Tunable modulation of antibody-antigen interaction by protease cleavage of protein M

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Abstract
While immunoglobulins find ubiquitous use in biotechnology as static binders, recent developments have created proantibodies that enable orthogonal switch-like behavior to antibody function. Previously, peptides with low binding affinity have been genetically fused to antibodies, to proteolytically control binding function by blocking the antigen-binding site. However, development of these artificial blockers requires panning for peptide sequences that reversibly affect antigen affinity for each antibody. Instead, a more general strategy to achieve dynamic control over antibody affinity may be feasible using protein M (ProtM) from Mycoplasma genitalium, a newly identified polyspecific immunity evasion protein that is capable of blocking antigen binding for a wide range of antibodies. Using C-terminus truncation to identify ProtM variants that are still capable of binding to antibodies without the ability to block antigens, we developed a novel and universal biological switch for antibodies. Using a site-specifically placed thrombin cut site, antibody affinity can be modulated by cleavage of the two distinct antibody-binding and antigen-blocking domains of ProtM. Because of the high affinity of ProtM toward a large variety of IgG subtypes, this strategy may be used as a universal approach to create proantibodies that are conditionally activated by disease-specific proteases such as matrix metalloproteinases.

KEYWORDS
antibody, antigen blocking, biological switch, protein M

1 | INTRODUCTION

Many naturally occurring and engineered proteins have the ability to respond to environmental stimuli, such as pH, temperature, small molecules, and enzymes (Dueber, Yeh, Chak, & Lim, 2003; Fong & Wood, 2010). These signal-responsive proteins are incredibly useful as they can provide control over protein function that can be tailored to a large variety of applications. For example, the mechanism for blood clotting depends on the switch-like behavior of pro-thrombin to trigger wound healing (Esmon, Owen, & Jackson, 1974). Developing antibodies to have tunable behavior could have widespread impact on biotechnology, given that antibodies are the fastest growing class of therapeutic drugs (Ecker, Jones, & Levine, 2015) and integral to countless biosensors and assays.

Recently, antibodies and antibody fragments have been further enhanced by developing proantibodies (Erster et al., 2012; Yang et al., 2016) and proantibody fragments (Thomas & Daugherty, 2009). In general, these antibody variants are initially inactivated by tethering a binding site-masking peptide to the antibody. Activation in vivo by a disease-specific protease, releases the antibody-binding domains and allows the antibodies to bind to antigens. For instance, a cancer-specific matrix metalloproteinase (MMP) yielded a 200-fold increase in binding affinity and restored anti-VCAM-1 binding in mouse tissues (Erster et al., 2012). Similarly, anti-EGFR activity was
tuned by creating a masked antibody (Donaldson, Kari, Fragoso, Rodeck, & Williams, 2009). ScFv versions of anti-EGFR antibodies, matuzumab and cetuximab, were genetically fused to a mutated EGFR fragment that has reduced affinity for the antibodies than wild-type EGFR. These domains were linked together with a MMP cleavable sequence and used to form heterodimers. These masked dimers exhibited an eight-fold decrease in anti-EGFR activity, and proteolysis using MMP-9 restored affinity. Although both of these methods hold considerable promise in enhancing the in vivo specificity, a different binding site-masking peptide with the required specificity and binding affinity must be selected by multiple rounds of panning for each antibody. Ideally, a technology that affords universal blocking and unblocking of antibody binding will greatly expand our flexibility in targeting any antigen of practical interest.

Many IgG-binding proteins have been reported and most of them bind either to the Fc region (protein A or G) or the light chain region (protein L) without blocking antigen binding sites in the complementarity determining region (CDR) (Hober, Nord, & Linhult, 2007). On the other hand, protein M (ProtM), a newly discovered antibody-binding domain found on the cell surface of *Mycoplasma genitalium*, binds tightly (Kd = 1–5 nM) to many human IgG subtypes by attaching to a conserved region of the variable light chain (Vλ) domain (Grover et al., 2014). Despite variability in the CDR, the C-terminus of ProtM has been shown to wrap loosely around the CDR, sterically clashing with antigens and blocking any interaction with antibodies as part of an immunity evasion system based on antibody neutralization (Grover et al., 2014).

Previously, Grover et al. (2014) identified a ProtM fragment composed of residues 78–468 (pM468) as a stable truncation though trypsin digestion of the ProtM/IgG complexes. The crystal structure of pM468 complexed with IgG revealed that several hydrogen bonds and salt bridges formed between residues 78–440 and the conserved IgG residues likely give ProtM its high binding affinity. In contrast, residues 441–468 were deemed to be interacting with antibodies in a largely disordered manner and extended over the CDR region, much like a cap that blocks entry to large antigens (Grover et al., 2014). Because pM468 still incorporates a significant portion of the reported antigen blocking motif (residues 441–556), additional C-terminal truncations are likely necessary to identify a stable ProtM variant capable of binding to antibodies without completely quenching the CDR.

Because of its broad specificity, ProtM has the potential to act as a universal antibody inhibitor. Since the antigen blocking mechanism is based on steric hindrance rather than tight binding to the CDR, it may be possible to create cleavable ProtM variants that provide the same tunable masking and unmasking function on therapeutic antibodies. This is supported by the two proposed distinct epitopes within ProtM that are responsible for either antibody binding (N-terminus amino acids 78–440) or antigen blocking (the C terminus amino acids 441–468; Grover et al., 2014). In this paper, we constructed several C-terminally truncated ProtM fragments that still maintain high antibody affinity. One particular truncation (termed pM420) retained the ability to bind to antibodies, but without the ability to block antigens (Figure 1). This pM420 fragment, when ligated with the missing C-terminal fragment using sortase A (SrTA; Chen et al., 2015), completely restored antigen blocking. Finally, proteolysis of a thrombin cleavable sequence in the domain junction abolished antigen blocking and restored antibody function, demonstrating the feasibility of a universal biological switch for antibodies.

2 | MATERIALS AND METHODS

2.1 | Materials

All primers (Table S1) were purchased from IDT (Coralville, IA). All ingredients for culturing media were purchased from Fisher Scientific (Pittsburgh, PA), all ingredients for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-rad (Hercules, CA), all enzymes related to DNA manipulation and cloning were purchased from New England Biolabs (Ipswich, MA), and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO). Zyppsy Plasmid Miniprep and Zymoclean Gel DNA Recovery kits were purchased from Zymo Research (Irvine, CA). Murine antimaltose binding protein (MBP) was purchased from New England Biolabs and murine anti-fluorescein isothiocyanate (FITC) was purchased from Fisher Scientific.

2.2 | Strains

*Escherichia coli* NEB5a (New England Biolabs, Ipswich, MA) [fhuA2 Δ(argF-lacZ)U169 phospho_αgluV44 φ80 Δ[lacZ]M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17] was used as the host for all genetic manipulations and plasmid maintenance. *E. coli* strain BLR (DE3; EMD Millipore, Madison, WI) [F- ompT hsdSB(rB- mB-) gal dcm (DE3) Δ(srl-recA)306::Tn10 (TetR)] and BL21 (DE3; EMD Millipore, Madison, WI) [fhuA2 [lon] ompT gal (Δ DE3) [dcm] ΔhsdS5 Δ DE3 = λ sBamH1o ΔEcoRI-B int::[lac::PlacUV5::T7 gene1] i21 Δnin5] were used as production hosts for all proteins.

2.3 | Expression of protein M truncations

All protM (accession number P47523) constructs were expressed in BL21 (DE3). Cell lines bearing the expression plasmids were grown in Terrific Broth (TB) media (12 g/L tryptone, 24 g/L yeast extract, 0.4% (v/v) glycerol, 9.4 g/L potassium phosphate monobasic, and 2.2 g/L potassium phosphate dibasic) supplemented with 50 µg/ml kanamycin. The cultures were initially inoculated from a preculture and grown to an OD600 = 0.8~1 at 37°C. The cultures were then shifted to 20°C and incubated without any inducer for 16 hr.

Cultures were pelleted using centrifugation at 4,000 × g for 10 min at 4°C, and the pellet was suspended in 1× TN150 (50 mM Tris-HCl, 150 mM NaCl pH 8.0) to an OD600 = 30. The cell suspension was sonicated to lyse the cells and clarified using centrifugation at 15,000g for 15 min at 4°C. The supernatant was decanted and stored at 4°C until use.
2.4 | Purification of ProtM truncations

Imidazole was added to clarified lysates to a concentration of 5 mM. Meanwhile, gravity columns were packed with Ni-NTA His-Bind Resin (Thermo Fisher Scientific, Waltham, MA). The column was used as instructed using TN150 + 10 mM imidazole as the equilibration buffer, TN150 + 20 mM imidazole as the wash buffer, and TN150 + 250 mM imidazole as the elution buffer. Eluates were dialyzed against TN150 to remove the excess imidazole.

2.5 | Expression of pMCT-ELP79 and (G4S)3-ELP79

Both pMCT-ELP79 and (G4S)3-ELP79 constructs were expressed in BLR (DE3). Cell lines bearing the expression plasmids were grown in Terrific Broth (TB) media (12 g/L tryptone, 24 g/L yeast extract, 0.4% (v/v) glycerol, 9.4 g/L potassium phosphate monobasic, and 2.2 g/L potassium phosphate dibasic) supplemented with 50 µg/mL kanamycin. The cultures were initially inoculated from a preculture to an OD600 = 0.05 and expressed overnight using leaky expression at 37°C.

Cultures were pelleted using centrifugation at 4,000 g for 10 min at 4°C, and the pellet was suspended in 1× TN150 (50 mM Tris-HCl, 150 mM NaCl pH 8.0) to an OD600 = 30. The cell suspension was sonicated to lyse the cells and clarified using centrifugation at 15,000 g for 15 min at 4°C. The supernatant was decanted and stored at 4°C until use.

2.6 | Purification of pMCT-ELP79 and (G4S)3-ELP79

All elastin-like polypeptide (ELP) constructs were purified using inverse transition cycling (Meyer, Trabbic-Carlson, & Chilkoti, 2001). The harvested lysate was incubated with 0.5 M (NH4)2SO4 diluted from a 3 M stock. After mixing, the solution turned turbid due to the formation of micron-sized aggregates. These ELP aggregates were then collected using centrifugation for 10 min at 12,000g at room temperature.
temperature (Liu, Tsai, Madan, & Chen, 2012). The supernatant was decanted from the pellet and the pellet was resolubilized using cold TN150 buffer. This pellet was incubated on ice with intermittent pipetting or overnight at 4°C with end-over-end rotation. This resuspension was then centrifuged for 5 min at 12,000g at 4°C. The supernatant, which contains purified ELP fusions, was decanted and stored at 4°C until use. This process, called inverse transition cycling (ITC), can be repeated to have a small incremental increase in purity.

## 2.9 Affinity assays for antibodies

Both dot blots and enzyme-linked immunosorbent assay formats can be used to analyze antibody affinities (Engvall & Perlmann, 1971; Towbin, Staehelin, & Gordon, 1979). All assays were performed using antibodies preincubated with protM constructs because protM cannot displace already formed antibody/antigen complexes in spite of its ability to block antibody/antigen interactions (Grover et al., 2014).

### 2.10 Dot blot using large molecule antigens (maltose binding protein)

Nitrocellulose membranes were wetted with 1× TBS (20 mM Tris-HCl, 150 mM NaCl; pH 7.5). The membrane was then dried on a stack of filter papers. Purified MBP was diluted to 1, 5, 10, 20, 50, and 100 ng/µl in 1× TBS (pH 7.5) and 1 µl of each sample was dotted onto the membrane. The membranes were incubated in blocking solution (1× TBS, 0.5% Tween 20; 0.8% milkfat) overnight at 4°C, followed by 2 hr incubation at room temperature with orbital shaking. During this time, the primary antibody, anti-MBP, was incubated at room temperature with 50-fold molar excess of protM at a 250 µl total volume. After blocking, the membranes were washed twice using 10 ml of TTBS (1× TBS, 0.5% Tween 20) for 5 min each. The membranes were then incubated with the protM/anti-MBP mixture, such that the anti-MBP was diluted 10,000-fold, on an orbital shaker at room temperature for 2 hr. These membranes were then washed three times using 10 ml of TTBS for 5 min each time. Then the membranes were incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse at a 5,000× dilution in 1× TBS for 30 min on an orbital shaker at room temperature. The membranes were washed twice using TTBS. The dot blots were visualized using a colorimetric reaction using the Bio-Rad AP Color development reagents NBT and BCIP according to the manufacturer’s instructions.

### 2.11 Using fluorescein isothiocyanate as the antigen

Unlike MBP, fluorescein does not bind to nitrocellulose membranes. Instead, anti-FITC incubated with protM was bound to the nitrocellulose membranes. In short, anti-FITC was incubated with 50-molar excess protM for about 1 hr. This mixture was dotted onto nitrocellulose membranes that were wetted in 1× TBS and dried using a stack of filter papers. The membranes were incubated in blocking solution for at least 2 hr and then washed twice using TTBS. The washed membranes were incubated with 1 µM FITC-oligonucleotide or fluorescein in 1× TBS for 1 hr on an orbital shaker. The membranes were washed twice using TTBS. FITC-oligo bound to anti-FITC was detected using fluorescence (Ex 488, Em 526) on a Typhoon Variable Mode Imager 9400 (GE Healthcare, Marlborough, MA).
2.12 | Quantification of dot blots using ImageJ

Images were converted to 8-bit after cropping to the relevant area. Background subtraction was performed by selecting the light background option along with the rolling ball radius set at 500 pixels. A circular area was drawn around a dot and the integrated pixel density was measured for each dot on the blot.

2.13 | Thrombin cleavage of ProtM variants and the effect on antibody deactivation

Ligated ProtM variants (10 µM) were incubated with 0.1, 0.5, or 1 mg/ml thrombin for 16 hr. The cleavage products were analyzed by SDS-PAGE. Ability of the cleaved products to inhibit MBP binding was tested as described above. For thrombin cleavage of the anti-MBP/pM420-lig-ThpMCT complex, pM420-lig-ThpMCT was first incubated with anti-MBP in 10-fold excess in a 100 µl total volume of TBS for 8 hr. Following the preincubation, thrombin was added to a concentration of 1 mg/ml to the appropriate sample, while the control sample was left untreated. Thrombin incubation occurred at room temperature overnight for 16 hr. Nitrocellulose membranes were dotted with 100 ng of purified MBP, blocked overnight, and washed as detailed above. After incubating with either anti-MBP or anti-MBP/pM420-lig-ThpMCT complex with and without thrombin cleavage, MBP binding was visualized as described above.

3 | RESULTS AND DISCUSSION

3.1 | Design of ProtM truncations

Since two proposed distinct regions within pM468 are responsible for antibody binding (N-terminus amino acids 78–440) and antigen blocking (the C-terminus amino acids 441–468; Grover et al., 2014), we reasoned that it may be possible to generate C-terminus truncation ProtM variants capable of antibody-binding without antigen blocking. Based on the proposed residues involved with antigen blocking, we constructed a series of C-terminus truncations of ProtM residues 78–556 (pM556) to residues 78–378, 78–439, 78–420, 78–440, and 78–468 to yield pM378, pM393, pM420, pM440, and pM468, respectively.

All ProtM variants were expressed with an N-terminal SH3 binding ligand (Kim & Chen, 2016) and a C-terminal hexahistidine tag in E. coli (Figure 2a). While the expression of full-length proteins was confirmed and purified using Ni-NTA columns for pM420, pM440, pM468, and pM556, significantly truncated products were detected for pM393 and pM378 and these constructs were excluded from further study (Figure S1). The ability of these truncated ProtM domains to retain IgG binding was first determined by mixing with a model human IgG. After incubation, ProtM-IgG complexes were recovered using the N-terminal proline-rich w3 ligands on the truncated ProtM domains by coprecipitation with their respective ELP binding partner fusions (SH3 domain) using the thermally induced phase transition property of ELP (Kim & Chen, 2016). All ProtM truncations were able to capture IgG while ELP-SH3 domains alone did not (Figure 2b,c). The binding affinity of pM420 appeared to be slightly compromised as a larger pM420 to IgG ratio was needed to achieve the same level of binding (Figure 2c).

3.2 | Evaluation of antigen binding by ProtM truncations

We next assessed the antigen-blocking ability of each truncation using dot blot analysis. A model antigen, MBP, was chosen because of ease of production, facile purification, and its relatively large size (42.5 kDa). Nitrocellulose membranes were dotted with purified MBP at 1, 5, 10, 20, 50, and 100 ng, respectively. These membranes were exposed to a murine anti-MBP antibody preincubated with either no

FIGURE 2  (a) Reduced sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of ProtM variants in cell lysates (L) and in purified fractions (P). (b) Nonreduced SDS-PAGE of elastin-like polypeptide (ELP)-2rSH3/ProtM complex pull-down of IgG. NC = Negative Control, 440 = pM440, 468 = pM468, 556 = pM556. (c) Nonreduced SDS-PAGE of ELP-3rSH3/pM420 complex ELP pull-down of IgG. M = Initial Mix, R = Resuspension after one ELP cycle.
ProtM (positive control), pM420, pM440, pM468, or pM556. The binding of anti-MBP to MBP was visualized in a colorimetric manner using an alkaline phosphatase (AP)-conjugated mouse-specific secondary antibody.

Compared to the positive control where highly intense spots were visible throughout the entire range of loaded MBP (1–100 ng), the addition of pM440, pM468, or pM556 clearly hindered anti-MBP from interacting with MBP (Figure 3a). Since pM440 still retained some level of antigen blocking ability, this suggests that the antigen blocking region may be more extensive than previously reported (Grover et al., 2014). This was further corroborated in the dot-blot analysis, which showed pM420 retained a small degree of antigen blocking (Figure 3a). Given that pM420 maintains affinity to anti-MBP (Figure 2c), this suggests that pM420 may be used as a minimized antibody-binding domain that has reduced interference with antigen binding (Figure S2).

Antigen blocking of smaller antigens, such as an oligonucleotide conjugated to FITC (FITC-oligo, MW = 18 kDa) was also investigated. Murine anti-FITC was preincipuated with either pM420, pM440, pM468, and pM556, and these mixtures were spotted onto a nitrocellulose membrane. After incubating with FITC-oligo, the membrane was imaged using a Typhoon Multi-Mode Imager (Amersham Biosciences) to detect fluorescence (Figure 3b). As with MBP, pM556 and pM468 showed virtually no FITC binding, confirming that these ProtM truncations can still successfully block the CDR/FITC-oligo interaction. On the other hand, complexing with pM440 and pM420 seemed to insufficiently disrupt antigen binding through steric clashes, and we hypothesize that the FITC-oligo/anti-FITC interface is likely smaller than the MBP/anti-MBP interface (Figure S2).

3.3 Design split ProtM modules for Srt-mediated reconstitution of antigen blocking

In order to use ProtM as a protease-responsive affinity switch to control antibody-antigen interaction, we needed to ensure that the C-terminal fragment of ProtM (residues 421–468, pMCT) responsible for antigen blocking can be reversibly added and removed from the C-terminus of pM420. To achieve reversibility, we turned to SrtA-mediated ligation (Levary et al., 2011; Mao, Hart, Schink, & Pollok, 2004) for the addition of pMCT, and thrombin cleavage for the subsequent removal of pMCT from pM420 (Figure 1 and Figure 3a). With this strategy, the following were some of the criteria for success: (a) Will the truncated C-terminal domain express and fold correctly without the rest of ProtM? (b) Once pMCT is added to pM420, will the ligated product regain the ability to block the antibody/antigen interaction? (c) Will cleavage of functional pM420-pMCT into two separate fragments cause the loss of this blocking capability?

To address these questions, a GGG tag and a thrombin cleavage site (Th) were added to the N-terminus of pMCT for SrtA ligation (Figure 1). For ease of purification before and after ligation, an ELPS tag was added to the C-terminus of pMCT. The resulting ThpMCT-ELP79 fusion expressed well in E. coli and was easily purified using the thermally induced phase transition property of ELP (Figure S3; Meyer & Chilkoti, 1999). Similarly, addition of a C-terminal (G4S)-LPETGG motif to pM420 allows the ligation of pM420 onto ThpMCT-ELP79 using SrtA (Figure 4a). The ligation reaction was performed with excess ThpMCT-ELP79 to ensure complete functionalization of pM420, and a new band (~90 kDa) corresponding to the expected ligation product was detected after ELP purification (Figure 4a). Unligated ThpMCT was also copurified due to the presence of ELP.

Utilizing the MBP/anti-MBP dot blot assay again, it was clear that the individual pMCT domains exhibited low impact on anti-MBP binding (Figure 4b,c). This aligns well with that theory that the C-terminal portion of ProtM binds loosely with the CDR in a disordered manner. Furthermore, while pM420 only slightly diminishes antigen blocking, the ligated pM420-lig-ThpMCT-ELP79 product completely inhibited antigen binding to a similar extent as pM468. This result...
indicated that antigen blocking can be reconstituted only when the amino acids 421–468 are restored in pM420.

3.4 | Tunable antibody blocking and unblocking using a protease-responsive ProtM module

While SrtA-mediated ligation can be used to attach pMCT to pM420, specific proteases, such as thrombin, can be used to remove pMCT from the ligation product, creating a signal-responsive way to modulate antibody function. Incorporating a thrombin cleavable sequence between pM420 and pMCT on the ligated product, we next investigated the effect of thrombin cleavage on antigen binding. The ligated pM420-lig-ThpMCT-ELP79 product was incubated with 0.1, 0.5, or 1 mg/ml thrombin and complete cleavage between pM420 and pMCT was confirmed using SDS-PAGE analysis through the disappearance of the 90 kDa ligation product band and the reappearance of the 49 kDa pM420 band (Figure 4c). Dot blot analysis of anti-MBP/MBP interactions using the thrombin-released pM420 showed that anti-MBP affinity was restored to almost the original pM420 levels. Despite complete cleavage of the ligation product at 1 mg/ml thrombin, antigen binding was not completely restored to the same level as with pM420 alone (Figure 4b,c). This may be due to the low affinity between pM420 and pMCT. This was further confirmed by detecting a significant drop in antigen binding by simply mixing an excess amount of pMCT with pM420 (Figure S4). However, considering the modest 1:1 ratio after complete thrombin cleavage, this is unlikely to pose any problem for practical antigen binding activation.

To assess whether the antibody-bound pM420-lig-ThpMCT-ELP79 could still be cleaved, we performed a similar dot blot experiment in which anti-MBP was preincubated with ligated pM420-lig-ThpMCT-ELP79 prior to the addition of thrombin. Thrombin cleavage was first verified using SDS-PAGE analysis (Figure 5a), a new band corresponding to the cleaved pM420 fragment was clearly visible. Several minor bands were also detected due to nonspecific thrombin cleavage. Again, minimum antigen blocking was detected using the antibody-pM420 complex. However, cleavage of bound pM420-lig-ThpMCT-ELP79 by thrombin significantly reduced antibody blocking, resulting in restoration of antigen binding (Figure 5b). Quantitative analysis of the dot blot results using ImageJ confirmed that antigen binding was restored by more than 70% after thrombin cleavage (Figure 5c). The slight reduction in binding is likely the result of a small amount of nonspecific cleavage of the anti-MBP antibody.
In summary, we identified a truncated ProtM fragment, pM420, which has significantly reduced antigen-blocking ability while maintaining functional binding for antibodies. Ligation of the antigen-blocking, C-terminal pMCT fragment to pM420 by SrtA ligation reconstituted antigen-blocking functionality to a level similar to that of pM468, a ProtM variant that has the complete functionality of full-length ProtM. Separation of the two protein fragments by thrombin cleavage restored antigen binding, creating a conditional proantibody complex that is activated by a specific protease. The flexibility to adapt this framework toward different antibodies to achieve protease-responsive modulation of antigen binding suggests that ProtM may be used as a universal strategy to create stimuli-responsive antibodies.

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