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Role of the molecular interactions between complement C3d and Factor H in regulating the complement cascade of innate immunity

Background

The complement system comprises a large number of plasma proteins which have the ability to opsonize pathogens and induce an inflammatory response. Complement is activated by three activation pathways; the classical pathway, lectin pathway and alternative pathway which all converge at C3. Initiation of the alternative pathway proceeds following the spontaneous hydrolysis of C3. This particular pathway generates a distinct convertase; C3bBb which cleaves C3 into active C3b. It is critical to have mechanisms in place to ensure that the complement response occurs in a targeted manner towards pathogenic surfaces and to prevent damage to host cells.

The regulatory protein being examined is Factor H which rapidly dissociates C3bBb to limit amplification of the complement response whilst competing with factor B for C3b binding. In addition, Factor H acts as a cofactor to Factor I, degrading C3b to iC3b and finally C3d. C3d is the final degradation product of C3 and a proteolytic fragment of C3b. C3d binds to host cell membranes through its thioester group providing additional binding sites for Factor H when further regulation is required. Ternary complex formation has been observed between Factor H, C3b/C3d and glycosaminoglycans. More specifically, Factor H SCR 19 and 20 are shown to be critical for discriminating self from non self.

Two conflicting crystal structures have been presented revealing a 1:1 complex of C3d and Factor H SCR 19/20 (FH 19/20) [1] as well as a 2:1 complex of C3d and FH 19/20 [2]. Determining the correct stoichiometry for Factor H interacting with its ligands is essential for fully understanding the regulation of complement.

Aims

To investigate which crystal structure is most representative of the complex formed between FH 19/20 and C3d under physiological conditions using isothermal titration calorimetry, fluorescence detection analytical ultracentrifugation and surface plasmon resonance studies. Furthermore, to establish a K_D for both binding sites; SCR 19 with C3d and SCR 20 with C3d.

Description of work

Expression and Purification of recombinant C3d

Recombinant C3d containing a glutathione S transferase tag was expressed using an *Escherichia coli* expression system. Cell culture preparations took place in Luria broth before transfer to 2xYT medium. Optical density measurements monitored the growth of *E. coli* prior to induction by Isopropyl β-D-1-thiogalactopyranoside (IPTG), an inducer of the Lac operon. Following overnight induction, the cells were harvested by centrifugation and re-suspended in Tris buffer for sonication. Vital protease inhibitors were added including leupeptin, pepstatin A and Pefabloc to prevent degradation of C3d. Further centrifugation placed recombinant C3d in the supernatant. The lysate was passed through a GSTrap FF column for affinity chromatography using an AKTA purification system. Glutathione and GST have relatively slow binding kinetics and so the flow rate was set to 0.5 ml/min to increase binding capacity. C3d now bound to glutathione in the GSTrap FF column was eluted using thrombin which cleaves the GST-tag. Finally, size exclusion chromatography using gel filtration further purified our protein by separation according to size.

Expression and Purification of FH 19/20

Expression of FH 19/20 was carried out in a *Pichia pastoris* expression system. *P. pastoris* cells transformed with a pPICZαA vector containing a zeocin resistance gene were streaked onto YPD agar plates. Incubation for 48 hours ensured cell growth had been achieved by observing colony growth. Inoculation of buffered complex glycerol media then allowed for the large scale growth of *P. pastoris* before transfer to buffered complex methanol media. pPICZαA contains an alcohol oxidase gene with a strongly inducible promoter, and so cells were fed with methanol to induce protein expression. Following 3 days' incubation, centrifugation of the media resulted in FH 19/20 being secreted into the supernatant. FH 19/20 purification was achieved using Heparin affinity purification in an AKTA purification system using Tris buffer with a NaCl gradient. Finally gel filtration removed other unwanted cell and media debris.

Fluorescence detection analytical ultracentrifugation

Analytical ultracentrifugation (AUC) is a non-destructive technique which monitors the sedimentation of a sample in real time as it is centrifuged at high speeds. Previous studies using conventional AUC experiments revealed that unbound C3d masks the 1:1 complex as these share a similar sedimentation coefficient and so complex formation could not be monitored. To overcome this, Factor H was labelled using fluorescein isothiocyanate (FITC). Labelled FH 19/20 and C3d were mixed at varying ratios and spun at 40k rpm in a fluorescence detection system, a novel technique in this laboratory. Results were analysed using SEDFIT.

Isothermal Titration Calorimetry (ITC)

ITC gives a highly sensitive thermodynamic analysis of interactions in solution. Protein samples were concentrated and C3d was injected into the sample cell containing FH19/20 and the power required to maintain both the reference cell and sample cell was measured.

Results and conclusions

C3d and FH 19/20 were successfully expressed and purified as confirmed using SDS-PAGE (Fig.1). Initially FH 19/20 yields were low (Fig.2). Altering the protocol for this and feeding *P. pastoris* cells twice daily was carried out in an attempt to improve yields which proved unsuccessful. Interestingly, changing and installing a new heparin trap FF column resulted in prosperous FH 19/20 purification. Purification of one C3d batch produced a promising 7 mg/ml of protein although further purification was required in order to meet experimental demands.

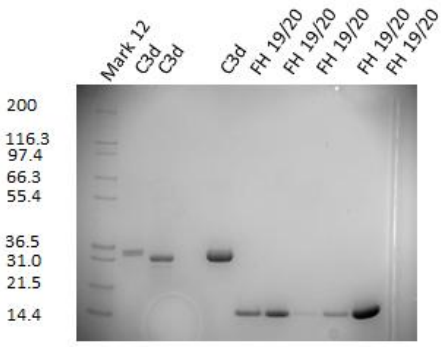

Figure 1 SDS-PAGE of purified C3d and FH 19/20 samples to determine their legitimacy. Well 1 contains our protein marker Mark 12, Wells 2,3 and 5 contain assumed C3d whilst wells 6-10 contain assumed FH 19/20. All Factor H samples can be used. Only well 2 yielded pure C3d, wells 3 and 5 were older samples to be discarded. Protein bands stained using coomassie blue.

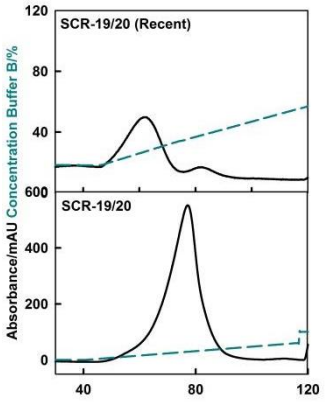

Figure 2- FH 19/20 purification AKTA chromatogram. The top graph shows recent purification results revealing low FH 19/20 yields whilst the bottom graph shows expected yields. Buffer B (50 mM Tris HCL, 1 M NaCl, 1 mM EDTA, pH 7.4) gradient is displayed as a dashed line.

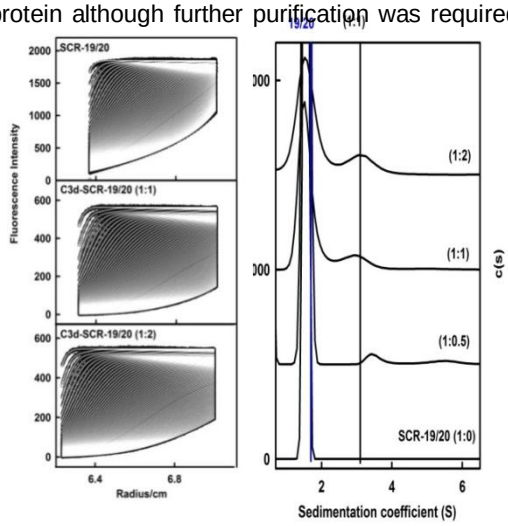

Figure 3- Preliminary FDS-AUC results using SEDFIT analysis. Fit curves on the left demonstrate that the data has been correctly fitted. This has yielded preliminary S values for FH 19/20 and our 1:1 complex as seen on the right giving $s_{20,w}$ values of 1.6 S and 3.6 S respectively.

Preliminary FDS results have been obtained and analysed using SEDFIT (Fig. 3). These revealed 1:1 complex formation in 137 mM NaCl at a range of ratios with an $s_{20,w}$ value of approximately 3.6 S. The observable peak must correspond to our complex as only FH 19/20 is fluorescently labelled whilst C3d is not. To confirm this, control experiments should be run to prove that C3d is not naturally fluorescent. Unfortunately, mechanical problems with the FDS stepper motors meant that no final results have been obtained.

Isothermal titration calorimetry required our titrant concentration to be tenfold higher than the sample concentration for a 1:1 interaction. When investigating a 2:1 interaction the titrant concentration should ideally be twentyfold higher. In this case, the titrant was C3d whilst the sample was FH 19/20. This has proven to be difficult to obtain despite the small volumes required. In addition, protein samples cannot be recovered so trial experiments have decreased the availability of our proteins. As a result, trial experiments showed saturation to be reached during initial injections whilst the transition point was not reliable. This indicated that further experiments would be required to obtain trustworthy data.

Departures from original proposal

Due to time constraints, further experiments on the FDS were not done. However, I observed the use of other techniques such as surface plasmon resonance in other projects.

Future directions

Further work is required on ITC as insufficient concentrations meant that saturation was reached too early. To support our results, a control experiment on the FDS should be run to indicate that C3d is not fluorescent and does not contribute to the signal observed, and a full set of experiments in 137 mM NaCl and 50 mM NaCl need to be done.

Value of studentship

My experience within the lab has been invaluable. The project has complemented my undergraduate studies and I have gained confidence within the laboratory. I have witnessed a variety of biophysical techniques and through much guidance, being involved in research has finalised my decision to pursue a PhD following my undergraduate studies. For the lab, the value of the studentship has been an extra pair of enthusiastic hands to help resolve the stoichiometry of the C3d complex with FH 19/20.

References

[1] Morgan, H. *et al.* 2011, Structural basis for engagement by complement factor H of C3b on self surface *Nat. struct.mol.biol.* [online] Available at: http://www.nature.com/nsmb/journal/v18/n4/full/nsmb.2018.html[Accessed: 29 June 2013]
[2] Kajander, T. *et al.* 2011, Dual interaction of Factor H with C3d and glycosaminoglycans in host-non host discrimination by complement *Proc.Natl.Acad.Sci.USA* [online] Available at: http://www.pnas.org/content/108/7/2897.long [Accessed: 29 June 2013]