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SUPPLEMENTAL MATERIALS for "The interaction between fibrinogen and zymogen FXIII-A_2B_2 is mediated by fibrinogen residues γ390-396 and the FXIII-B subunits," James R. Byrnes, Clare Wilson, Anthony M. Boutelle, Chase B. Brandner, Matthew J. Flick, Helen Philippou, and Alisa S. Wolberg

SUPPLEMENTAL METHODS

Materials. Taq polymerase was from New England Biolabs (Ipswich, MA). Dulbecco's modification of Eagle's medium/Ham's F12, 50/50 mix was from Corning (Manassas, VA). Glycine was from Sigma (St. Louis, MO). Polyvinylidene fluoride membranes were from Millipore (Billerica, MA), 6X SDS loading dye was from Boston Bioproducts (Ashland, MA), and 10% Tris-glycine gels were from Bio-Rad (Hercules, CA).

Generation of recombinant Fibγ$^{390\text{-}396A}$. The fibrinogen γ-chain expression vector pMLP-γ[1,2] was used as a template to generate two overlapping fragments of cDNA corresponding to the C-terminal region of the γ-chain containing γ390-396A. The 5′ fragment was amplified using a forward primer containing a BstXI site (5′-CCATCTCAATGGAGTTTATT-3′) and a reverse mutagenesis primer (5′-TTGCTGTCCAGCTGCAGCCGCAGCTGCGGCGAATGGGAT-3′). The 3′ fragment was amplified using a forward mutagenesis primer (5′-ATCCCATTCGCCGCAGCTGCGGCTGCAGCTGGACAGCAA-3′) and a reverse primer containing a NotI site (5′-CTCTATATTACCCGCCGGCG-3′). These two fragments were gel-purified, thermally-denatured, and reannealed. The resulting hybrid fragment was extended and amplified using the BstXI and NotI primers before digestion with BstXI and NotI. This digested fragment was then ligated into BstXI- and NotI-digested pMLP-γ with T4 DNA Ligase (Thermo Fisher, Waltham, MA). Competent DH5α Escherichia coli were transformed with the ligated DNA and the γ390-396A mutation was confirmed by Sanger sequencing (Eton Bioscience, Research Triangle Park, NC). The pMLP-γ390-396A expression vector was then transfected into Chinese Hamster Ovary cells containing expression vectors for normal fibrinogen Aα- and Bβ-chains, as previously described.[2] Positive clones were identified by dot blotting with anti-human fibrinogen antibody.

Preparation of SPR analytes. FXIII ligands and fibrinogen analytes were dialyzed into running buffer at 4°C for 16 hours with rotation and one buffer change. Dialysis was performed in a volume 10,000-times that of the ligand volume using a Slide-A-Lyzer® MINI dialysis unit (MWCO 7,000 Daltons, Life Technologies, Grand Island, NY). Protein concentration was determined using a Nanodrop1000 (Thermo Fisher, Waltham, MA).

A COOH-V chip (SensiQ Technologies, Oklahoma City, OK) was installed into the SensiQ Pioneer per the manufacturer's instructions and the prime function was performed three times using filter-sterilized, degassed running buffer (10 mM HEPES, 140 mM NaCl, 0.05% Tween-20, pH 7.4). The chip surface was preconditioned for adsorption by injecting 2 bursts each of 10 mM HCl, 50 mM NaOH and 0.1% SDS (100 µL/min, 10 seconds). The chip was primed a further 3 times using running buffer. All chips were checked to ensure they were aligned for each flow channel; if they were not well aligned a normalize function was performed using 100% DMSO, followed by 3 prime steps. The chip surface was then activated by injecting a 50:50 solution of EDC/NHS (25 µL/min, 4 minutes), resulting in o-acylisourea active ester groups. The dialyzed FXIII ligands were flowed across the activated chip surface at 5 µL/min flow rate at minimum concentration of 50 µg/mL, diluted into 10 mM sodium acetate with a pH one order of magnitude lower than the pI of the protein. The target ligands were allowed to couple to the active esters via primary amine groups until an R_{MAX} of at least 50 was reached according to Equation 1. Any uncoupled ester groups were blocked by injecting ethanolamine (20 µL/minute, 5 minutes). Each protein target was immobilized on either flow-channel (FC) 1 or 3, and FC2 was always used as a reference channel.

Equation 1.

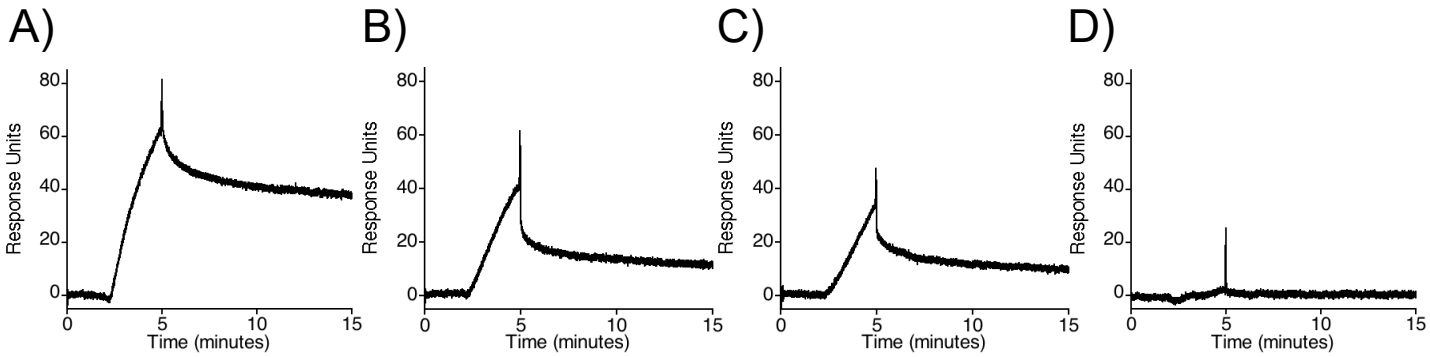
$$R_{MAX} = \frac{M_w\ Analyte}{M_w\ Ligand} \times R_L \times S_M$$

Where:
R_{MAX} = The maximum binding capacity assigned between the immobilized ligand and the analyte, in resonance units (RU).
M_w Analyte = Molecular mass of the molecule in solution in Daltons.
M_w Ligand = Molecular mass of the immobilized ligand in Daltons.
R_L = Immobilized ligand, in RU.
S_M = Predicted molecular stoichiometry of analyte to ligand.

Prior to analyte injections, the chip matrix was conditioned for regeneration using 3 bursts each of 1, 2, and 3 M NaCl (30 µL/min for 2 minutes), interspersed with 3 bursts of running buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, 1.5 mM $CaCl_2$) at the same rate. Salt was washed from the chip using 3 bursts of running buffer (30 µL/min for 2 minutes), and the loops were purged 3 times. All kinetic binding assays were performed with both the analysis and sample rack temperatures at 20°C. Three prime functions were performed prior to analysis using running buffer.

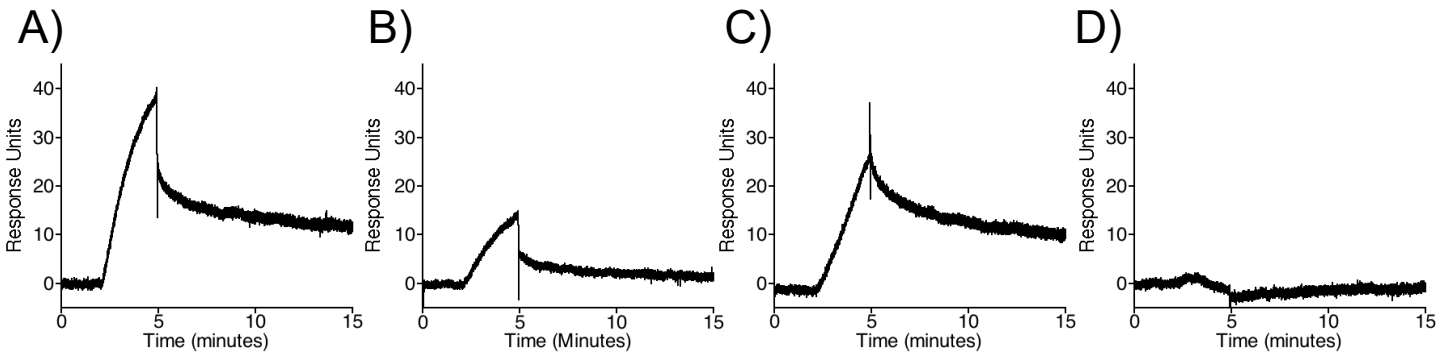
SPR using Taylor dispersion injections. SPR was performed using a OneStep® titration function based on Taylor dispersion injection (TDi) theory.[3,4] Compared to traditional (fixed concentration) injections that record analyte binding with respect to injection time of a series of samples of different concentrations, TDi analyzes a continuous analyte concentration gradient formed after a single injection in a capillary tube before the sample enters the SPR detector. This approach encodes a second, independent time domain into an analyte gradient, which permits the use of a single binding curve that does not need to reach steady-state to obtain binding affinities. Thus, compared to fixed concentration injections, OneStep® titration reduces the time required to analyze a given analyte, lowers the dependence of parameters on one another and on experimental variability, increases resolving power, and enables analysis of interactions from a fewer number of injections. This approach was particularly important for analyzing high concentrations of limited amounts of recombinant fibrinogen variants.

SUPPLEMENTAL FIGURES



Supplemental Figure 1. Recombinant fibrinogen binding to FXIII-A_2B_2. Surface plasmon resonance (SPR) was performed using OneStep® titration as described in the Methods and Supplemental Methods. Representative SPR binding curves using 1 µM fibrinogen (maximum) for (A) γA/γA, (B) γ′/γ′, (C) Aα251, and

(D) Fib$\gamma^{390-396}$ binding to FXIII-A_2B_2. Curves representative of n=3-6 experiments as indicated in Table 1.



Supplemental Figure 2. Recombinant fibrinogen binding to FXIII-B_2. SPR was performed as described in the Methods and Supplemental Methods. Representative SPR binding curves using 1 µM fibrinogen (maximum) for (A) γA/γA, (B) γ′/γ′, (C) Aα251, and (D) Fib$\gamma^{390-396A}$ binding to recombinant FXIII-B_2. Curves representative of n=4-6 experiments as indicated in Table 1.

SUPPLEMENTAL REFERENCES

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