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Comparison of mammalian and bacterial expression library screening to detect recombinant alpha-1 proteinase inhibitor variants with enhanced thrombin inhibitory capacity☆

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ABSTRACT

Serpins are a widely distributed family of serine proteases. A key determinant of their specificity is the reactive centre loop (RCL), a surface motif of ~20 amino acids in length. Expression libraries of variant serpins could be rapidly probed with proteases to develop novel inhibitors if optimal systems were available. The serpin variant alpha-1 proteinase inhibitor M358R (API M358R) inhibits the coagulation protease thrombin, but at sub-maximal rates compared to other serpins. Here we compared two approaches to isolate functional API variants from serpin expression libraries, using the same small library of API randomized at residue 358 (M358X): flow cytometry of transfected HEK 293 cells expressing membrane-displayed API; and a thrombin capture assay (TCA) performed on pools of bacterial lysates expressing soluble API. No enrichment for specific P1 residues was observed when the RCL codons of the 1% of sorted transfected 293 cells with the highest fluorescent thrombin-binding signals were subcloned and sequenced. In contrast, screening of 16 pools of bacterial API-expressing transformants led to the facile identification of API M358R and M358K as functional variants. Kinetic characterization showed that API M358R inhibited thrombin 17-fold more rapidly than API M358K. Reducing the incubation time with immobilized thrombin improved the sensitivity of TCA to detect supra-active API M358R variants and was used to screen a hypervariable library of API variants expressing 16 different amino acids at residues 352–357. The most active variant isolated, with TLSATP substituted for FLEAI, inhibited thrombin 2.9-fold more rapidly than API M358R. Our results indicate that flow cytometric approaches used in protein engineering of antibodies are not appropriate for serpins, and highlight the utility of the optimized TCA for serpin protein engineering.

1. Introduction

Members of the serpin superfamily that act as serine protease inhibitors contain a scissile bond called the reactive centre (reviewed in (Gettins, 2002; Huntington, 2011)). When a cognate protease attacks the reactive centre, its cleavage releases stored energy (Im et al., 2000; Lee et al., 2000) which powers a massive conformational change, one that results in translocation of protease to the opposite pole of the serpin (Stratikos and Gettins, 1997; Stratikos and Gettins, 1999; Wilczynska et al., 1997). The distortion of the protease active site traps it in acyl intermediate form, covalently attached to the cleaved serpin (Dementiev et al., 2006). The reactive centre is found on the serpin surface, housed within a region known as the reactive centre loop (RCL), of approximately 20 amino acids in length (Huber and Carrell, 1989). Aligned serpin RCL sequences show little consensus (Huber and Carrell, 1989). Nevertheless, crystallized encounter complexes of serpins with active site-mutated proteases confirm that, for those serpins lacking binding exosites, the RCL is the first point of contact between serpin and protease (Baglin et al., 2002; Dementiev et al., 2003; Lin et al., 2011; Ye et al., 2001). As such, it represents a compelling target for serpin protein engineering; by varying the sequence of the RCL, serpins of novel specificity could be generated.

The serpin alpha-1 proteinase inhibitor (API, also known as alpha-1 antitrypsin) is the most abundant serpin found in mammalian plasma (Ferrarotti et al., 2012). Its chief physiological role as an inhibitor of neutrophil elastase in lung tissue is supported by the increased risk of emphysema borne by individuals genetically deficient in API (Brantly et al., 1988; Lomas and Silverman, 2001). The API reactive centre is M358-S359 (Kurachi et al., 1981), or P1–P1' using the Schechter and Berger conventional numbering scheme

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(Schechter and Berger, 1967). The naturally occurring M358R Pittsburgh mutation re-directs API from inhibiting elastase to inhibiting thrombin and other activated coagulation proteases (Scott et al., 1986; Travis et al., 1986). While this change elicited a bleeding tendency in the index case (Owen et al., 1983), it sparked interest in using the API scaffold to generate novel protease inhibitors by protein engineering. Directed mutagenesis approaches have met with some success in enhancing the specificity of API for non-physiological targets, but typically at the cost of elevating the reaction stoichiometry (Dufour et al., 2001; Dufour et al., 2005; Filion et al., 2004; Sutherland et al., 2007). Random mutagenesis approaches involving the screening of hypervariable serpin expression libraries has until recently been employed only in the case of one serpin, plasminogen activator inhibitor 1 (PAI-1) (Pannekoek et al., 1993; Stoop et al., 2001, 2000; van Meijer et al., 1996).

We previously described the expression of antithrombin and API M358R as membrane proteins tethered to the surface of mammalian cells in culture, and the retention of thrombin inhibitory activity by the tethered serpins (Gierczak et al., 2011). This finding suggested a strategy for selection of novel API variants by cell sorting of transfected cells acquiring the ability to bind thrombin (Gierczak et al., 2011). We also described a thrombin capture assay and its use to select functional serpin variants from pools of candidates expressed in libraries expressed in *Escherichia coli* (Bhakta et al., 2013). Our objectives in the current study were: (1) to adapt the mammalian cell expression of tethered API M358R for library screening; (2) to screen the same API P1 expression library in both mammalian cell and bacterial systems; and (3) to optimize the more efficient system of the two to favor the selection of variants inhibiting thrombin with improved kinetics compared to API M358R. We report that the bacterial, but not the mammalian expression strategy, enriched for thrombin-inhibitory API P1 variants in a single round of gene transfer, and the isolation of a novel API M358R variant hyper-reactive with thrombin through optimization of the screening of the bacterial expression library.

2. Materials and methods

2.1. Construction of pCEP4 constructs directing the expression of AR-API proteins

Plasmid DNA from plasmids pC3-AR-API (M358R) and pC3-AR-API (T345R/M358R) (Gierczak et al., 2011) was restricted with HindIII and XhoI, and the 1492 bp minor fragment was inserted between these sites in pCEP4 (Invitrogen/Life Technologies, Carlsbad, CA), forming pCEP4-AR-API M358R and pCEP4-AR-API T345R/M358R, respectively (both 11,661 bp).

2.2. Construction of hypervariable API expression libraries in pCEP4-AR-API background

Overlap extension was used to position unique restriction sites upstream and downstream of RCL codons in pCEP4-AR-API M358R, using PCR mediated by Phusion HF heat-stable DNA polymerase under conditions recommended by the manufacturer (Finnzymes/Fisher Scientific, Ottawa, ON). Deoxyribonucleotide primer sequences are shown in Table 1. Amplicons generated by PCR using primers A and B (430 bp) and primers C and D (177 bp) were denatured, annealed, and extended to yield a 583 bp product ABCD. ABCD was restricted with PmlI and XhoI and the resulting 523 bp fragment was inserted between these sites in pCEP4-AR-API M358R, yielding pCEP4-AR-API T339Stop, which contained unique restriction sites BsiWI and AflII between codons P23 and P6'. Restriction of pCEP4-AR-API T339Stop with BsiWI and AflII allowed insertion of annealed oligonucleotides to restore RCL codons; the BsiWI and AflII sites were not regenerated, in order to restore wild-type residues at P21 (L340) and P6' (V364). Annealing and insertion of degenerate oligonucleotide pairs E and F and G and H produced pCEP4-AR-API-based hypervariable plasmid libraries with (a) all possible codons at M358 (P1) (in pCEP4-AR-API M358X) or (b) all possible codons between P7 and P2 (ran for randomized), and a subset of possible codons not encoding Arg in pCEP4-AR-API (P7-P2ran-P1 not Arg).

2.3. Construction of hypervariable API expression libraries in pBAD-H_6-API background

The hypervariable region in pCEP4-AR-API (P7–P2-ran/P1 not Arg) was transferred into the pBAD-H_6-API background by inserting the 559 bp PmlI-EcoRI M358R restriction endonuclease digestion fragment of pCEP4-AR-API (P7–P2-ran/P1 not Arg) between the corresponding sites of pBAD-H_6-API T345R/M358R, replacing all RCL codons and forming pBAD-H_6-API (P7–P2ran-P1 no Arg). A second hypervariable library for bacterial expression screening was constructed de novo, via PCR using primers A and I. The resulting amplicon was digested with PmlI and Bsu36I to yield a 458 bp digestion product, which was inserted between these sites in pBAD-H_6-API M358R, replacing the RCL codons and forming pBAD-H_6-API (P7–P2 ran No Stop/M358R). In this API hypervariable library, the first codon position encoding P7–P2 residues inclusive was limited to A, C, or G. This limitation imposed an absence of Phe, Tyr, Trp, Cys, and termination codons at P7–P2 inclusive among the possible API mutant proteins encoded in the library. A third library was constructed via PCR using primers A and J by analogous steps as described above, forming pBAD-H_6-API M358X.

2.4. Transfection and cell sorting of HEK 293 cells

HEK 293 cells were transfected with pCEP4-based AR-API expression plasmids as previously described for pCDNA 3.1-based AR-API constructs, using Lipofectamine 2000 reagent at 90–95% confluency (Gierczak et al., 2014). Following transfection, cells were resuspended in serum-free culture medium and reacted with thrombin, affinity-purified sheep anti-human prothrombin IgG, and Alexa Fluor488-conjugated donkey anti-sheep IgG as previously described (Gierczak et al., 2011). They were then characterized by flow cytometry and 0.5 to 1.0×10^6 transfected cells were sorted using a BD LSR II bench-top flow cytometer operated by the McMaster Flow Cytometry Facility powered by FACSDiva 6.0 software (BD Biosciences, San Diego, CA). The resulting data was analyzed using FlowJo version 10.0.7 (FlowJo LLC, Ashland, OR). In some experiments, sub-populations (~5–10,000 sorted cells) with the lowest or highest fluorescent signals were recovered and re-plated in cell culture flasks or pooled and subjected to DNA extraction (Qiagen Miniprep kit, Qiagen, Chatsworth, CA). Recovered plasmid DNA was amplified by PCR using oligonucleotides AX and K, and the resulting 592 bp amplicon was purified (Qiaquick PCR Purification Kit, Qiagen) and inserted into pJET1.2 via blunt-ended ligation as directed by the manufacturer (Thermo/Fisher Scientific, Ottawa, ON). The DNA sequence of inserted DNA in pJET1.2 subclone plasmids was determined using commercial pJET1.2 forward and reverse primers at the Molecular Biology Institute (MOBIX) Central Facility, McMaster University. Other constructs were also verified analogously.

2.5. Screening of pBAD-H_6-API-based expression libraries using a thrombin capture assay

Screening of libraries of bacterial colonies harbouring pBAD-H6-API plasmids varying at one or more API codons was performed as previously described (Bhakta et al., 2013). Briefly, single bacterial colonies or pools of ten colonies were used to inoculate archive