Autolytic Cleavage of C3

Autolytic cleavage was performed by adding 10 µl of C3 (5 ng/µl) to 10 µl 0.1 M Tris acetate buffer pH 8.3 and 2 µl 10% SDS. After 1 h incubation at 80°C, 11 µl of 3× reducing electrophoresis buffer was added. The sample was heated for 5 min at 95°C and then loaded onto a 10% polyacrylamide gel. Western blot using a rabbit polyclonal antibody to C3 was employed to monitor the α chain fragments.

C3 ELISA

This was developed to quantitate the concentration of C3 in the transfection supernatants. Briefly, wells (Nunc Immuno Modules, Rochester, NY) were coated with a monoclonal anti-C3d antibody (Quidel, San Diego, CA) at 2 µg/ml in PBS overnight at 4°C and then blocked with 1% BSA and 0.01% Tween-20 in TBS for 60 min at 37°C. Wild-type and mutant C3 proteins and purified C3 (Complement Technologies, Tyler, TX) were added and the samples incubated for 60 min. After washing with PBS containing 0.05% Tween 20, affinity purified chicken anti-human C3 (1:10,000) (Biodesign International, Saco, ME) was applied for 1 h at 37°C. After washing, an HRP-linked donkey anti-chicken IgY (1:10,000) (Jackson Immunoresearch, West Grove, PA) was added for 1 h at 37°C. Following washing, TMB substrate (Pierce, Rockford, IL) was added and absorbance was read at OD 630.

Recombinant MCP production

MCP repeats 1-4 were cloned into the EcoR1 and Not1 sites of pET28a+-2 (modification of PET28a+, EMD Biosciences, La Jolla, CA). The MCP insert was generated by PCR using the template MCP-BC1/pSG5 [1] and the following oligos:
5′-CCGGAATTCATGTGTGAGGAGCCACCAACAT-3′ and
5′-ATAAGAATGCGGCCGCTTACACTTTAAGACACTTTGGAAC-3′
For recombinant protein induction, 500 ml of LB containing kanamycin (30 µg/ml) and chloramphenicol (34 µg/ml) were inoculated with 25 ml of an overnight culture of the MCP-expressing clone and grown to an OD 600 of 0.6-0.8. Protein synthesis was induced with 1 mM IPTG at 37°C for an additional 3-5 hrs (according to EMD Biosciences directions). Cells were harvested and pellets were frozen at -80°C.

For inclusion body protein purification, pellets were thawed and resuspended in 50 ml of Solution Buffer (50 mM Tris pH 8.0, 25% sucrose, 1 mM EDTA, 0.01% NaN_3, and 10 mM DTT) to which 0.8 ml of freshly prepared 50 mg/ml lysozyme, 1250 units of benzonase nuclease (Novagen, LaJolla, CA), and 1 ml of 1M $MgCl_2$ were added. An equal volume of lysis buffer (50 mM Tris pH8.0, 1% Triton X-100, 0.1 M NaCl, 0.01% NaN_3, and 10 mM DTT) was added and the solution was stirred gently at room temperature for one h. After cooling, the suspension was sonicated with three 15 sec bursts (Fisher Scientific Model 500 Sonic Dismembrator) at 50% amplitude followed by the addition of 5 ml of 0.5 M EDTA. The lysate was then centrifuged at 6000 rpm for 30 min at 4°C (Beckman JA-14). The resulting inclusion body pellet was washed (Wash Buffer: 50 mM Tris pH 8.0, 0.5% Triton X-100, 0.1 M NaCl, 1 mM EDTA, 0.01% NaN_3, and 1 mM DTT) followed by washing with same buffer as above, except lacking Triton X-100.

For solubilization of the inclusion bodies the pellet was resuspended in 6 M Guanidine HCl, 10 mM Tris pH 8.0, and 20 mM β-mercaptoethanol and centrifuged at 14,000 × g for 10 min. A

second high speed spin at 100,000 × g for 30 min at 4°C was performed to remove any insoluble material.

For protein refolding, solubilized inclusion body protein was added drop wise in three injections over 36 hrs at a final concentration of 10-100 µg/ml in refolding buffer (100 mM Trizma Base, 400 mM L-arginine-HCl, 2 mM EDTA, 0.02 M ethanolamine, 0.5 mM oxidized glutathione, and 5 mM reduced glutathione). The refolding solution was concentrated in a Millipore Stirred Filtration Cell followed by buffer exchange.

Ligand binding assays

Proteins were coated on wells using 2 µg/ml of sMCP (prepared as above), factor H (Complement Technologies), soluble CR1 (gift of H. Marsh, Avant Immunotherapeutics, INC, Needham, MA) or factor B (Complement Technologies) in PBS overnight at 4°C followed by blocking at 37°C for 60 min (1% BSA, 0.1% Tween in 10 mM Tris, 150 mM NaCl). Dilutions of wild-type and mutant C3 proteins were prepared in a low salt ELISA buffer for assessing binding to MCP and factor H (10 mM Tris pH 7.2, 25 mM sodium chloride, 0.05% Tween 20, 4% BSA) or, for assessing binding to factor B, in low salt buffer containing 10 mM magnesium chloride. Following an incubation at 37°C for 1 h, the sample was washed (10 mM Tris, 25 mM NaCl, 0.05% Tween), and affinity purified chicken anti-human C3 (1:10,000) (Biodesign International, Saco, ME) was applied for 1 h at 37°C. After washing an HRP-linked donkey anti-chicken IgY (1:10,000) (Jackson Immunoresearch, West Grove, PA) was applied for 1 h at 37°C. Following washing, TMB substrate (Pierce) was added and absorbance was read at OD 630. Values are given as percent of binding to wild-type C3. These binding assays are typically carried out under low ionic strength conditions because of the low affinity of ligands to monomeric iC3 or C3b at physiologic ionic strength.

Cofactor assays

C3 preparations were incubated for 0 to 30 min at 37°C with factor I (5 ng in MCP assays and 20 ng in factor H assays) (Complement Technologies) and a cofactor protein MCP (50 ng recombinant) or factor H (200 ng) (Complement Technologies) in 15 µl of buffer (10 mM Tris, pH 7.4, 150 mM NaCl). To stop the reaction, 7 µl of 3× reducing Laemmli sample buffer was added. Samples were boiled at 95°C for 5 min, electrophoresed on 10% Tris-glycine polyacrylamide gels, transferred to nitrocellulose and blocked overnight with 5% non-fat dry milk in PBS. These blots were probed with either a 1:10,000 dilution of chicken anti-human C3 (Biodesign International) followed by HRP-conjugated donkey anti-chicken IgG (Jackson Immunoresearch Laboratories) or, after stripping (see below), a 1:5000 dilution of goat anti-human C3 (Complement Technologies) followed by HRP-conjugated donkey anti-goat IgG (Jackson Innumoresearch Laboratories). The blots were developed with SuperSignal substrate (Pierce).

Membranes were stripped by washing with double-distilled water for 5 min, followed by washing in 0.2 M sodium hydroxide for 5 min and a final water wash of 5 min.

REFERENCES

1. Liszewski MK, Leung M, Cui W, et al. Dissecting sites important for complement regulatory activity in membrane cofactor protein (MCP; CD46). *J Biol Chem*. 2000;275:37692-37701.