*Cryo-Electron Microscopy of FVIII bound to Phospholipid Vesicles

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Abstract – Membrane-binding of a human blood clotting protein, factor VIII (FVIII), was studied by cryo-electron microscopy. Well visible gap junctions between adjacent phosphatydyl serine containing phospholipid vesicles were observed upon FVIII association. Digital images acquired with a JEOL2010FEG transmission electron microscope equipped with a 4x4k CCD camera were analysed with the Digital Micrograph software to study the lipid protein interactions and shed light onto the mechanism of FVIII membrane-binding. This study has been carried out in conjunction with ongoing structural electron microscopy studies of membrane-bound FVIII to better understand its role in blood coagulation and haemophilia.

1. Introduction

Human coagulation factor VIII (FVIII) is an essential protein in the blood clotting process, which after proteolytic activation acts as a co-factor to the serine protease factor IXa (FIXa) in the membrane-bound X-ase complex. Naturally occurring mutations of this protein result in mild to severe haemophilia. This life threatening blood disorder affects 1 in 5000 of the male population [1]. Binding of FVIII to Phosphatidylserine (PS) containing phospholipid (PL) membranes and to FIXa amplifies the production of Factor X and consecutively of Thrombin, 100,000 times.

We have investigated the association of full length human recombinant FVIII (rFVIII-FL provided by Baxter USA) to L-α-Phosphatidylcholine (PC) vesicles containing 10% L-α-Phosphatidylserine (PS) by Cryo-Electron microscopy (Cryo-EM) in the closest to *in vivo* conditions. The strong affinity of FVIII to PS containing PL membranes (dissociation constant, Kd ~ 10 nM), has so far allowed structural studies of FVIII membrane-bound conformation by electron microscopy [2, 3]. We have analysed in detail the protein-lipid interactions from the digital micrographs acquired with a 200kV and field emission gun (FEG) equipped microscope (JEM2010F), using a 4096 x 4096 pixels CCD camera (Ultrascan 4000, GATAN Inc, UK). The obtained results were combined with previous and ongoing structural and biophysical studies of the rFVIII-FL membrane-binding mechanism and conformation(s) in context of its crucial role in the blood coagulation process [4].

Cryo-Electron microscopy is a versatile tool in the study of lipid vesicles and lipid-protein interactions at the membrane level, ensuring that the samples remain fully hydrated and in the closest to the native state [5]. We have further explored the application of digital Cryo-EM microscopy using a 200 kV- FEG and high resolution CCD camera to follow protein interaction with a lipid bilayer. Images were captured at 15 μm/pixel resolution, in low-doses of electrons (< 16 e.Å^{-2}) and magnification range between 20,000x and 50,000x and analysed with the Digital Micrograph software (GATAN Inc, UK).

2. Results and Discusion

Cryo-EM of PL vesicles: 10% PS : 90% PC in 20mM Tris, 500mM NaCl, 3mM NaN_3, 2mM $CaCl_2$, pH 7.2 which were extruded through a 100nm polycarbonate filter, showed a heterogeneous solution of vesicles ranging from 100 nm up to 500 nm. The vesicles were often multilamellar, without pronounced fusion or aggregation and the separate bilayers were well resolved within multilayer vesicles (Figure 1A). Upon addition of the protein, rFVIII-FL in 1:1 w/w protein:lipid ratio, well defined gap junctions were observed between adjacent vesicles. The vesicles observed, were smaller in size and less multilamellar (Figure 1B). FVIII molecules have been shown to bind 2 -4 molecules PS and cover a surface of 50 lipid molecules [2]. From SDS-PAA gels it has been shown that the rFVIII-FL molecular mass is 270kDa, which at 1:1 w/w ratio of lipid to protein and 10% of PS contain of the membrane, ensures a saturation of all surface exposed PS molecules. The presence of gap-junction upon adding of rFVIII-FL suggests a deeper insertion of the protein than previously suggested [3], as well as tight interaction between adjacent protein molecules when bound to the membrane, and between molecules bound to opposite bilayers. Based on the presented Cryo-EM study we can elaborate further the mechanism of FVIII membrane-binding in conjunction of our ongoing structural electron microscopy and molecular modelling studies. Our data give strong evidence that gap junctions are formed by the interaction of FVIII molecules on the surface of the vesicles, when they come into contact. and we will continue to further improve our knowledge collecting more and better data at different ion concentration and lipid compositions.

Cryo-EM is a method of excellence to study PL vesicles, capable of following changes in the membrane organisation at different lipid compositions and in the presence or absence of proteins [6]. PS containing PL vesicles have been seen to aggregate in the presence of high concentrations of divalent ions *i.e.* Ca^{2+} or when fusenogenic peptides are included into the membrane [5, 7] . The Ca^{2+} and NaCl concentration as well as the PS

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content of the vesicles in the present study was kept outside the theoretical values required to induce fusion and aggregation, confirming that he observed gap junctions are as a result of FVIII binding.

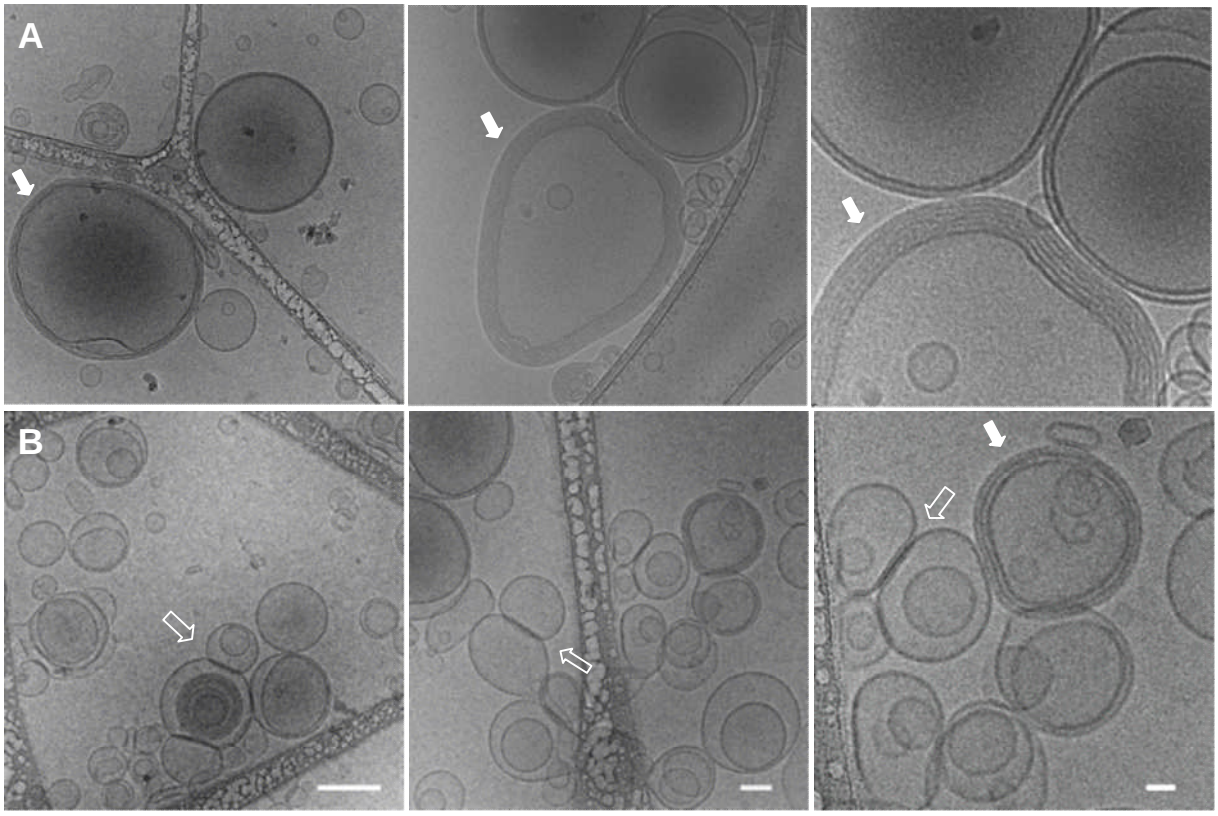

Figure 1 Cryo-EM micrographs of vesicles displaying "gap junction" structures. Row A. PCPS vesicles. Row B. Cryo-EM of PCPS vesicles + FVIII-FL. Full arrows indicate multilamellar vesicles. Hollow arrows indicate "gap junctions".

3. Conclusion

Binding of FVIII to PS containing PL vesicles has been studied by digital Cryo-TEM. Upon adding of the protein significant changes in the vesicle interactions and shape has been observed and the presence of gap junction unambiguously visualised. In comparison, cholesterol containing PC/PS vesicles did not show such a pronounced formation of gap junctions upon adding of the protein. Cryo-EM has been proven to be a unique and direct method to see protein-lipid interactions at the molecular level. We have used a high-resolution 200 kV FEG cryo-transmission electron microscope (JEM2010F) and a high resolution CCD camera (GATAN Ultrascan 4000) to routinely resolve a lipid bilayer and the effect and nature of protein binding to it, by Cryo-EM.

4. References

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