Binding Analysis of Factor H Related Proteins with C3d

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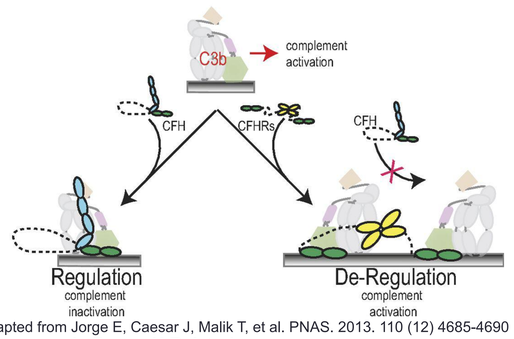
ABSTRACT

The complement system is a major arm of the innate immune system which helps recognize foreign particles and clear them. Recognition of foreign particles is mediated in part by Factor H and Complement Factor H Related (CFHR) proteins.[1] Dysregulation of Factor H and CFHR proteins can result in multiple diseases such as atypical hemolytic uremic syndrome (aHUS), C3 glomerulopathies, as well as other kidney diseases.[1] aHUS is a rare condition that leads to end-stage renal disease in approximately 60% of patients.[1] Here we produced modified versions of mouse CFHR proteins using transfected mammalian cell lines. The CFHR proteins were then tested in binding affinity assays against a well characterized complement pathway protein, C3d. Initial studies showing binding indicate the need for further development of these related protein variants. These experiments can help us better understand how inflammation is controlled in the kidney and may help to identify novel therapeutic strategies.

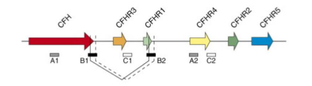
BACKGROUND

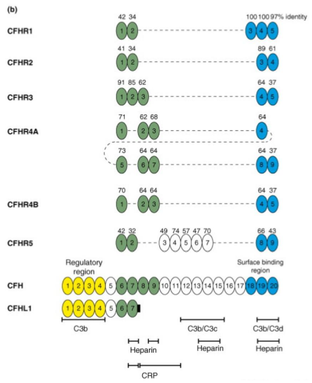


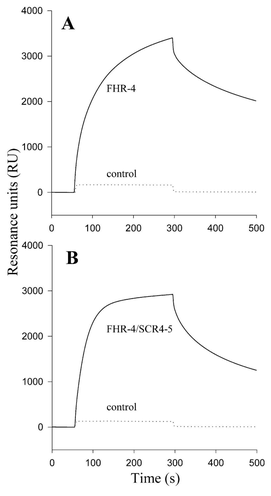
Adapted from Jorge E, Caesar J, Malik T, et al. PNAS. 2013. 110 (12) 4685-4690

Overview of the genomic Factor H Related Proteins. (a) Relative location of all FHR proteins and Complement Factor H on Chromosome 1. (b) All FHR proteins contain repeated sequences from Complement Factor H indicating that FHR proteins may interact with the same proteins as Complement Factor H.

Adapted from Jozsi M, Zipfel P. Trends in Immunology. 2008. 29:8 380-387







Binding of Factor H Related Proteins with C3d. Previous research has characterized binding of the genome coded FHR proteins with C3d. This research indicates that since interactions exist then FHR proteins may have a regulatory role in the alternative compliment pathway.

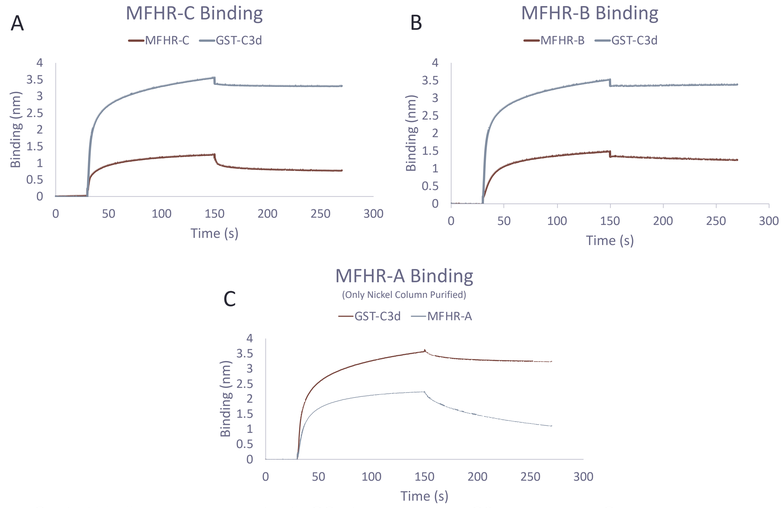
Adapted from Hellwage J, Sakari Jokiranta T, Friese M, et al. J Immunol. Dec 15, 2002, 169 (12) 6935-6944;

RESEARCH QUESTION

- **If we produce forms of FHR proteins which strongly bind to C3d, can we artificially induce function of this protein and restore compliment activation and function in mutated individuals?**

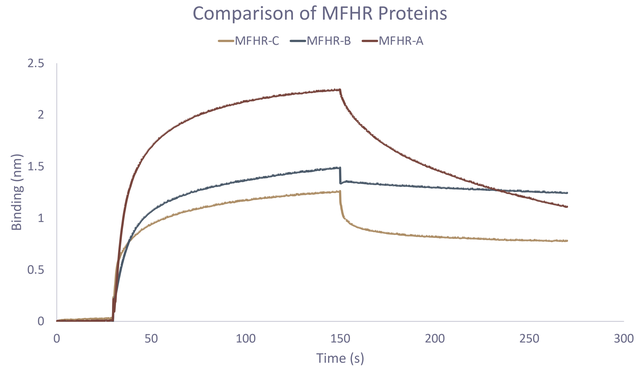
RESULTS

Figure 1: (A) Mouse FHR protein form C initial binding analysis with GST tagged human C3d. (B) Mouse FHR protein form B initial binding analysis with GST tagged human C3d. (C) Mouse FHR protein form A initial binding analysis with GST tagged human C3d. (FHR-A was only purified by nickel column)



Control binding measurement with GST tagged human C3d is shown in Gray.

Figure 2: Comparison of the two Mouse FHR proteins binding to Human C3d



Preliminary binding analysis seems to show that MFHR-B is able to bind better to C3d than MFHR-C. Nickel column purified MFHR-A appears to show significant affinity but significant dissociation.

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METHODS

Amplification of FHR protein DNA plasmids:
Competent E. coli cells were transformed with desired DNA containing an E. coli gene for ampicillin resistance. Transformed cells were plated on a minimal media plate containing ampicillin and incubated for 16 hours. Individual colonies were picked from the plates and placed in a 5 mL LB+Amp solution and cultured for 16 hours at 37 °C in a shaker. The 5 mL culture was then placed in 500 mL of LB+Amp solution and cultured 16 hours at 37 °C in a shaker. DNA was isolated using the Zymopure II Plasmid Maxiprep Kit (Zymo Research).

Mammalian Cell Culture and Transfection:
An established line of 293T cells were thawed and 1.5 mL of the cells were resuspended in 8.5 mL of complete DMEM (Prepared with 500 mL DMEM, 55 mL of FBS 1 μg/mL, 5.5 mL of 1000x pen/strep) and centrifuged down for 5 mins at 1500 rpm and 22 °C. The cell pellet was then resuspended in 10 mL of complete DMEM and plated in a 75 cm^2 flask. Cells were incubated at 37 °C, 8% CO_2 until cells reached 90% confluency in the flask. Cells were then split by removing original DMEM, washing with PBS, and removing the cells from the flask with 10 mL of new complete DMEM cells, which were then distributed into 9 new 225 cm^2 flasks in a 1:25 dilution with a final volume of 50 mL in each flask. The remaining volume of cells were placed in two new 75 cm^2 flasks in a 1:10 dilution. Cells were then incubated until 90% confluency and transfected. Transfection media was prepared by placing DMEM (no additives) in two tubes equal to 1/20 culture volume in each tube. To one tube, 1μg of DNA per 5cm^2 of culture area was added. To the other tube, PEI was added in a 1:4 DNA:PEI ratio. The tubes were equilibrated for 15 mins then the PEI tube was poured into the DNA tube. After another 15 minute incubation, equal amounts of the DNA/PEI solution was added to each flask. Flasks were incubated overnight then media was removed, cells were washed with PBS, and 50 mL of Complete freestyle media (1% pen/strep) was added.

Supernatant Harvesting:
After incubation for 5 days in freestyle media, freestyle media was removed and placed in 50 mL falcon tubes. Tubes were centrifuged down at 9000 rpm for 30 mins at 4 °C. Clarified supernatant was placed in a sterile bottle and stored at 4 °C until purification. 50 mL of pre-warmed Freestyle was placed in the harvested flasks. Flasks were harvested a second time after 4 days.

Nickel Column Purification:
Supernatant was clarified again by centrifuging at 3500 rpm for 22 mins at 4 °C. Clarified supernatant was run through an equilibrated 5 mL His-trap nickel column. After the majority of supernatant was through the column, the column was washed with 5 column volumes of 20 mM imidazole. Protein was then eluted into 12 fractions of 1.5 mL using 300 mM imidazole. The presence of protein was confirmed using NanoDrop 2000c Spectrophotometer (Thermo Scientific).

FPLC purification:
Fractions that contained protein were concentrated down to 0.5 mL and injected into a s200 increase sucrose column.

Expression and Purification of Human C3d:
C3d was expressed using the pGex system, thus tagging the C3d with Glutathione S-Transferase (GST). Bacterial clones were incubated with shaking at 37 °C until OD600 was 0.3. Subsequently, the culture was induced with IPTG, and cultures were grown overnight at 25 °C. Bacterial cells were harvested by centrifugation, lysed by sonication, and supernatant passed over a GST affinity column followed by bulk elution using Glutathione. The concentrated eluent was further purified using FPLC as above.

BLItz Binding Analysis:
Protein samples were run through BLItz analyzer (ForteBio) using the Simple Kinetics protocol. Specifically, C3d was bound to Anti-GST analyzer tips and then the same tip was subjected to binding analysis with the relevant protein.

FUTURE WORK/ ACKNOWLEDGEMENTS

- Perform binding analysis for FPLC purified MFHR-A protein with C3d
- Test binding of MFHR proteins with Simian Monkey C3d

Acknowledgements

- University of Colorado Colorado Springs Chemistry and Biochemistry Department and the Biochemistry Research Lab of Dr. James M Kovacs

Sources:

1. Skerka C, Chen Q, et al. Complement Factor H Related Proteins (CFHRs). Molecular Immunology. 2013. Volume 56 Issue 3. Pages 170-180