Fab slowed down the crystallization optimization process. We used streak-seeding in trying to speed up the crystallization. Streak-seeding is quite effective both in increasing the crystal size (50 × 50 × 200 µm; Fig. 2*a*) and reducing the crystallization time from 3–4 weeks to about 2 d.

It is interesting to note that sometimes (especially when 10C12 Fab was aged) large yellowish oily droplets appeared in the crystallization setup. These droplets often served as a nucleation surface, with 10C12 Fab crystals growing out from the surface of the droplets (Fig. 3). These yellowish oil droplets contained concentrated but not denatured protein, as adding water dissolved the droplets leaving a clear solution.

The complex of 10C12 Fab with factor IX Gla was crystallized at 2.3 M ammonium sulfate, 0.1 M HEPES pH 7.5, 5 mM calcium chloride and 2% MPD (Fig. 2*b*). MPD, albeit at a low concentration, is absolutely necessary for the formation of the crystals. MPD is the most popular additive for protein crystallization and has a positive effect on protein stabilization and shielding the hydrophobic groove (Anand *et al.*, 2002). At a synchrotron source, the complex crystals diffracted to 2.1 Å with strong signal-to-noise ratio (I/σ of 25.7), high data redundancy (8.4) and low merging R factor (0.05).

We are analyzing the structures of 10C12 and its complex with factor IX Gla domain. The results will provide insights about the characteristics of the anticoagulant antibody 10C12, especially the conformational flexibility of its CDR region and shed light on new strategies to target coagulation factors.

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