Supplement

Methods

Generation of iC3

iC3 was generated by treating purified C3 with 200 mM methylamine pH 8.0 for 3 hours at 37°C. The solution was then diluted 1:20 in 25 mM Tris pH 8.0 and 5 mM EDTA. The reaction mixture was loaded onto a Mono Q column equilibrated in 20 mM Tris pH 8.0 containing 5 mM EDTA and eluted with a 0.0 - 0.5 M NaCl gradient. The iC3 peak was pooled, concentrated and frozen at -80°C.

Affinity maturation of YW144.2.45

To generate the library template for affinity maturation of YW144.2.45, the GCN4 leucine zipper of the parental phagemid was removed using Kunkel mutagenesis to provide a monovalent display Fab format. Three stop codons were incorporated in CDR-L3. A soft randomization strategy was used for affinity maturation, which introduced a mutation rate of approximately 50% at selected positions by using a poisoned oligonucleotide strategy with 70-10-10-10 mixtures of bases favoring the wild type nucleotides. Three different libraries were constructed based on combinations of CDR loops L1/L2/L3, L3/H1/H2 and L3/H3. Mutations were generated at positions 28-33 of CDRL1, 50 and 53-55 of CDRL2, 91-94 and 96 of CDRL3, 28-35 of CDRH1, 50-58 of CDR-H2, and 95-100 of CDRH3 (Fig. S1).

For selecting affinity-matured clones, phage libraries were subjected to plate sorting for the first round and followed by four rounds of solution phase sorting as described (1). After the first round of plate panning, four rounds of solution phase sorting were performed to enhance the efficiency of affinity-based selection with increased stringency achieved by decreasing the amount of biotinylated C3b from 10 nM to 0.5 nM and by adding non-biotinylated C3b as competitor during round 4 through 6.

After six rounds of panning, a high throughput single-point competitive phage ELISA was used to rapidly screen for high affinity clones as described (2). Clones were selected for further analysis based on the ratio of binding affinity in the presence or absence of 1 nM C3b.

Surface Plasmon Resonance

The anti-C3 antibody (S77) was amine coupled to a Biacore CM5 chip and blocked with ethanolamine. Complement proteins were individually tested at varying concentrations in HBS-P buffer (0.01 M HEPES, pH 7.4/0.15 M NaCl/0.005% Surfactant P20). All SPR analyses were performed at 25°C by using a flow rate of 20 µl/min. Sensorgrams were run in order from low to high concentration and in duplicate. All sensor chips were regenerated, without loss of activity, by using two injections (30 seconds) of 10 mM glycine, pH 1.5. Analysis of the binding curves and determination of Kd values were done using evaluation software (version 4.1; Biacore).

***Generation of CR1 LHR**AC*

Long Homologous Repeat (LHR) A and C of CR1 were generated by PCR amplification of a CR1 splice variant lacking LHR B (Origene Technologies). The PCR fragment was subcloned in a pRK vector containing a (6) His at the N-terminus. CHO cells were transiently transfected with the plasmid, and CR1 LHRAC was isolated using a nickel affinity column. The protein was further purified by ion-exchange chromatography and dialyzed against PBS buffer.

C3b ELISA for testing specificity of anti C3b antibody S77 IgG

To determine the selectivity of S77 and the concentration of S77-reactive C3 proteins in serum, S77 IgG was used as a capture antibody. To determine the concentration of total C3 proteins, a polyclonal antibody to C3 (C3 pAb, Cappel, Solon, OH, Cat. 55033) reactive with all C3 proteins was used as a capture antibody. Capture antibody was diluted in PBS to 2 µg/mL and added to wells of a 384-well MaxiSorp plate (Nunc). After a blocking step (PBS/0.5% BSA), C3b (Complement Technology, Inc.) or human plasma samples were diluted at a minimum dilution of 1:300, serial 1:4 in sample buffer (PBS, pH 7.4/0.5% BSA/0.05% Tween 20/0.2% bovine gamma globulin/0.25% CHAPS/5 mM EDTA/15ppm Proclin) and added to the plates. Plasma was obtained with informed consent from the donors. Following a 2 hr incubation, detection antibody (HRP-conjugated goat $F(ab')_2$ anti-human C3 (Protos Immunoresearch, Burlingame, CA, Cat. 765) diluted 1:7,000 in assay buffer (PBS/0.5% BSA/0.05% Tween-20) was added, and the plates were incubated for a final 2 hr. Color was then developed for 10 min using tetramethyl

benzidine (KPL, Gaithersburg, MD), and the reaction was stopped by adding 25 µl/well 1.0 M phosphoric acid. The absorbance was read at 450 and 630 nm wavelength.

Factor B, factor H, and CR1 binding assays

384-well MaxiSorp plates were coated overnight at 4°C with 3 µg/ml C3b in PBS (20 µl/well) prepared as described previously (3). The plates were washed 6 times with 100 µl PBS 0.1% Tween (PBST) (BioTek EL405 washer) and blocked for 2 hrs at RT with 4% BSA / 0.05% Tween / PBS (PBST). S77 or control Fab was added for 30 minutes at room temperature with shaking. CR1 (LHRAC, 50 nM), fH (0.33 µM), and fB (1 µM) were added to wells for 1 hr then washed 6x with PBST. To detect bound proteins, 2 µg/ml monoclonal anti-CD35 (CR1, BD), 2 µg/ml monoclonal anti-factor H (Quidel, San Diego), 1:5000 polyclonal-anti-factor B (Kent Labs, Bellingham, WA) in PBST were added to respective wells for 30 minutes then washed 6x PBST. Wells were subsequently incubated for 30 min with 1:7000 donkey-anti-mouse IgG (H+L) or -anti-goat IgG (H+L) HRPO (Jackson, West Grove, PA) then washed 9 x with PBST. Reaction was developed with 20 µl TMB substrate (BD) and stopped with 10 µl 2 N sulfuric acid. Plates were read at 450 nm.

Properdin binding assay

384-well MaxiSorp plates were coated overnight at 4°C with 10 µg/ml factor P (CompTech) in PBS (20 µl/well). The plates were washed 6 times with 100 µl PBS 0.1% Tween (PBST) (BioTek EL405 washer) and blocked for 2 hrs at RT with 4% BSA / 0.05% Tween / PBS (PBST). 50 nM C3b plus increasing concentrations of S77 or control Fab were mixed for 30 min @room temperature with shaking. Twenty ul C3b/inhibitor mix was added to properdin coated pates for 1 hour @room temperature. Anti-properdin antibody (Quidel #1) was added directly to properdin-coated plate for 30 min then 50 nM C3b was added. Plate was washed 6x with PBST and 20 ul 1:5000 anti-C3 HRPO (MP Biomedical) was added for 30 minutes, then washed 6x PBST. Reaction was developed with 20 µl TMB substrate (BD) and stopped with 10 µl 2N sulfuric acid. Plates were read at 450 nm.

Decay assays

The microtiter plate assay for the alternative pathway decay accelerating activity was performed as described previously (4) with the following modifications. 384-well Maxisorp plates were coated overnight at 4ºC with 20 µl of 3 µg/ml C3b in PBS. Plates were washed 6 times in PBST and blocked for 2 hrs at room temperature with PBST containing 4% BSA. Plates were incubated for 1 hr at room temperature with 400 ng/ml of factor B and 25 ng/ml of factor D in AP convertase buffer (veronal buffer (BioWhittaker), 0.15 mM $CaCl_2$, 2 mM $NiCl_2$, 25 mM NaCl, 0.05% Tween 20 and 4% BSA) , and washed 3x in PBST, followed by incubation for 15 min with either S77, control Fab or CR1 in AP convertase buffer, then washed 6x with PBST. To determine the effect of S77 on convertase formation, increasing concentrations of S77 or control Fab was added and incubated for 30 minutes. Next, factor B and factor D diluted in AP convertase buffer was added and incubated for 1 hr followed by washing 6 times with PBST. Factor Bb was detected with sequential 30 minute incubations of 1:5,000 dilution of goat anti-human factor B polyclonal antibody (Kent) in AP and 1:7,000 dilution of donkey anti-goat antibody conjugated to HRPO (Jackson) in AP convertase buffer. Color was developed with TMB substrate (BD), stopped in 2N H_2SO_4 and absorbance read at 450 nm.

Factor H and CR1 co-factor gel assay

All dilutions were made in 0.1% gelatin veronal buffer containing 0.15 mM $CaCl_2$ 1 mM $MgCl_2$. 0.4 µM C3b plus increasing concentrations of S77 or control Fab were incubated for 20 minutes at room temperature. Then 20 nM fI and 20 nM fH or 12 nM CR1 were added. The mixture was incubated for 60 min at 37 °C, stopped with Laemmeli's buffer (Bio-Rad, Hercules, CA) and the samples visualized on an 8% gel with Simply Blue Safe Stain (Invitrogen, Carlsbad, CA).

C3 convertase assays

The effect of S77 on fluid phase C3 convertase activity was determined by incubating 0.067 µM purified C3 with increasing concentrations of S77 or control Fab in GVB (veronal buffer [BioWhittaker]/0.5% bovine skin gelatin [Sigma]) at room temperature for 10 min. Thereafter, 0.4 µM each factor B and factor D were added in the presence of 33 mM MgEGTA in a total volume of 75 µl to activate

the pathway. After a 10 min incubation at room temperature, C3a produced in the reaction mixtures was converted to the inactive C3a des Arg by the addition of rabbit serum (25 µl, diluted 1:2 with GVB), followed by the addition of 0.25 M EDTA (100 µL). C3a des Arg was measured by an ELISA in which anti-C3a/C3a des Arg (Abcam) was used as the capture antibody and biotinylated anti-C3a (Fitzgerald) was used as the detection antibody.

For analysis of C3 convertase activity by gel-electrophoresis, 0.4 µM purified C3 and S77 or control Fab were incubated for 20 minutes at room temperature in PBST/0.15 mM $CaCl_2$/1 mM $MgCl_2$. 0.4 µM factor B and 0.04 µM factor D were added and incubated for 30 minutes at 37ºC. The reaction was stopped by adding Laemmli's sample buffer containing 2-mercaptoethanol, boiled for 3 min, and electrophoresed on an 8% SDS-PAGE gel (Invitrogen). Proteins were visualized by staining the gel with Simply Blue (Invitrogen). The difference in IC50 of S77 Fab inhibition of fluid-phase C3 convertase using the ELISA versus gel electrophoresis can be explained by the differences in C3 concentrations used in each assay.

C5 convertase assay

C5 convertase was assembled on zymosan particles (CompTech) according to Rawal and Pangburn (5) with the following modifications. Activated zymosan particles (CompTech) were washed in 1x GVB (0.1% gelatin veronal buffer). 10^{10} zymosan particles, 2 mg purified C3 and 5 µg trypsin in 200 µl GVB were incubated for 10 minutes at 22º C, repeating the C3-trypsin step. Zymosan-C3b was washed 6x in GVB, resuspended in 100 µl GVB, and 50 µl containing 35 µg factor B and 0.5 µg factor D plus 50 µl of 10 mM $NiCl_2$ were added and incubated for 5 minutes at 22ºC, washed in GVB and repeated 3x.

Convertase activity was determined by adding C5 to zymosan-C3b as follows. C5 was preincubated for 20 minutes at 37ºC to eliminate any C5b,6 activity. A 25 µl reaction mix in siliconized tubes containing 5 µM C5, 258 nM factor B, 167 nM factor D, 833 nM C6, 0.5 mM $NiCl_2$, increasing concentrations of S77 or control Fab and $2x10^6$ zymosan-C3b was incubated for 15 minutes at 37ºC. Reaction was stopped with 200 µl ice cold GVB-10 mM EDTA (GVBE). C5 converatse activity was measured by C5b,6-mediated lysis of chicken erythrocytes (Ec). 25 µl aliquot of C5 convertase reaction was added to $1x10^7$ Ec and 500 µl human serum in 225 GVBE on ice, transferred to 37ºC water bath and

incubated for 10 minutes. Unlysed Ec were pelleted for 1 minute at 10,000 x g and 200 ul supernatant was read at 414 nm for Ec lysis.

C5 binding assay

To determine whether S77 blocked C5 binding to C3b, 3 µg/ml C3b in PBS was coated in 384-well Maxisorp plates overnight at 4ºC the washed 6x in TBST (20 mM Tris pH7.5, 150 mM NaCl, 0.05% Twee-20) and blocked in TBST plus 4% BSA for 2 hours at room temperature. S77 or control Fab plus 400 nM C5 (CompTech) were added in TBST containing 20 mM $CaCl_2$, 20 mM $MgCl_2$, and 1% BSA for 1 hour at room temperature and Washed 6x in TBST. Bound C5 was detected with 2 µg/ml monoclonal anti-human C5 (clone 7D12, Genentech) incubated for 30 minutes, washed 6 times, and incubated with 1:5000 anti-mouse IgG HRPO (Jackson) for 30 minutes at room temperature. After washing 6 times with TBST, reaction was developed with TMB (KPL) and stopped with 2 N H_2SO_4.

Hemolysis assays

To determine the effect of S77 on alternative pathway activity, rabbit erythrocytes (Er, Colorado Serum) were washed 3x in GVB and resuspended to 2 x 10^9/ml. Increasing concentrations of S77 or control Fab (50 µl) were mixed with 20 µl of the Er suspension which was diluted 1:1 with GVB/0.1M EGTA/0.1M $MgCl_2$ (GVB^{++}) just prior to addition. Complement activation was initiated by the addition of C1q-depleted human serum (Quidel; 30 µl diluted 1:3 in GVB). After a 30 minute incubation at room temperature, 200 µl GVB/10 mM EDTA were added to stop the reaction and samples were centrifuged for 5 min at 500 g. Hemolysis was determined in 200 µl supernatant by measuring absorbance at 412 nm. Data were expressed as % of hemolysis induced in the absence of the inhibitor. To determine the effect of CRIg on the classical pathway of complement, a similar procedure was followed except that Er were replaced with IgM-coated sheep erythrocytes (E-IgM, Complement Technologies) and the assay was performed in factor B deficient human serum in GVB^{++}. Anti C5 antibody, generated by immunizing mice with human C5 and which block cleavage of C5, were used as a positive control in the classical pathway hemolytic assay.

References

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Supplemental Figures

Fig. S1. Alignment of the light-chain (LC) and heavy-chain (HC) variable region amino acid residues of the naïve (YW144.2.45) and affinity matured (YW144.2.45.S77 or S77) phage antibodies. Orange indicated the amino acid substitutions introduced for affinity maturation of S77. Boxes indicate CDR sequences by Kabat definition (6).

Fig. S2A. ***Alignment of human and rhesus (Macaca mulatta) C3***. Indicated in green are residues in the beta chain that are in contact with S77. Purple highlights indicate residues in the alpha chain that are in contact with S77. Arrow indicates amino acid substitution in Rhesus (Macaca Mulatta) C3 (H898F) crucial for S77 binding to C3b.

Fig. S2B. ***Complex of S77with C3b***.

S77 is depicted as a molecular surface, heavy chain in yellow and the light chain in grey. The main chain of C3b is depicted as a grey ribbon. Residues important for CR1 cofactor activity and fH binding (7) are color coded as indicated in Fig. S2A.

Fig. S3. ***S77 does not affect binding of properdin to C3b.***

fP was coated on microwell plates followed by addition of C3b with increasing concentrations of S77 or a

fP blocking antibody. Binding of C3b to fP was determined with an HRPO-conjugated anti C3 polyclonal antibody. Data are expressed as mean ± SD of triplicate measurements.

Supplemental Table 1: Data collection and refinement statistics

Data collection	C3b/S77 Fab complex
Space group	C2
Cell parameters	a=216.4, b=180.4, c=154.6, β=115.7
Resolution (Å)	50-3.1
R_{sym}[b]	0.135 (0.667)
Number of observations	379428
Unique reflections	101495
Completeness (%)	100.0 (100.0)
Refinement	
Content of asymmetric unit	2 C3b/S77-Fab complexes
Resolution (Å)	20-3.1
Number of reflections	91568
Final R[c], R_{free} (F>0)	0.21.7, 0.283
Number of residues	3959
Number of non-H atoms	31007
Rmsd bonds (Å)	0.007
Rmsd angles (°)	1.1

[a] Numbers in parentheses refer to the highest resolution shell.

[b] $R_{sym} = \Sigma|I-\langle I\rangle| / \Sigma I$. <I> is the average intensity of symmetry related observations of a unique reflection.

[c] $R = \Sigma|F_o-F_c| / \Sigma F_o$. R_{free} is calculated as R, but for 5 % of the reflections excluded from all refinement.

LC

Kabat - CDR L1: Kabat# 24–34

Kabat#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
YW144.2.45	D	I	Q	M	T	Q	S	P	S	S	L	S	A	S	V	G	D	R	V	T	I	T	C	R	A	S	Q	D	V	S	T	A	V	A	W	Y	Q
YW144.2.45.S77	D	I	Q	M	T	Q	S	P	S	S	L	S	A	S	V	G	D	R	V	T	I	T	C	R	A	S	Q	D	V	S	T	A	V	A	W	Y	Q

Kabat - CDR L2: Kabat# 50–56

Kabat#	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74
YW144.2.45	Q	K	P	G	K	A	P	K	L	L	I	Y	S	A	S	F	L	Y	S	G	V	P	S	R	F	S	G	S	G	S	G	T	D	F	T	L	T
YW144.2.45.S77	Q	K	P	G	K	A	P	K	L	L	I	Y	S	A	S	F	L	Y	S	G	V	P	S	R	F	S	G	S	G	S	G	T	D	F	T	L	T

Kabat - CDR L3: Kabat# 89–97

Kabat#	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108
YW144.2.45	I	S	S	L	Q	P	E	D	F	A	T	Y	Y	C	Q	Q	S	Y	T	T	P	P	T	F	G	Q	G	T	K	V	E	I	K	R
YW144.2.45.S77	I	S	S	L	Q	P	E	D	F	A	T	Y	Y	C	Q	Q	S	Y	A	T	L	P	T	F	E	Q	G	T	K	V	E	I	K	R

HC

Kabat - CDR H1: Kabat# 31–35

Kabat#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
YW144.2.45	E	V	Q	L	V	E	S	G	G	G	L	V	Q	P	G	G	S	L	R	L	S	C	A	A	S	G	F	T	F	S	S	S	S	I	S
YW144.2.45.S77	E	V	Q	L	V	E	S	G	G	G	L	V	Q	P	G	G	S	L	R	L	S	C	A	A	S	G	F	S	F	T	S	S	S	V	S

Kabat - CDR H2: Kabat# 50–65

Kabat#	41	42	43	44	45	46	47	48	49	50	51	52	A	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74
YW144.2.45	P	G	K	G	L	E	W	V	G	I	I	Y	P	Y	D	G	S	T	Y	Y	A	D	S	V	K	G	R	F	T	I	S	A	D	T	S
YW144.2.45.S77	P	G	K	G	L	E	W	V	G	L	I	Y	P	Y	N	G	F	N	Y	Y	A	D	S	V	K	G	R	F	T	I	S	A	D	T	S

Kabat - CDR H3: Kabat# 95–102

Kabat#	80	81	82	A	B	C	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	A	B	C	D	E	F	101	102	103	104	105
YW144.2.45	L	Q	M	N	S	L	R	A	E	D	T	A	V	Y	Y	C	A	R	N	A	L	Y	G	S	G	G	Y	Y	A	M	D	Y	W	G	Q
YW144.2.45.S77	L	Q	M	N	S	L	R	A	E	D	T	A	V	Y	Y	C	A	R	N	A	L	Y	G	S	G	G	Y	Y	A	M	D	Y	W	G	Q

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             520       530       540       550       560       570
CO3_HUMAN    GQREVVADSVWVDVKDSCVGSLVVKSGQSEDRQPVPGQQMTLKIEGDHGARVVLVAVDKG
CO3_MACACAM  GQREVVADSVWVDVKDSCVGSLVVKSGQSEDRQPLPGQQMTLKIEGDHGARVGLVAVDKG
             ********************************** ***************** *******
                 MG5                      MG6

             580       590       600       610       620       630
CO3_HUMAN    VFVLNKKNKLTQSKIWDVVEKADIGCTPGSGKDYAGVFSDAGLTFTSSSGQQTAQRAELQ
CO3_MACACAM  VFVLNKKNKLTQSKIWDVVEKADIGCTPGSGKDYAGVFSDAGLTFASSSGQQTAQRAELQ
             ********************************************* **************
                                           LNK

             640       650       660       670       680       690
CO3_HUMAN    CPQPAARRRRSVQLTEKRMDKVGKYPKELRKCCEDGMRENPMRFSCQRRTRFISLGEACK
CO3_MACACAM  CPQPATRRRRSVQLAEKRMDKVGQYPKELRKCCEHGMRENPMRFSCQRRTRYITLDEACK
             ***** ******** ******** ********** **************** * * ****
                                           ANA

             700       710       720       730       740       750
CO3_HUMAN    KVFLDCCNYITELRRQHARASHLGLARSNLDEDIIAEENIVSRSEFPESWLWNVEDLKEP
CO3_MACACAM  KAFLDCCNYITELRRQHARASHLGLARSNLDEDIIAEENIVSRSEFPESWLWKIEELKEA
             * **************************************************  * *** 
                         ANA                       α'NT         MG6

             760       870       880       890       800       810
CO3_HUMAN    PKNGISTKLMNIFLKDSITTWEILAVSMSDKKGICVADPFEVTVMQDFFIDLRLPYSVVR
CO3_MACACAM  PKNGISTKLMNIFLKDSITTWEILAVSLSDKKGICVADPFEVTVMQDFFIDLRLPYSVVR
             *************************** ********************************
                         MG6                                 MG7

             820       830       840       850       860       870
CO3_HUMAN    NEQVEIRAVLYNYRQNQELKVRVELLHNPAFCSLATTKRRHQQTVTIPPKSSLSVPYVI
CO3_MACACAM  NEQVEIRAVLYNYRQNQELKVRVELLHNPAFCSLATAKRRHQQTVTIPPKSSLSVPYVI
             ************************************ **********************
                                        MG7

             880       890       900       910
CO3_HUMAN    VPLKTGLQEVEVKAAVYHHFISDGVRKSLKVVPE
CO3_MACACAM  VPLKTGQQEVEVKAAVYHFFISDGVRKSLKVV--
             ****** *********** *************
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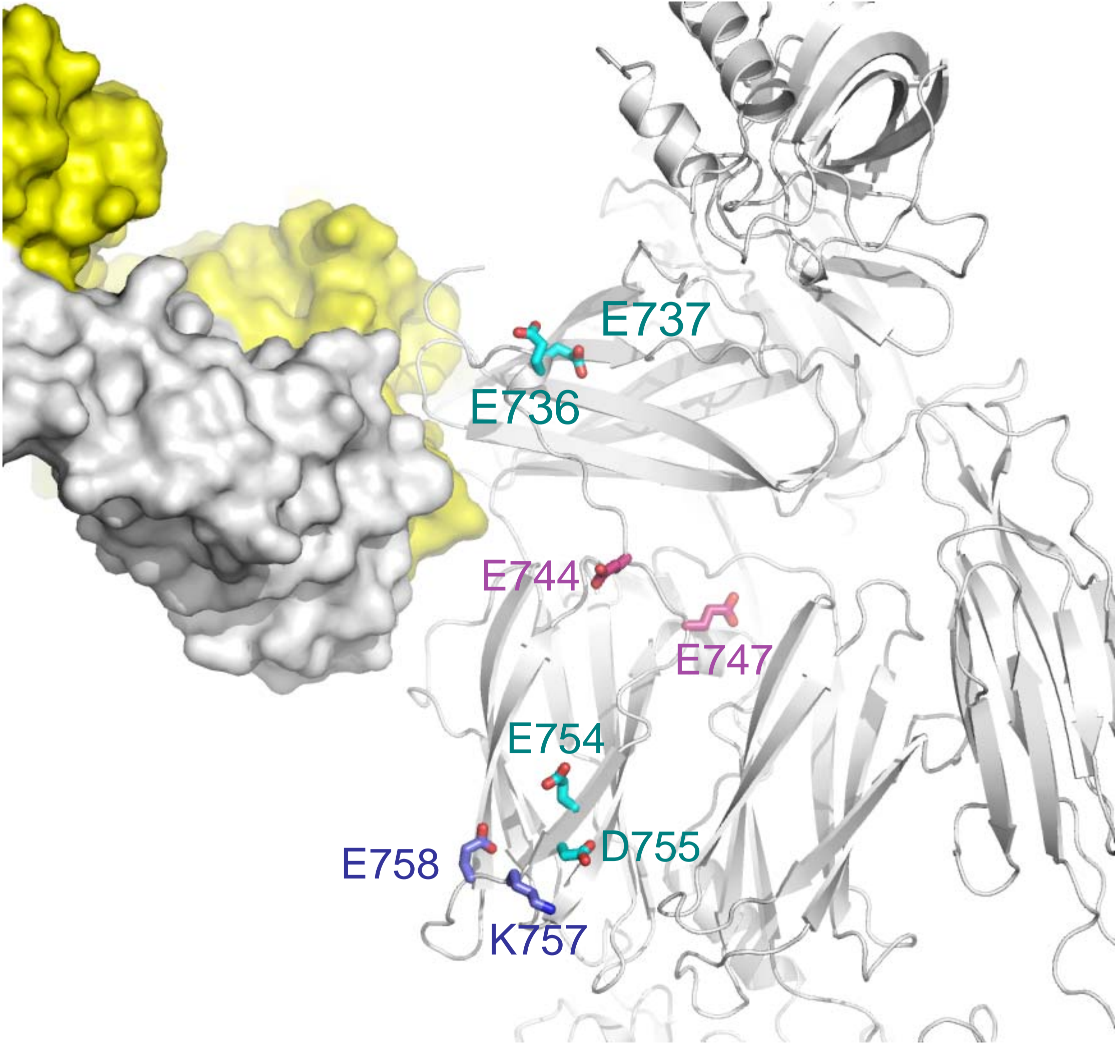
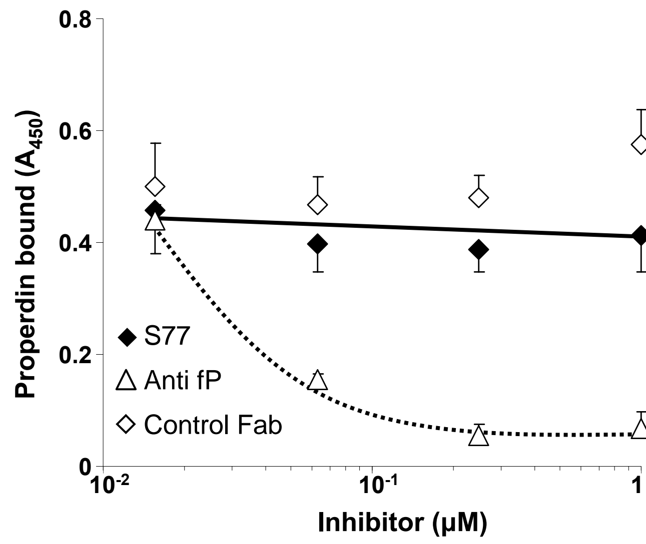
- (red circle) Important for binding fH to $C3H_2O$
- (light blue circle) Important for CR1 cofactor activity for the first 2 fI-mediated cleavages
- (dark blue circle) Important for CR1 cofactor activity for the third fI-mediated cleavages

S2A



S2B


0.8
0.6
0.4
0.2
0
Properdin bound (A_{450})
S77
Anti fP
Control Fab
10^{-2}
10^{-1}
1
Inhibitor (µM)