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Research paper

Enhancement of antibody fragment secretion into the *Escherichia coli* periplasm by co-expression with the peptidyl prolyl isomerase, FkpA, in the cytoplasm☆

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ABSTRACT

Improper protein folding or aggregation can frequently be responsible for low expression and poor functional activity of antibody fragments secreted into the *Escherichia coli* periplasm. Expression issues also can affect selection of antibody candidates from phage libraries, since antibody fragments displayed on phage also are secreted into the *E. coli* periplasm. To improve secretion of properly folded antibody fragments into the periplasm, we have developed a novel approach that involves co-expressing the antibody fragments with the peptidyl prolyl *cis-trans* isomerase, FkpA, lacking its signal sequence (cytFkpA) which consequently is expressed in the *E. coli* cytosol. Cytoplasmic expression of cytFkpA improved secretion of functional Fab fragments into the periplasm, exceeding even the benefits from co-expressing Fab fragments with native, FkpA localized in the periplasm. In addition, panning and subsequent screening of large Fab and scFv naïve phage libraries in the presence of cytFkpA significantly increased the number of unique clones selected, as well as their functional expression levels and diversity.

1. Introduction

Several groups have attempted with varying degrees of success to improve bacterial production of antibody fragments by co-expressing them with molecular chaperones or folding catalysts (Bothmann and Pluckthun, 1998; Strachan et al., 1999; Bothmann and Pluckthun, 2000; Levy et al., 2001; Mavrangelos et al., 2001; Maynard et al., 2005). The correct folding of scFv and Fab antibody fragments is highly dependent on the activity of peptidyl prolyl *cis-trans* isomerases (PPIases). Following the formation of variable and constant domain intra-chain disulphide bonds, peptidyl prolyl *cis-trans* isomerization reactions drive folding into the native conformation, allowing formation of the interchain disulphide bonds. PPIases also prevent aggregation of antibody fragments (Feige et al., 2010). Kappa light chain variable domains (Vκ) contain two conserved prolines in the *cis* conformation at positions L8 and L95 (Bothmann and Pluckthun, 2000) unlike the frameworks of heavy chain variable (VH) and lambda light chain variable (Vλ) antibody domains which, based on evaluation of sequences in the PDB database, do not contain any *cis*-prolines (Horne and Young, 1995). *Cis-trans* isomerization at Pro-L95 is a rate limiting step in the folding of Vκ domains and is essential for VL/VH docking and therefore for native protein conformation

Abbreviations: Fab, fragment-antigen-binding; Fc, fragment crystallizable; Fd, fragment of antibody consisting of CH1 and VH; ScFv, single-chain variable fragment; VH, variable heavy; VL, variable light; PPIase, peptidyl prolyl *cis-trans* isomerase; PCR, polymerase chain reaction; IPTG, isopropyl β-D-1-thiogalactopyranoside; OD, optical density; PBS, phosphate buffer saline; M.O.I., multiplicity of infection; PEG, polyethylene glycol; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; TMB, 3,3′,5,5′-tetramethylbenzidine; huINSR, human insulin receptor; BSA, bovine serum albumin; kd, dissociation constant; RT, room temperature; EC_{50}, half maximal effective concentration; CFU, colony-forming units; SD, Shine–Dalgarno sequence; PVDF, polyvinylidene fluoride; SPR, Surface Plasmon Resonance; SDS, sodium dodecyl sulfate; SEM, Standard Error of the Mean.

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(Suominen et al., 1987; Knappik and Pluckthun, 1995; Forsberg et al., 1997; Ramm and Pluckthun, 2000). Interestingly, co-expression of the periplasmic *Escherichia coli* PPIase, FkpA, resulted in a significant improvement in secretion into the bacterial periplasm of functional scFv fragments containing either Vκ chains, which contain *cis* prolines, or Vλ chains which do not contain *cis*-prolines, suggesting that it has both molecular chaperone and PPIase enzymatic activities (Bothmann and Pluckthun, 2000). Employing FkpA deletion mutants and functional assays, Saul et al. (2004) established that the FkpA carboxy and amino terminal domains carry independent PPIase and chaperone activities, respectively.

Previously, Missiakas et al. (1996) demonstrated that FkpA can act as a "global folding catalyst" that limits the levels of unfolded proteins in the outer membrane and periplasm. Periplasmic overexpression of FkpA facilitates the expression of multiple heterologous proteins, including an *E. coli* maltose binding protein misfolding mutant (Arie et al., 2001), single-chain antibodies and antibody fusions (Arie et al., 2001; Zhang et al., 2003; Padiolleau-Lefevre et al., 2006; Sonoda et al., 2010).

Another molecular chaperone in the *E. coli* periplasm is the 17 kDa Skp protein which forms a trimer with a central cavity. This cavity allows Skp to engulf native polypeptide substrates and prevents their subsequent aggregation (Walton et al., 2009). Co-expression of Skp with a poorly soluble single chain Ab resulted in its secretion into the *E. coli* periplasm as well as improved solubility and phage display of the antibody fragment and diminished the toxicity of the antibody for the host cells (Hayhurst and Harris, 1999). As observed with FkpA, other groups have demonstrated that co-expression of scFvs with Skp increased their secretion in *E. coli* (Sonoda et al., 2010). Previously, it also was shown that overexpression of Skp lacking its signal sequence significantly improved the yield of a correctly folded Fab produced by a *trxBgor* mutant *E. coli* strain that enables the production of disulphide bonds in the bacterial cytoplasm (Levy et al., 2001).

We report here improvement in functional Fab expression into the *E. coli* periplasm as a result of its co-expression with FkpA lacking a signal sequence (cytFkpA). The secretion of active Fabs into the periplasm was higher when co-expressed with cytFkpA either on a separate vector under control of an L-arabinose-inducible promoter, or as part of a tricistronic message that includes the chaperone, Fd and light chains on a single plasmid. We also examined the effect of cytFkpA expression on selection of scFv or Fab candidates from large phage libraries and have demonstrated increased expression levels and diversity of displayed antibodies targeting the selected antigens, resulting in selection of a larger number of functional, sequence-unique antibody fragments with slower dissociation constants.

2. Materials and methods

2.1. Strains

XL1-Blue cells (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F′ proAB lacI*qZΔ*M15* Tn*10* (Tetr)]) and TG1 cells (*supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)*5* (rK-mK–) [F′ *traD36 proAB lacI*qZΔ*M15*]) were purchased from Agilent (Santa Clara, CA).

2.2. Plasmids expressing bacterial chaperones

In order to generate the plasmids responsible for cytoplasmic expression of chaperones, the native signal sequences were excised from the genes encoding the chaperones FkpA (Swiss-Prot accession no. P65764) and Skp (Swiss-Prot accession no. P0AEU7). Chaperones were also allowed to express in the bacterial periplasm with their native signal sequences. To generate the plasmid constructs of the cytoplasmic or periplasmic versions of the chaperones Skp and FkpA, and the bicistronic Skp-FkpA, the chaperone gene fragments were amplified by PCR and then cloned into the plasmid vector pAR3 (ATCC accession no. 87026). The vector pAR3 (Perez-Perez and Gutierrez, 1995) contains the pBAD promoter and the *cat* gene which confers chloramphenicol antibiotic resistance. This plasmid harbors the p15A origin of replication which is compatible with the origin ColE1 included in all the vectors co-expressing Fabs or scFvs in our experiments. Two different forward primers and one reverse primer were designed in order to amplify FkpA from XL-1Blue cells by PCR amplification with or without the native leader peptide. Similarly, two forward primers and one reverse primer were designed to amplify Skp from XL1Blue cells by PCR with or without its native signal sequence.

To generate the chaperone plasmid constructs pAR3-FkpA and pAR3-Skp for periplasmic expression and pAR3-cytFkpA and pAR3-cytSkp for cytoplasmic expression, the products of the previous PCR reactions were used as templates for PCR re-amplification using forward primers to incorporate a BglII restriction site followed by the enhancer sequence GAATTCA TTAAAGAGGAGAAATTAACT upstream from the chaperone encoding gene fragment. Reverse primers were used to incorporate the V5 tag sequence (GGTAAGCCTATCCCTAACC CTCTCCTCGGTCTCGATTCTACG) into pAR3-Skp and pAR3-cytSkp and the FLAG tag sequence (GACTACAAGGACGATGACGACAAG) into the pAR3-FkpA and pAR3-cytFkpA, followed by the restriction site HindIII.

To generate the bicistronic periplasmic pAR3-[Skp + FkpA] and cytoplasmic pAR3-cyt[Skp + FkpA] constructs, the monocistronic PCR products were reamplified. To reamplify Skp, forward primers were used to incorporate BglII, followed by the enhancer GAATTCATTAAAGAGGAGA AATTAACT and the periplasmic or cytoplasmic versions of Skp. A reverse primer was designed that anneals to the entire V5 sequence and to an optimized Shine–Dalgarno (SD) sequence driving the translation initiation of FkpA. To reamplify FkpA, forward primers were designed to anneal to the C-terminal portion of V5, the optimized SD, and the periplasmic or cytoplasmic versions of FkpA. A reverse primer was designed to add a HindIII-FLAG tag sequence to the C-terminal portion of FkpA. The Skp and FkpA PCR products were then gel-purified using Qiagen gel extraction kits (Valencia, CA) and used as templates for an overlap extension PCR reaction using the external forward Skp primer and an external reverse FkpA primer. Fig. 1a illustrates the resulting chaperone inserts. Ligations of the BglII–HindIII digested PCR products to the BglII–HindIII digested pAR3 vector were then transformed by electroporation into XL1-Blue cells. The final constructs were confirmed by DNA sequencing.