Engineering toward a bacterial “endoplasmic reticulum” for the rapid expression of immunoglobulin proteins

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Abbreviations: Open Cell Free Synthesis, OCFS; heavy chain, HC; light chain, LC; immunoglobulin, IgG; peptidyl prolyl isomerase, PPIase; protein disulfide isomerase, PDI; endoplasmic reticulum, ER; binding immunoglobulin protein, BiP; heat shock protein 70, hsp70; single chain Fragment variable, scFv; fragment antigen-binding, Fab; Fragment constant, Fc

Antibodies are well-established as therapeutics, and the preclinical and clinical pipeline of these important biologics is growing rapidly. Consequently, there is considerable interest in technologies to engineer and manufacture them. Mammalian cell culture is commonly used for production because eukaryotic expression systems have evolved complex and efficient chaperone systems for the folding of antibodies. However, given the ease and manipulability of bacteria, antibody discovery efforts often employ bacterial expression systems despite their limitations in generating high titers of functional antibody. Open-Cell Free Synthesis (OCFS) is a coupled transcription-translation system that has the advantages of prokaryotic systems while achieving high titers of antibody expression. Due to the open nature of OCFS, it is easily modified by chemical or protein additives to improve the folding of select proteins. As such, we undertook a protein additive screen to identify chaperone proteins that improve the folding and assembly of trastuzumab in OCFS. From the screen, we identified the disulfide isomerase DsbC and the prolyl isomerase FkpA as important positive effectors of IgG folding. These periplasmic chaperones function synergistically for the folding and assembly of IgG, and, when present in sufficient quantities, gram per liter IgG titers can be produced. This technological advancement allows the rapid development and manufacturing of immunoglobulin proteins and pushes OCFS to the forefront of production technologies for biologics.

Introduction

Antibodies are remarkable proteins that function to specifically recognize and neutralize antigens associated with invading organisms and other diseases. Antibodies, or immunoglobulins, are higher order protein complexes that form a quaternary structure stabilized by interchain disulfide bridges. Immunoglobulin gamma (IgG) is a class of antibodies composed of two heavy chain (HC) and two light chain (LC) polypeptides. They are further modified by glycosylation in the conserved Fc region of the HC, which can help mediate interactions with other components of the immune system. Both HCs and LCs are composed of protein domains characterized by the β-stranded immunoglobulin fold. B cells or plasma cells are optimized for the folding and assembly of these complex molecules in the endoplasmic reticulum (ER).1 The lumen of the ER is an oxidative environment, which is required to form and stabilize native intra and interchain disulfide bonds. In the ER, special chaperone proteins such as protein disulfide isomerases (PDI) catalyze disulfide bond formation and shuffling until the correct cysteine linkages are formed.2,3 Other chaperone families participate in the folding of IgG molecules, in particular of the HC polypeptide, which is difficult to fold.1,2,4 BiP, an immunoglobulin HC binding protein, is a member of the hsp70 superfamily that associates to HC molecules during folding.4,5 In the monomeric state, HC proteins are prone to misfolding and require BiP for stabilization against aggregation. Once HC is disulfide bonded to LC, a stable protein complex is formed. Prolyl isomerization has also been proposed to be a

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We thus undertook a protein additive screen to identify chaperone proteins that may improve the folding and assembly of the human epidermal growth factor receptor (HER)2-targeted IgG trastuzumab in OCFS. We chose a limited subset of chaperones to screen based on their potential ability to functionally replace chaperones critical for the folding of IgGs in the eukaryotic ER. From the screen, we identified the disulfide isomerase DsbC and the prolyl isomerase FkpA as important positive effectors of IgG folding. These periplasmic chaperones function synergistically for the folding and assembly of IgG, and, when present in sufficient quantities, gram per liter IgG titers can be obtained in OCFS. This is in contrast to other bacterial systems that may produce 10–100 times less protein. This technological advancement may facilitate the rapid development and manufacturing of a variety of immunological proteins and contribute to the burgeoning therapeutic antibody business, which is projected to generate annual revenues of $58 billion by 2016.9

Results

Chaperone sequential expression screen

In vivo, eukaryotic chaperones are known to play an important role in the folding and assembly of IgGs. Therefore, expression of IgG molecules in bacterial systems that lack these physiological foldases has been challenging.10,11 Given that bacterial expression systems still have several advantages over eukaryotic systems, we undertook a screening approach to identify positive effectors of IgG folding or assembly in a prokaryotic expression system (Fig. 1A). Candidate chaperones were expressed in a coupled prokaryotic in vitro transcription/translation system (hereafter referred to as Open Cell-Free Synthesis, or OCFS) and expressed chaperones were subsequently titrated into new cell-free reactions for the expression of trastuzumab, a HER2-binding IgG1 molecule. IgG was translated in the presence of 14C-Leucine as a tracer for heavy and light chain folding and assembly. (B and C) Positive effectors of IgG synthesis were identified by their ability to improve the folding or assembly of IgG as compared with a GFP-expressed negative control. DsbC, SlyD, and Skp are representative examples of positive effectors from functional groups controlling redox, prolyl isomerization, and deaggregation. (C) Improvements in IgG folding were expressed as a fold-change over the GFP control (20% v/v shown).
were added to IgG reactions in the presence of spent extract. To ensure that chaperone DNA was not being transcribed and expressed in subsequent IgG reactions, chaperone proteins were expressed from PCR template that is significantly more labile than plasmid DNA. The addition of GamS protein helped preserve the PCR template so that sufficient levels of chaperone protein could be synthesized from the PCR amplified DNA templates. This PCR DNA was still degraded by the end of the chaperone synthesis reaction as demonstrated by the lack of chaperone synthesis in the subsequent cell-free reactions (Fig. S1).

Several families of chaperones were of particular interest given their role in folding IgG in vivo. Redox proteins, PPIases, foldases and deaggregases from bacterial, yeast, and human species were tested (Table 1). Among the redox chaperones, we found that yeast PDI (yPDI) and DsbC significantly aided IgG formation, consistent with previous findings (Fig. 1C; ref. 7). Notably, human PDI (hPDI) did not significantly affect IgG folding, probably due to its poor expression in cell extract, which did not allow sufficient quantities to be added to IgG expression reactions (Fig. 1C; ref. 7). By contrast, the bacterial protein DsbC expressed very well in the extract, allowing the addition of ~5 μM DsbC to the IgG reaction (DsbC was expressed at ~25 μM and it was added at 5%, 10%, and 20% v/v to an IgG reaction in Figure 1B; 20% chaperone addition reactions were tabulated in Figure 1C). Among the PPIases tested, several proved to be beneficial to IgG expression as well (Fig. 1C).

Table 1. Expression titers of positive effectors in the screen

| Chaperone class       | Chaperone | Molecular weight (kDa) | Chaperone expressed (μM) |
|-----------------------|-----------|-----------------------|-------------------------|
| Redox proteins        | DsbC      | 23.6                  | 26                      |
|                       | DsbG      | 29.8                  | 11                      |
|                       | yPDI      | 55.8                  | 3                       |
|                       | hPDI      | 55.3                  | 3                       |
|                       | yTrr1     | 34.2                  | 18                      |
|                       | yGlr1     | 53.4                  | 3                       |
| Prolyl isomerases     | SlyD      | 20.9                  | 43                      |
|                       | tig       | 48.2                  | 15                      |
|                       | SurA      | 45.1                  | 8                       |
|                       | FkpA      | 28.9                  | 48                      |
|                       | hPPIB     | 20.3                  | 23                      |
|                       | yCpr1     | 17.4                  | 23                      |
|                       | yCpr6     | 42.1                  | 12                      |
|                       | yFpr1     | 12.2                  | 63                      |
| Deaggregating proteins| hERdj3    | 38.2                  | 12                      |
|                       | hBiP      | 70.5                  | 3                       |
|                       | yHsc82    | 80.9                  | 7                       |
|                       | IbPA      | 15.8                  | 52                      |
|                       | IbPB      | 16.1                  | 69                      |
|                       | Skp       | 17.7                  | 71                      |

Interchangeability of PDI and DsbC

From the screen, as well as from previous work,7,8,13 we had observed that both yeast PDI and bacterial DsbC had the ability to promote disulfide bond formation and IgG assembly in cell-free reactions. While both proteins are disulfide bond isomerases, it is possible that eukaryotic and prokaryotic isomerases may have evolved to specifically fold proteins found in their respective proteomes. This would predict that PDI would be superior at folding a eukaryotic substrate protein such as IgG. To better understand the dependence of IgG folding and assembly on eukaryotic and prokaryotic disulfide bond isomerases, IgG cell-free protein synthesis reactions were run at varying concentrations of PDI and DsbC. IgG was expressed in cell-free reactions in the presence of 0–5 μM PDI in combination with 0–13 μM DsbC. Expressed IgG-His was purified by Ni2+ resin and quantified by capillary electrophoresis (Fig. 2). Ni2+ -based purification was utilized to avoid acidic elution conditions employed in Protein A purifications that might dissociate IgG complexes. In the absence of DsbC, IgG was highly dependent on PDI for folding (Fig. 2, closed circles). As the concentration of DsbC in the reaction increased, however, the dependence on PDI decreased such that, at 6.4 μM DsbC, there was no additional benefit attributable to PDI in the reaction (Fig. 2, open triangles). Furthermore, by increasing the concentration of DsbC in the reaction, we saw marked improvements in IgG titers beyond what we had previously observed (Fig. 2, open circles and ref. 7). In effect, we observed the efficient substitution of a eukaryotic disulfide bond isomerase with a bacterial
chaperone of a similar function in the folding of a eukaryotic protein.

Purified Skp, SlyD, and FkpA can improve IgG titers

Among the positive effectors identified in the chaperone screen, we decided to further evaluate the bacterial proteins Skp, SlyD, and FkpA. To confirm that they benefited IgG folding, we expressed and purified Skp, SlyD, and FkpA and added them back into IgG cell-free protein synthesis reactions in the presence of 13 μM DsbC (Figs. 3 and S2). The chaperone Skp aided the solubility of HC and LC, but did not increase the amount of assembled IgG significantly. However, for the prolyl isomerases, SlyD and FkpA, we observed that the solubility of expressed proteins, as well as the proportion of assembled IgG, increased dramatically with added chaperone protein. We reasoned that prolyl isomerization was a function that may have been limiting for IgG formation in OCFS, and the addition of these exogenous proteins substantially improved IgG folding and assembly, consistent with previous observations. 

FkpA and DsbC have different roles in the folding and assembly of IgG

To better understand the roles that FkpA and DsbC play in IgG formation, we independently evaluated their contributions to IgG folding (Fig. 4). Purified FkpA and DsbC protein were titrated into cell extract derived from strain SBJY001, and trastuzumab HC and LC were expressed in the presence of [14C]-Leucine followed by visualization with SDS-PAGE and autoradiography. Notably, the addition of FkpA significantly reduced the degree of higher molecular weight aggregates formed during HC and LC synthesis. With increasing amounts of FkpA, we also observed the formation of IgG, as well as a number of partially assembled products. These proteins migrated as indistinct bands, suggesting that they may represent mixed populations of cross-disulfide bonded proteins. The addition of DsbC, on the other hand, generated IgG that migrated in clear, defined bands by SDS-PAGE. However, without FkpA, a significant proportion of the expressed proteins formed higher order aggregates that could not completely enter the gel. Together, these results suggest that FkpA and DsbC have distinct roles in the folding of IgGs.

Developing extract with high levels of DsbC and FkpA

Adding high concentrations of purified chaperone proteins is expensive and labor-intensive, and we wanted to move toward a strain-engineered solution. As such, we undertook to develop bacterial strains that overexpressed DsbC and FkpA, which would then be present at high concentrations in the subsequent cell-free extract. Plasmids carrying single (1xDsbC) or double (2xDsbC) copies of the dsbC gene behind the constitutive promoter Mt-cons-10 were constructed and transformed into bacteria. ELISA quantitation of these bacterial extracts suggested that we had 1–2 g/L and 3–4 g/L of DsbC (or ~65 and ~150 μM) expressed in the 1xDsbC and 2xDsbC extracts, respectively (Fig. 5A). IgG expression in OCFS with DsbC-containing extracts (2xDsbC) was very efficient, with a high proportion of soluble proteins forming assembled IgG (Fig. 5B, lane 1).

We then evaluated the synergy of combined DsbC and FkpA action on the folding of IgG (Fig. 5B). Trastuzumab HC and LC were expressed in a DsbC-containing extract with exogenous FkpA protein added into the reactions. Addition of 50 μM FkpA significantly improved IgG folding and assembly (Fig. 5B). Accordingly, we developed bacterial strains and the corresponding cell extracts that overexpress FkpA (Fig. 5A). 2–3 g/L and 5 g/L FkpA (or ~90 and ~180 μM) could be achieved in the 1xFkpA and 2xFkpA cell extracts, respectively, which corresponds to intracellular concentrations as high as 15 g/L FkpA. However, given that FkpA alone is inadequate for IgG assembly (Fig. 4),
A strain engineering solution was desired for both chaperones. Two tandem copies of the *dsbC* gene were chromosomally integrated, and the resultant strain was transformed with the 2xFkpA plasmid (2xD + 2xF). Although extract made from this strain had lower overall titers of DsbC compared with strains overexpressing only one chaperone (Fig. 5A), the extract was nonetheless able to support gram per liter titers of trastuzumab in an overnight cell-free synthesis reaction (Fig. 5C).

DsbC and FkpA-overexpressing extract can improve the expression and assembly of multiple IgGs

Having identified chaperones that dramatically improve the folding and assembly of trastuzumab in OCFS, we wanted to understand if our engineered extracts could also benefit the folding of other IgGs. Accordingly, we expressed a panel of IgGs in a control extract (SBJY001), DsbC extract (2xDsbC extract), and DsbC + FkpA extract (2xD + 2xF) (Fig. 6). Included in the panel were the therapeutic antibodies trastuzumab (an anti-HER2 IgG1) and brentuximab (an anti-CD30 IgG1), in addition to two germ-line HCs VH3–7 and VH3–23 in combination with the LC Vk3–20. Notably, DsbC was essential for the expression of all the IgGs tested. Further improvements were observed in the DsbC + FkpA extract, bringing expression levels to 1 g/L for both trastuzumab and brentuximab, and nearly 1.5 g/L for the germline IgGs. Together, these results suggest that the engineered extracts are likely to benefit the expression of a wide range of immunoglobulin proteins in OCFS.

**Discussion**

The mammalian ER is a powerful system that can properly fold and assemble complex disulfide-bonded molecules such as IgGs. In an attempt to mimic the suite of different chaperone activities in the mammalian ER, chaperones from the same functional classes implicated in IgG folding were screened. In agreement with other reports, addition of disulfide isomerase dramatically improved IgG assembly.7,1513 In fact, addition of > 10 μM DsbC to our bacterial transcription/translation system improved IgG expression by more than 20-fold (Fig. 2).

We identified two disulfide isomerases, DsbC from *E. coli* and PDI from *S. cerevisiae* that were capable of enhancing the production of IgG in OCFS. Surprisingly, DsbC was able to boost IgG assembly to a similar extent as PDI, despite having evolved as a redox catalyst for proteins in the bacterial periplasm. This
suggests a striking functional overlap for these proteins despite limited sequence or substrate similarity.

Similar to the mammalian ER, high level IgG production also required the presence of a PPlase. From a panel of eight PPlases, the *E. coli* proteins FkpA and SlyD were identified for their ability to augment IgG folding and assembly during OCFS (Figs. 1 and 3). The hsp70 BiP is another chaperone reported to be highly upregulated during plasma cell differentiation and contributes to HC stabilization during IgG folding. We did not, however, observe any benefit with the addition of human BiP or its cofactor human Erdj3, possibly due to the relatively low levels that were added into the IgG synthesis reactions (Fig. 1 and Table 1). Given that these human chaperones expressed more poorly than bacterial proteins whose sequences have evolved to translate and fold efficiently in *E. coli* systems (Table 1), we were not able to test the effect of these chaperones on IgG folding over a wide range of concentrations. Another possibility for why a BiP-associated improvement was not observed is that the bacterial hsp70 DnaK and cofactor DnaJ are at sufficiently high concentrations in the extract that addition of another hsp70 would have little impact. Indeed, hsp70 proteins are highly conserved and abundant. The hsp70 DnaK has been estimated to be 1–2% of total protein (or 2–5 g/L DnaK) in *E. coli*. One deaggregase from our screen that did have a positive effect on IgG solubility was Skp, which modestly increased IgG titers. Because FkpA markedly increased HC solubility while also improving IgG assembly, we further investigated the role of FkpA.

We evaluated the different roles of FkpA and DsbC and found that the chaperones act synergistically to fold IgG. DsbC is critical for the formation of properly assembled and disulfide-bonded IgG (Fig. 4B). The addition of FkpA further enhances the degree of IgG folding, likely through improved HC solubility and perhaps prolyl isomerization (Fig. 3B and 5B). Indeed, we have observed FkpA addition to significantly improve solubility in both small-scale and large-scale reactions (data not shown). FkpA has been reported to improve protein solubility as well as catalyze prolyl isomerization for several model proteins. In addition, FkpA localized to the cytoplasm has also been shown to enhance secretion of both Fabs and scFv into the *E. coli* periplasm, possibly by helping each stay soluble long enough for export from the cytoplasm. However, FkpA alone is insufficient to support IgG assembly (Fig. 4A), highlighting the critical role of disulfide isomerases in IgG formation.

Addition of exogenous chaperones such as DsbC and FkpA is a powerful metric for understanding the functional requirements of IgG maturation. Keeping with our goal of modeling the eukaryotic ER, we were curious about whether DsbC and FkpA could be produced in vivo at titers sufficient to support high level IgG production in a cell-free reaction. While the concentration of PDI is estimated to be as high as 2 mM in the mammalian ER, the above experiments indicate that disulfide isomerase concentrations 2 orders of magnitude lower are sufficient for proper disulfide formation in OCFS (Figs. 2 and 5B). To determine if the required chaperone levels could be achieved, we constructed a low copy plasmid carrying two copies of *dsbC* or *fkpA* behind a constitutive promoter. Bacteria transformed with these plasmids had intracellular concentrations of 450 μM DsbC (10 mg/mL) or 550 μM FkpA (15 mg/mL), approximately three times the concentration of our assessed extracts. This falls in the range of chaperone proteins required for efficient IgG folding (Fig. 5). Therefore, strain 2xD + 2xF was engineered to produce high concentrations of both chaperones, thus allowing for the rapid production and folding of IgG without the addition of large amounts of exogenous protein. Traditional methods of intracellular IgG expression in bacterial cultures often result in cellular toxicity, especially if the cells are also generating high levels of chaperone to support IgG folding. However, in OCFS, the cell’s resources are first allocated for the production of high concentrations of chaperone protein during bacterial fermentation. Once the cells are harvested and used to generate cell extract, the cellular machinery, which now includes high chaperone concentrations, can be reallocated and fully dedicated to the production of one protein of interest—in our case, IgG. In this manner, we are able to achieve rapid production of extremely high antibody titers.

In this study, the versatility of open cell free protein synthesis was utilized to quickly screen a large variety of chaperones to determine which might boost production of native IgGs in the context of prokaryotic protein synthesis machinery. By expressing these
factors in the extract-production strain, it was possible to achieve gram per liter titers in an overnight reaction. These titers are on par with high expressing stable cell lines, without the risk, time, and cost of developing the cell lines. This advancement pushes OCFS to the forefront of production technologies for IgGs.

Materials and Methods

Small-scale cell-free expression

100 μl cell-free reactions were run at 30°C for 12 h in a 96-well microtiter plate at 650 rpm in a VWR Thermomixer in the presence of 10 μg/mL DNA (2.5 μg/mL trastuzumab LC DNA, 7.5 μg/mL trastuzumab HC DNA in the expression vector pYD3177,8). To facilitate disulfide bond formation, cell-free extracts were treated with 50 μM iodoacetamide for 30 min at RT (20 °C) to quench reactive thiol groups and then added to a premix of components. The final concentration in the protein synthesis reaction was 30% cell extract (v/v), 2 mM GSSG, 8 mM magnesium glutamte, 10 mM ammonium glutamate, 130 mM potassium glutamate, 35 mM sodium pyruvate, 1.2 mM AMP, 0.86 mM each of GMP, UMP, and CMP, 2 mM amino acids (except 1 mM for Tyrosine) mM potassium acetate.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/mabs/article/28172
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