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Inefficient ribosomal skipping enables simultaneous secretion and display of proteins in *Saccharomyces cerevisiae*

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Abstract

The need for recombinant expression of soluble protein slows down validation of engineered proteins isolated from combinatorial libraries, and limits the number of protein variants evaluated. To overcome this bottleneck, we describe a system based on inefficient ribosomal skipping, for simultaneous cell surface display and soluble secretion of proteins in *Saccharomyces cerevisiae*. Ribosomal skipping mediated by "self-cleaving" 2A peptides produces two proteins from a single open reading frame. Incorporation of the F2A peptide sequence – with ~ 50% efficiency of ribosomal skipping – between the protein of interest and the yeast cell wall protein Aga2 results in simultaneous expression of both solubly secreted protein, and the protein-Aga2 fusion that is tethered to the yeast cell surface. We show that binding proteins derived from the Sso7d scaffold and the homodimeric enzyme glucose oxidase (GOx) can be simultaneously secreted solubly, and expressed as yeast cell surface fusions, using the F2A-based system. Further, a combinatorial library of Sso7d mutants can be screened to isolate binders with higher affinity for a model target (lysozyme), and the pool of higher affinity binders can be characterized in soluble form. Significantly, we show that both N- and C-terminal fusions to Aga2 can be simultaneously secreted solubly and displayed on the cell surface; this is particularly advantageous because protein functionality can be affected by the specific position of Aga2 in the protein fusion. We expect that the F2A-based yeast surface display and secretion system will be a useful tool for protein engineering and enable efficient characterization of individual clones isolated from combinatorial libraries.

Graphical abstract

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Author Contribution

CCT, KT, and BMR conceived the study and designed the experiments. CCT, KT, and AM conducted the experiments. CCT, KT, and BMR analyzed and interpreted the data. CCT and BMR wrote the manuscript, with input from the other authors.

Conflict of Interest

The authors have no conflict of interest to declare.

Supporting information

Supporting information included with this manuscript includes:

Supplementary Figures (7)

Supplementary Tables (3)

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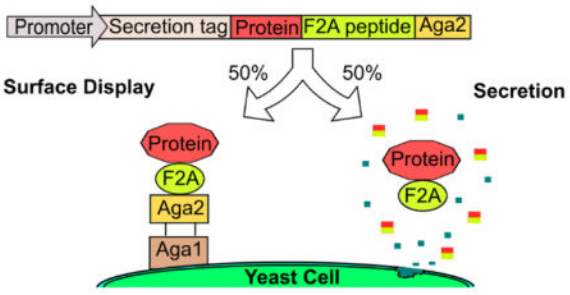

Keywords

yeast surface display; secretion; ribosomal skipping; 2A peptide; Sso7d; glucose oxidase

Yeast surface display is a powerful tool for screening combinatorial protein libraries, and has been widely used for isolating proteins – including antibodies and antibody fragments, non-immunoglobulin protein scaffolds, and enzymes[1] – with new or improved properties. In the most commonly used configuration of yeast surface display, the protein of interest is expressed as an N- or C-terminal fusion to the Aga2 subunit of the yeast mating protein a-agglutinin. The Aga2 subunit in turn is linked to the yeast cell wall-associated Aga1 subunit by two disulfide bonds, resulting in tethering of the protein of interest to the yeast cell wall[2]. The yeast cell serves to link the protein of interest to its genetic code, thereby enabling "bar-coding" of mutant proteins. Mutants with desired properties – often binding affinity and specificity – are isolated from a combinatorial library using magnetic selection and/or fluorescence activated cell sorting (FACS), and the mutant proteins are identified by DNA sequencing.

Mutants identified by yeast surface display screening – and combinatorial protein library screening platforms in general including phage display and mRNA display – typically need to be recombinantly produced as soluble proteins for further validation and characterization. The recombinant expression of mutant proteins often involves time-consuming subcloning steps; this slows down the validation process and limits the number of individual mutant clones that can be characterized. Therefore, a screening platform that allows for cell surface display of a protein of interest, as well as soluble secretion of the same protein without additional subcloning steps is highly desirable. Towards this end, the homodimerization of the Fc region of immunoglobulin G (IgG) molecules has been exploited to secrete full-length antibody molecules, and simultaneously display monovalent IgG molecules in *Pichia pastoris*[3]. Deventer *et al.* have developed a system in *Saccharomyces cerevisiae* wherein cells expressing a solubly secreted protein can switch to expressing the protein as a cell surface fusion to the Aga2 protein by amber codon suppression and addition of a non-canonical amino acid into cell culture medium[4]. Effectively simultaneous soluble secretion and surface display of proteins in yeast has also been achieved using a "secrete and capture" approach, wherein protein solubly secreted by a yeast cell is captured by a binding moiety present on the cell surface[5,6]. In mammalian cells, Horlick *et al.* have demonstrated the use of alternate splicing of pre-mRNA for simultaneous cell surface display and secretion of monoclonal antibodies[7]. Here, we present a simple and effective system for simultaneous

secretion and display of proteins in yeast, based on ribosomal skipping mediated by a “self-cleaving” 2A peptide derived from a foot-and-mouth disease virus.

2A peptides are amino acid sequences that were first identified in the foot-and-mouth disease virus, where viral polyprotein processing was observed without the assistance of proteinases or post-translation modifications[8,9]. Since then, other 2A peptides have been identified in other viruses[10,11]. Although the family of 2A peptide sequences are regarded as self-cleaving peptides, no cleavage reaction occurs. Instead, the peptide bond between a glycine and proline at the C-terminus of the peptide fails to form inside the ribosome during mRNA translation[12]. This effectively results in the translation of two proteins from the same open reading frame (ORF). Importantly, the efficiency of the skipping reaction depends on the sequence of the self-cleaving peptide[11,13]. Therefore, ribosomal skipping efficiency can be tailored as desired; for example, Yu *et al.*[14] used 2A peptides with different skipping efficiencies to tune the levels of membrane-bound and solubly secreted IgG in B cells. We sought to exploit this peptide sequence dependence of ribosomal skipping efficiency for simultaneous secretion and cell surface display of proteins in yeast *Saccharomyces cerevisiae.*

Ribosomal skipping in yeast has been previously reported. De Felipe *et al.* showed that the 2A peptide is active in *Saccharomyces cerevisiae*[12]. Subsequently, Sun *et al.* have used a self-cleaving peptide to display an enzyme fused to two different surface anchor proteins from a single ORF in *Pichia pastoris*[15]. A 2A peptide has also been used to develop a fluorescent reporter system for the presence of yeast surface displayed proteins in *Saccharomyces cerevisiae*. The surface-displayed protein and a green fluorescent protein (GFP) reporter are translated from the same ORF; GFP fluorescence is used as a proxy for the expression level of surface-displayed protein[16]. Here we describe the use of an 18 amino acid 2A peptide sequence from the foot-and-mouth disease virus, referred hereafter as the F2A peptide, in the context of simultaneous yeast surface display and soluble secretion of proteins in *Saccharomyces cerevisiae*.

In a typical yeast surface display system, a protein fused to the Aga2 subunit of the yeast mating protein a-agglutinin, and which contains a secretory signal peptide, is expressed as a yeast cell surface fusion[2]; in the absence of Aga2, a protein fused to a secretory signal peptide is solubly secreted into the cell culture medium. Since the F2A peptide shows 50% efficiency of ribosomal skipping[13], we hypothesized that a single ORF wherein the protein of interest and Aga2 are separated by the F2A peptide, and wherein the protein of interest is fused to a secretory signal peptide (i.e. signal peptide-protein-F2A-Aga2), will result in the formation of two distinct products that each contain a secretory signal peptide – the protein of interest, and a protein-Aga2 fusion. Consequently, the protein of interest will be simultaneously expressed as a cell surface fusion and solubly secreted into the culture medium.

We tested and confirmed our hypothesis in the context of simultaneous display and secretion of mutants derived from the Sso7d protein scaffold, and the homodimeric enzyme glucose oxidase (GOx). The Sso7d protein from the hyperthermophilic archaeon *Sulfolobus solfataricus* has been used as a versatile scaffold for generation of binding proteins to a wide

spectrum of targets, and in the context of several different applications including affinity-based bioseparation and biosensing[17–26]. Further, we show that yeast surface display libraries of mutant proteins containing F2A peptide fusions can be screened to isolate binders with higher affinity. Finally, we show that the protein of interest can be expressed as an N- or C-terminal fusion to Aga2, provided the ORF includes suitable secretory signal sequences; this is an important advantage because the functionality of yeast surface displayed proteins may be affected by its mode of fusion (i.e. N-terminal or C-terminal) to Aga2[27].

Results and Discussion

Design of a plasmid vector for simultaneous secretion and cell surface display

Fig. 1 shows the plasmid construct designed to obtain simultaneous cell surface display and secretion of proteins in yeast *Saccharomyces cerevisiae* (pCT-NT-F2A). A synthetic alpha-maturation factor (prepro) is used as the secretory signal peptide[28]. The protein of interest – here denoted as Sso7d – is placed after the prepro sequence, and is followed by the F2A peptide and the surface anchor protein Aga2. Since the ribosomal skipping efficiency of F2A is ~ 50%, translation of mRNA encoded by this ORF is expected to result in three species: (N-term) prepro-Sso7d-F2A (C-term), (N-term) prepro-Sso7d-F2A-Aga2 (C-term), and Aga2. The first two species containing the prepro sequence are directed to the secretory pathway where the prepro sequence is cleaved. Sso7d fused to Aga2 is expressed as a cell surface fusion, whereas the protein is solubly secreted in the absence of Aga2. Both solubly secreted and cell surface displayed fusions contain an N-terminal 6x histidine (His) tag and a C-terminal c-myc epitope tag (Fig. 1). Additionally, a $(G_4S)_4$ polypeptide linker and an HA epitope tag are included between the F2A peptide and Aga2. Addition of a flexible polypeptide linker between the yeast cell wall and the protein has been shown to increase enrichment of binders when screening yeast display libraries by cell panning[29]. The HA and c-myc epitope tags enable detection of cell surface displayed proteins by immunofluorescent labeling; the His tag allows purification of solubly secreted protein by immobilized metal affinity chromatography (IMAC). A detailed vector map of pCT-NT-F2A is included in Fig. S1.

F2A peptide enables simultaneous cell surface display and soluble secretion of Sso7d mutants

To test our proposed system for simultaneous yeast cell surface display and soluble secretion of proteins (Fig. 1), we cloned two Sso7d mutants into the pCT-NT-F2A plasmid vector: Sso7d-streptavidin (Sso7dstrep), an Sso7d mutant with affinity for streptavidin[17]; and Sso7d-hfc (Sso7dhFc), an Sso7d mutant that binds to the Fc region of human IgG (hFc)[19]. Upon plasmid transformation into yeast, protein expression was induced by culturing overnight in SGCAA medium. Figs. 2A, C confirm that both Sso7dhFc and Sso7dstrep are expressed as yeast cell surface fusions, and can bind to their respective targets in the N-terminal configuration (Sso7d-Aga2). To test if the Sso7d mutants were being solubly secreted into the media, protein expression was induced by culturing yeast cells in SGCAA at 30°C for 3 days. Subsequently, streptavidin-coated or human IgG-coated beads were incubated with 100 μL supernatant from cells expressing Sso7dstrep or Sso7dhFc,

respectively, and bead-bound protein was detected with an anti-his antibody; the presence of immobilized IgG on streptavidin beads was confirmed using an antibody against human IgG (Fig. S2). Flow cytometry analysis showed presence of bead-bound protein and confirmed that the solubly secreted proteins were functional (Figs. 2B, D). The difference in signal between Sso7dstrep and Sso7dhFc is likely due to the difference in binding affinities for their respective targets. The binding affinity (K_D) of Sso7dstrep for streptavidin of 12 nM, as determined in experiments where Sso7dstrep was immobilized[17]; on the other hand, the K_D of Sso7dhFc for hFc as determined in experiments where hFc is immobilized is 400 nM[19].

Pulldown of proteins using His-Tag isolation beads followed by detection of the c-myc epitope tag further confirmed the secretion of Sso7dstrep and Sso7dhFc into cell culture supernatants. (Fig. 3A). A basal level of secretion of Aga2-fusion proteins has been previously reported[30]. In our case, solubly secreted Sso7d-Aga2 fusions will contain HA and His tags, and will be pulled down by the His-Tag isolation beads. To investigate the extent of secretion of Sso7d-Aga2 fusions, His-Tag isolation beads incubated with supernatant from yeast cells were labeled with an anti-HA antibody and analyzed using flow cytometry. Negligible binding of the anti-HA antibody was observed, indicating that the basal secretion of Sso7d-Aga2 fusions is not significant (Fig. 3B). Additional immunoblotting analysis of the supernatant from Sso7dstrep cultures using an anti-c-myc antibody showed the presence of a ~ 13 kDa protein (Fig. 3C); this corresponds to the expected molecular weight of the Sso7d-F2A fusion. Also, the 13 kDa protein containing a c-myc tag is the major solubly secreted product, confirming that most of the solubly secreted protein lacks Aga2. A band between 40 and 50 kDa is also observed, which may correspond to a glycosylated Sso7d-Aga2 fusion; the expected molecular weight of an Sso7d-F2A-Aga2 fusion is approximately 44 kDa (14.3 kDa from Sso7d-F2A-linker and 30 kDa from glycosylated Aga2[30]). We further purified Sso7dstrep from cell culture supernatant with Ni-NTA beads and obtained a yield of 7–8 mg/L from a 5 mL culture, comparable to the secretion of scFvs reported by Deventer *et al.*[4] in their system for switchable yeast surface display and secretion of proteins. SDS-PAGE analysis on the purified protein showed a single band corresponding to Sso7dstrep-F2A; a band for the Sso7d-Aga2 fusion was not observed (Fig. 3D). Taken together, our results in Figs. 2 and 3 confirm the F2A peptide sequence can mediate ribosomal skipping in yeast *Saccharomyces cerevisiae*. Importantly, the pCT-NT-F2A vector can be used for simultaneous expression of Sso7d mutants as functional yeast cell surface fusions, as well as solubly secreted proteins with reasonable yields.

A combinatorial library of Sso7d mutants incorporating the F2A peptide can be screened for high affinity binders

A yeast surface display system that enables simultaneous cell surface display and secretion of proteins is very useful in the context of screening combinatorial libraries of proteins. Therefore, we investigated if the F2A peptide-based yeast surface display system can be used for screening a combinatorial protein library. Towards this end, we sought to isolate binders with higher affinity for a model target protein (hen egg lysozyme) from a library generated by random mutagenesis of a pool of low affinity binders. DNA from a pool of low affinity binders to lysozyme obtained by magnet activated cell sorting (MACS) of an Sso7d library of ~ 10^8 mutants[17] was used to generate a yeast display library as previously

described[19], using the pCT-NT-F2A vector; the library diversity was estimated as ~ 5×10^7 mutants.

To isolate high affinity binders from this library, we used MACS, which is routinely employed in screening yeast display libraries[31]. However, initial experiments failed to isolate cells that bound to lysozyme-coated magnetic beads. A likely explanation is that the presence of the F2A peptide or N-terminal fusion of Sso7d mutants to Aga2 hinders interaction with biotinylated lysozyme immobilized on streptavidin-coated magnetic beads. An alternative explanation is that the lower cell surface density of binders on the yeast cell surface may reduce avidity of interaction between the beads and the yeast cells; the F2A-based yeast display system is expected to have fewer cell surface fusions than the conventional system because not all translated proteins are fused to Aga2. However, we did not find evidence of any significant reduction in levels of surface expression in the F2A-based system (Fig. S3). Also, we do not observe any effect of the F2A-based system on cell viability.

To explore an alternative strategy for immobilization of lysozyme, and to increase the surface density of immobilized target, we coated ELISA plates with lysozyme. The library was sorted using a panning strategy similar to the approach described by Xin *et al*[32]. The pool of yeast cells obtained after one round was labeled at 1 μM lysozyme and sorted further using a single round of fluorescence activated cell sorting (FACS). The pools of yeast cells before and after FACS were labeled with 1 μM lysozyme and an anti-HA antibody, and analyzed by flow cytometry (Figs. 4A, B). The ratio of the fluorescence due to target binding to the fluorescence due to signal from the anti-HA antibody (i.e. cell surface expression of protein), referred to as fluorescence ratio hereafter, can be used as a quantitative metric to compare relative binding affinities of yeast displayed proteins for a given target[21]. The mean fluorescence ratio was computed for the pools of binders after one round of panning, and after one round of FACS, for a range of lysozyme concentrations, and found to be significantly greater for the latter pool at all concentrations (Fig. 4C); this shows enrichment of high affinity binders after one round of FACS. Additionally, we computed the fluorescence ratio for single cells in pre- and post-FACS populations, for lysozyme concentrations of 1 μM. The fluorescence ratio was computed for cells exhibiting a fluorescence above background due to signal from the anti-HA antibody, and cells were placed into different bins based on this ratio, as previously described[21]. The population of cells after one round of FACS has a significantly greater frequency of cells with higher fluorescence ratio (Fig. 4D, Fig. S4). This analysis further confirms that binders with higher target binding affinity in the F2A-based yeast display library can be enriched by FACS.

We further investigated if soluble protein in the supernatant from yeast cultures of the heterogeneous population of cells obtained after one round of FACS retains binding to lysozyme. Towards this end, cells were cultured in SGCAA medium for 3 days at 30°C to induce protein expression, and solubly secreted proteins in the supernatant was purified using Ni-NTA resin. Yields of 4.7–7 mg/L of pure protein were obtained in different runs. SDS-PAGE analysis showed a major band at 13 kDa as expected (Fig. 5B), and immunoblotting analysis with an anti-c-myc antibody confirmed the presence of the desired protein species (Fig. 5C). Binding of the purified proteins to lysozyme was evaluated as

follows. Biotinylated lysozyme was immobilized on streptavidin-coated magnetic beads. Subsequently, the beads were incubated with 3.6 μM purified protein, and bead-bound protein was detected by labeling with an anti-His antibody (Fig. 5A). Flow cytometry analysis confirmed binding of the purified protein mix to immobilized lysozyme. Finally, plasmid DNA was isolated from 10 individual clones from the pool of cells after FACS. DNA sequencing revealed no sequence convergence; 10 distinct sequences were identified (Table S1). DNA sequencing results and flow cytometry analysis (Fig. 4B) indicate that additional rounds of FACS are needed to isolate the pool of binders with the highest affinity of lysozyme. However, isolation and characterization of the highest affinity lysozyme binders are beyond the scope of this study. Rather, taken together, our results in Figs 4 and 5 demonstrate two important points: first, a yeast surface display library of Sso7d mutants incorporating the F2A peptide can be screened to isolate a population enriched in binders with higher affinity for a given target. Second, the surface displayed proteins can be expressed in soluble form in cell culture supernatant at reasonable yields, enabling further characterization of the pool of binders isolated.

F2A peptide enables simultaneous yeast surface display and secretion of a dimeric enzyme

To assess its broader applicability, we chose to test the F2A-based yeast display system in the context of simultaneous surface display and secretion of glucose oxidase (GOx). GOx is a homodimeric enzyme that is only active in its dimeric state[33], making it a challenging test protein. Accordingly, we cloned GOx from *Aspergillus niger* into pCT-NT-F2A. As a control, GOx was also cloned into the pCTCON vector, which lacks the F2A peptide and results in yeast surface display of the protein as a C-terminal fusion to Aga2. Cell surface expression of GOx is significantly reduced when expressed as an N-terminal fusion to Aga2, relative to GOx expression in pCTCON (Fig. 6A). Nevertheless, when GOx was cloned into pCT-NT-F2A, 3-fold and 4-fold greater GOx activity was observed in the cell culture supernatant after 24 and 48 hours of protein induction, relative to the basal level of GOx secretion using pCTCON (Fig. 6C). GOx activity in the supernatant from yeast cells with pCT-NT-F2A decreases at 72 hours, likely due to enzyme degradation in supernatant.

One possible explanation for the higher cell surface expression of GOx in pCTCON is that Aga2 fused to the N-terminus of GOx may act as a solubility-enhancing fusion partner. Therefore, we investigated the effect of Sso7dhFc as an N-terminal fusion partner in pCT-NT-F2A (Sso7dhFc-Gox-F2A-Aga2). Strikingly, presence of N-terminal Sso7dhFc increased the display levels of GOx (Fig. 6B). Further, as seen in Fig. 6C, ~ 8-fold greater GOx activity was detected in the supernatant from pCT-NT-F2A cultures (Sso7dhFc-Gox-F2A-Aga2) after 48 and 72 hours, relative to the pCTCON control (Aga2-GOx). In these experiments, protein expression was induced by culture in SGCAA medium at 20°C, since lower GOx activity was observed at 30°C (data not shown).

GOx and Sso7d-GOx expressed on the yeast cell surface using the pCT-NT-F2A vector was found to be enzymatically active. (Fig. S5). Interestingly, despite the significantly reduced surface expression level, enzymatic activity of cells expressing GOx through pCT-NT-F2A was comparable to that of cells expressing GOx through pCTCON. It is possible that the

presence of the Aga2 protein at the N-terminus of GOx affects its enzymatic activity. Alternatively, the higher surface density of GOx in case of pCTCON may result in lower observed activity due to local depletion of oxygen or inactivation of GOx due to local accumulation of hydrogen peroxide, as observed in the case of sensors employing immobilized GOx[34–36]. However, further investigation into the apparent decrease in enzymatic activity of surface displayed GOx when the pCTCON vector is used is outside the scope of this study.

After induction of protein expression in SGCAA medium for 72 hours, Sso7dhFc-Gox in the supernatant was purified by ion exchange chromatography. Yields of 13–33 mg/L were obtained in different runs. Immunoblotting using an anti-c-myc antibody showed a single band ~ 100 kDa (Fig. 6D). The expected molecular weight of Sso7dhFc-GOx including the F2A peptide is ~ 80 kDa. It is likely that glycosylation of GOx increases its apparent molecular weight, as reported elsewhere[37,38].

F2A peptide enables simultaneous cell surface display and secretion of proteins when expressed as N- or C-terminal fusions to Aga2

Proteins on the surface of yeast can be displayed as either N-terminal (protein-Aga2) or C-terminal (Aga2-protein) fusions to Aga2. Importantly, functionality of the cell surface displayed protein may depend on the position of Aga2. For example, binding of an scFv to its target is greatly enhanced when expressed N-terminal to Aga2 rather than as a C-terminal fusion[39]. On the other hand, Stern *et al.*[29] observed that expressing some fibronectin domain mutants as N-terminal fusions to Aga2 hindered target binding. In this study, expression of GOx as an N-terminal fusion to Aga2 greatly decreased the cell surface expression of GOx, compared to the case when GOx is expressed C-terminal to Aga2 (Fig. 6A). Therefore, a system for simultaneous yeast surface display and secretion of proteins that allows the protein of interest to be expressed as an N- or C-terminal fusion to Aga2 will have a significant advantage. We hypothesized that incorporation of an additional secretory signal peptide downstream of the F2A peptide sequence will allow the protein of interest to be solubly secreted even in the absence of Aga2; the prepro sequence is N-terminal to Aga2 when the protein of interest is expressed as a C-terminal fusion to Aga2. Our proposed system is shown in Fig. 7A. The presence of a second secretory signal peptide is critical; in its absence, the protein of interest will remain in the cytoplasm. Indeed, as reported by Grzeschik *et al.*[16], when GFP was placed C-terminal to a 2A peptide sequence without a secretory tag (prepro-Aga2-protein-2A-GFP), GFP accumulated inside the yeast cell.

To test our hypothesis, GOx was cloned into pCT-CT-F2A (Fig. S1B), a modified pCTCON vector containing the F2A peptide between Aga2 and GOx (Aga2-F2A-Gox). GOx was chosen as a test protein because the first 23 amino acids of GOx are predicted to be a secretory signal (preGOx)[40]. Upon yeast transformation and induction of protein expression in SGCAA medium, 8–11 fold higher GOx activity was observed in the cell culture supernatant over 24–72 hours, relative to the pCTCON control (Fig. 7D). Furthermore, cell surface expression of GOx improved considerably when pCT-CT-F2A was used, when compared with the use of pCT-NT-F2A. (Fig. 7C). It is interesting to note that the cell surface expression levels of both Sso7d-GOx using pCT-NT-F2A, and GOx using pCT-CT-

F2A, are higher than those of GOx using pCT-NT-F2A. One potential explanation consistent with this observation is that the presence of preGOx in tandem with prepro may hinder protein processing in the secretory pathway, but not when another protein is present directly upstream of the preGOx, as is the case with Sso7d-GOx in the pCT-NT-F2A construct, and GOx in the pCT-CT-F2A construct. Another interesting observation is that a decrease in GOx activity in the supernatant was observed in case of pCT-NT-F2A at 72 hours, but not pCT-CT-F2A (Fig. 6C vs. Fig. 7D). A likely explanation for this observation is that higher levels of protein secretion in pCT-CT-F2A results in higher GOx activity in the supernatant at 72 hours vs. 48 hours, despite protein degradation. Notably, cell surface expression of Aga2-GOx in pCT-CT-F2A is greater than that of GOx-Aga2 in pCT-NT-F2A (Fig. 7C vs. Fig. 6A); therefore, one would reasonably expect greater level of secretion of GOx in pCT-CT-F2A.

After protein induction in SGCAA medium 72 hours at 20°C, solubly secreted GOx in yeast cell culture supernatant was purified by anion exchange chromatography and yields of 8–13 mg/L were obtained in different runs. Immunoblotting analysis of purified protein with an anti-c-myc antibody showed the presence of a single band at >100 kDa (Fig. 7E). As discussed earlier, the apparent molecular weight of GOx is greater than expected, likely due to glycosylation[37,38]. Frederick et al.[40] showed that GOx processed with the endogenous secretory signal peptide (preGOx) in *Saccharomyces cerevisiae* results in protein with higher apparent molecular weight than GOx processed with the α-maturation factor (α-mat) signal peptide. GOx secreted solubly with preGOx showed a band at molecular weight above 100 kDa in SDS-PAGE, whereas GOx produced using α-mat resulted in a band with molecular weight between 70 and 100 kDa. It was proposed that preGOx and α-mat secretion signals in GOx may be processed differently in the yeast secretory pathway. Notably, Sso7dhFcGox in pCT-NT-F2A utilizes a prepro secretory signal peptide, whereas GOx in pCT-CT-F2A uses preGOx; this difference in secretory signal peptide usage may explain the higher apparent molecular weight of GOx in pCT-CT-F2A relative to Sso7d-GOx in pCT-NT-F2A. Thus, simultaneous yeast cell surface display and secretion of GOx can be achieved using pCT-CT-F2A, where GOx is expressed as a C-terminal fusion to Aga2.

We further sought to investigate if Sso7d mutants can be simultaneously expressed as cell surface fusions and solubly secreted when expressed as C-terminal fusions to Aga2. Towards this end, Sso7dstrep and Sso7dhFc were cloned into pct-CT-F2A with the secretory leader sequence SED1SP[41] placed after the F2A peptide (Fig. 7A). SED1SP was chosen because of its smaller size (1.9 kDa) and lower hydrophobicity compared to other secretory signal peptides. Additionally, a $(PT)_5$ polypeptide linker was placed between the F2A and Sso7d. The resulting vector is referred to as pCT-CT-F2A-SED1SP (Fig. S1C).

Both Sso7dhFc and Sso7dstrep could be displayed on the surface of yeast in this construct (Fig. S6). However, binding of Sso7dstrep to streptavidin in pCT-CT-F2A is significantly reduced in comparison with the pCTCON control, likely due to the presence of the F2A peptide (Figs. S6A–C). On the other hand, binding of Sso7dhFc to hFc is affected to a lesser extent (Figs. S6D–F). Therefore, the effect of the F2A peptide on binding functionality of surface-displayed Sso7d mutants appears to be protein-specific.

Flow cytometry analysis confirmed the secretion of Sso7dstrep in the supernatant when protein expression was induced by culture in SGCAA medium for 72 hours. Solubly secreted protein was captured on His-Tag isolation beads and bead-bound protein was detected using an anti-c-myc antibody (Fig. 7B). Notably, secretion of Sso7dstrep was observed when SED1SP was fused to the N-terminus of Sso7dstrep, but not in its absence, confirming that an additional secretory signal peptide is necessary to enable soluble protein secretion when the protein is fused to the C-terminus of Aga2. Similar results were obtained for Sso7dhFc (Fig. S7). Although Sso7dstrep secretion was detected by flow cytometry, the levels of solubly secreted Sso7dstrep were too low to be detected by SDS-PAGE and immunoblotting analysis. Sso7dhFc was also undetectable by SDS-PAGE, but a protein band at the correct molecular weight was detected by immunoblotting analysis using an anti-c-myc antibody (Fig. S7).

We further investigated if a different secretory signal peptide could increase levels of solubly secreted Sso7d mutants in cell culture supernatant, when expressed as C-terminal fusions to Aga2. Towards that end, SED1SP was replaced with the synthetic prepro secretory leader used in pCT-NT-F2A. The resulting vector is referred to pCT-CT-F2A-prepro (Fig. S1D); this vector also lacks the $(PT)_5$ linker. Sso7dhFc was displayed on the surface of yeast in pCT-CT-F2A-prepro; however, binding to hFc was significantly reduced when compared with pCT-CT-F2A-SED1P (Fig. S7A vs. Fig. S6F). Thus, in addition to the F2A sequence, the specific secretory signal peptide chosen may affect binding functionality of the displayed protein. Furthermore, solubly secreted Sso7dhFc was detected by flow cytometry and immunoblotting analysis using an anti-c-myc antibody (Figs. S7B, C). However, the purified protein could not be detected by SDS-PAGE analysis, indicating low solubly secreted protein yields.

Upon protein translation, a proline residue and a histidine residue are inherently introduced at the N-terminus of SED1SP and prepro when the F2A peptide is present N-terminal to the secretory signal peptide. Proline results from ribosomal skipping of the F2A peptide and histidine from a translated endonuclease restriction site. It is possible that the presence of these additional amino acids inhibits the effectiveness of SED1SP and prepro as secretory signal peptides. Indeed, it has been observed that introduction of a single N-terminal mutation decreases secretion of yeast prepro alpha factor[42]. However, it is important to note that secretion of GOx is not hindered despite the introduction of additional amino acids to the GOx secretory tag. Therefore, it is likely that optimization of the secretory leader sequence or introduction of a secretory fusion partner, as described elsewhere[43,44], may enable higher secretion yield of Sso7d mutants expressed as C-terminal fusions to Aga2. Notably, Sso7d mutants can be obtained at reasonable yields when expressed as N-terminal fusions to Aga2. Indeed, though outside the scope of this study, the simultaneous secretion and surface display system described here is an ideal platform for efficiently identifying secretory peptide sequences that result in higher levels of protein secretion while retaining high levels of cell surface display.

Taken together, our result show that with the introduction of a suitable secretory peptide sequence, the F2A peptide can be used for simultaneous yeast cell surface display and soluble secretion of proteins expressed as C-terminal fusions to Aga2.

Conclusion

The F2A peptide from the foot-and-mouth disease virus causes ribosomal skipping during protein translation with an efficiency of ~ 50%. We have shown that this inefficient ribosomal skipping can be exploited to design a yeast surface display system in *Saccharomyces cerevisiae* wherein the protein of interest can be expressed simultaneously as a cell surface fusion and secreted in soluble form. Using this system, we have demonstrated simultaneous cell surface display and secretion of Sso7d mutants, and the homodimeric enzyme GOx. Further, we have shown that the F2A-based display and secretion system can be used to screen a combinatorial protein library. A major advantage of the F2A-based system is that the protein of interest can be expressed as a C- or N-terminal fusion to Aga2, as long as an appropriate secretory signal peptide is incorporated. This is particularly important because functionality of cell surface displayed proteins – including examples discussed in this study – can be affected by the mode of attachment (C-terminal vs. N-terminal) to the cell surface. The F2A peptide sequence, and/or the secretory signal peptide in cases where the protein of interest is C-terminal to Aga2, may affect functionality of a specific cell surface displayed proteins. Also, yield of solubly secreted protein will likely be protein specific. Nevertheless, use of a suitable polypeptide linker, secretory signal peptide, or secretory fusion partner, may overcome these limitations. Overall, we expect that the F2A-based system will be a useful tool for protein engineering and facilitate efficient validation of individual clones isolated from combinatorial protein libraries.

Materials and Methods

Reagents

Biotinylated human IgG, goat-anti-chicken DyLight®633 (GAC633), goat-anti-chicken DyLight®487 (GAC487), donkey-anti-rabbit DyLight®633 (DAC633), goat-anti-human IgG DyLight®633, and goat-anti-chicken HRP conjugate were purchased from Immunoreagents (Raleigh, NC). Chicken anti-c-myc antibody, rabbit-anti-HA antibody, streptavidin R-phycoerythrin conjugate (SA-PE), Dynabeads™ Biotin Binder (streptavidin-coated beads), Dynabeads™ His-Tag Isolation and Pulldown beads, High-fidelity Phusion™ polymerase, and CloneJET™ PCR cloning kit (K1231) were purchased from Thermo-Fisher (Waltham, MA). Mouse anti-penta-His antibody-Alexa Fluor 647 conjugate and Ni-NTA agarose were obtained from Qiagen (Valencia, CA). Frozen-EZ Yeast Transformation Kit™, Zymoprep™ Yeast plasmid Miniprep II kit, Quick-DNA™ Miniprep Plus Kit were purchased from Zymo Research (Irvine, CA). All restriction enzymes were purchased from New England BioLabs (Ipswich, MA). Gene fragments were purchased from Integrated DNA Technologies (IDT; Coralville, IA). Oligonucleotide primers were purchased from IDT or Eton Biosciences (Raleigh, NC). Sequences of all primers and G-blocks are included in Table S1 (primers) and Table S2 (gene fragments). The composition of yeast culture media used was as previously described[31].

Construction of plasmid vectors containing the F2A peptide sequence

To construct pCT-NT-F2A, gene block 1 with phosphorylated ends, containing the sequence corresponding to prepro-6xHis-Sso7dstrep-c-myc-F2A-Aga2, was cloned into the pJET

vector by blunt-end cloning using a CloneJET™ PCR cloning kit. The insert was then subcloned into the yeast surface display vector pCTCON between the EcoRI and XhoI restriction sites to generate pct-NT-F2A-0. To introduce a linker between Aga2 and F2A, gene block 2 was amplified by PCR with primers Pf1.1 and Pr1.1, and cloned into pct-NT-F2A-0 between BamHI and XhoI sites. An HA tag was then introduced into pCT-NT-F2A-0 between the linker sequence and Aga2 by amplifying the Aga2 from gene block 2 by PCR with primers Pf1.2 (containing the sequence corresponding to HA) and Pr1.1, and cloning in the fragment between AvrII and XhoI restriction sites. This vector was denoted pct-NT-F2A (Fig. S1A). To clone Sso7dhFc into pct-NT-F2A, the Sso7dhFc gene was amplified from a pCTCON-based plasmid with primers Pf2 and Pr2, and cloned into pCT-NT-F2A between NheI and BamHI restriction sites.

To construct pCT-CT-F2A (Fig. S1B), a gene block 3 containing the sequence corresponding to prepro-Aga2-F2A-6xHis-Sso7dstrep-c-myc was amplified by PCR with primer Pf3.1 and Pr3.1, and cloned into pCTCON between EcoRI and XhoI sites. This construct lacks a secretory signal peptide sequence before Sso7d. To construct pCT-F2A-SED1SP (Fig. S1C), gene block 4 was amplified by PCR using primers Pf3.2 and Pr3.2, and the PCR product was cloned in between AvrII and NheI restriction sites of pct-CT-F2A. Sso7dhFc was amplified from a pCTCON-based plasmid with primers Pf2 and Pr2, and cloned into pct-CT-F2A-SED1SP and pCT-CT-F2A using the NheI and BamHI restriction sites. SED1P and $(PT)_5$ linker sequences in pCT-CT-F2A-SED1SP were replaced by the synthetic prepro sequence to construct pCT-CT-F2A-Prepro (Fig. S1D); gene block 5 containing prepro and Sso7dhFc was amplified by PCR with primers Pf3.3 and Pr2, and cloned into pct-CT-F2A-SED1P between the NdeI and BamHI restriction sites.

All PCR reactions were performed in 50 μL reactions with high-fidelity Phusion™ polymerase, following the manufacturer's protocol; annealing temperature of 62°C and extension time of 15 seconds were used.

Yeast surface display of proteins in F2A for flow cytometry analysis

To induce cell surface protein expression, yeast cells containing pCTCON, pCT-NT-F2A, pCT-CT-F2A, pCT-CT-F2A-SED1SP, or pCT-CT-F2A-Prepro, were cultured in SGCAA media overnight, in an incubator-shaker at 20°C and 250 RPM. Subsequently, $5x10^6$ cells were washed with PBS containing 0.1% BSA (PBS-BSA), and labeled with a chicken-anti-c-myc antibody (1:100 dilution) in 50 μl PBS-BSA for 20 minutes at room temperature. Secondary labeling was carried out using GAC633 (1:250 dilution) in 100 μL PBS-BSA for 12 minutes on ice. For simultaneous detection of cell surface expression and target binding, yeast cells expressing Sso7dhFc and Sso7dstrep were labeled with 500 nM biotinylated IgG and a 1:200 dilution of SA-PE, respectively, in a 50 μL reaction along with 1:100 dilution of chicken-anti-c-myc antibody. Secondary labeling was performed in a 100 μL reaction with a 1:250 dilution of GAC633 and 1:250 dilution SA-PE for Sso7dhFc, and 1:250 dilution GAC633 for Sso7dstrep. Samples were analyzed by a BD Accuri™ C6 flow cytometer (Franklin Lakes, NJ). Three independent replicate experiments were conducted, and fluorescence data for 50,000 cells was collected in each instance.

Capture and detection of solubly secreted Sso7dstrep and Sso7dhFc on His-Tag isolation beads

To induce protein expression, yeast cells were cultured for three days in SGCAA media, in an incubator-shaker at 30°C and 250 RPM for 3 days. Subsequently, 1 mL of culture was centrifuged at 15,000 g, and 500 μL of supernatant was mixed with 1.5 mL of 1 M sodium phosphate pH 9. 10 μL of His-Tag isolation beads were added to the mix and incubated for 1 hour at room temperature with rotation. Subsequently, beads were washed once with PBS, and incubated for 3 hours in PBS + 1% BSA at room temperature. After this blocking step, buffer was removed and the beads were split into two identical aliquots. 50 μL of PBS-BSA with 1:100 dilution of chicken anti-c-myc antibody, or 1:100 dilution of rabbit-anti-HA antibody was added to each aliquot, and incubated for 20 minutes at room temperature, followed by one wash with 1 mL of PBS-BSA. Subsequently, 100 μL of a 1:300 dilution of GAC488 or DAR633 in PBS-BSA was added to beads labeled with the anti-c-myc or anti-HA antibodies, respectively, and beads were incubated for 12 minutes on ice. After a wash step with PBS-BSA, fluorescence of 100,000 beads was quantified with a BD Accuri™ C6 flow cytometer. To quantify levels of non-specific binding of the antibodies, beads that were not incubated with supernatant were labeled using an identical protocol.

Capture of solubly secreted Sso7dhFc and Sso7dstrep with streptavidin-coated beads

75 μL of streptavidin-coated beads (Dynabeads™ Biotin Binder) were functionalized with IgG by overnight incubation at 4°C in 100 μL of PBS-BSA solution containing 3 μL of 1.5 mg/mL biotinylated-human IgG. Plain streptavidin beads or IgG functionalized beads were blocked with 1% BSA in PBS buffer for 3 hours, and subsequently resuspended in a 100 μL of PBS with 0.2% BSA. 100 μL of supernatant from yeast cultures expressing Sso7dstrep or Sso7dhFc, wherein protein induction was carried out over a 3 day period as described earlier, was added to the bead suspension and incubated at room temperature for 20 minutes. Beads were then washed once with PBS-BSA, and labeled with 50 μL of a 1:100 dilution of anti-His Alexa-647 antibody in PBS-BSA, for 12 minutes on ice. After another wash step, fluorescence of 100,000 beads was quantified using a BD Accuri™ C6 flow cytometer. IgG-coated beads were labeled with a 1:250 dilution of goat-anti-human IgG DyLight®633 in 100 μL of PBS-BSA to confirm the presence of immobilized human IgG.

Purification of solubly secreted Sso7dstrep from yeast culture supernatant

Expression of Sso7dstrep was induced in a 5 mL yeast culture in SGCAA media at 30°C, for 72 hours and 250 RPM. Culture medium was centrifuged at 15,000 g for 5 minutes, and 4.5 mL of supernatant was filtered through a 0.22 μm syringe filter. 0.5 mL of 1 M sodium phosphate buffer was added to the media, and the pH was adjusted to 7.4 using 1M sodium hydroxide. 25 μL of His-Tag isolation beads was added and incubated for 1 hour, with rotation. The beads were recover with a magnet, washed 3 times with PBS buffer pH 7.4, and resuspended in 150 μL of PBS with 500 mM imidazole to elute the protein. Purity of the elution product was analyzed by SDS-PAGE. Protein concentration was measured by BCA assay, and the yield was estimated from this measurement. Two independent replicates of this experiment were conducted.

Generation of a combinatorial library using error-prone PCR

DNA from a population of yeast cells expressing Sso7d-based binders for lysozyme – previously obtained by screening a library of Sso7d mutants using one round of MACS[17] – was recovered using a Zymoprep™ Yeast plasmid Miniprep II kit. Sso7d sequences from the isolated DNA were amplified by error-prone PCR using nucleotide analogs, with primers Pf2 and Pr2, as detailed by Gera *et al*[19]. Products of the error-prone PCR were combined, and further amplified by PCR using primers Pf4 and Pr4 in six identical 50 μL reactions. The PCR products were combined and purified by ethanol precipitation. Briefly, potassium acetate was added to the combined PCR reactions in a 1:10 ratio, followed by 2.5 volumes of ice cold ethanol. The mix was incubated at −20°C overnight, centrifuged at 15,000 g for 10 minutes, and the supernatant was removed. The pellet was washed once with 70% ethanol, allowed to dry out, and resuspended in water. In parallel, 40 μg of pCT-NT-F2A-Ssod7strep was digested with NheI and BamHI restriction enzymes, and purified by ethanol precipitation. To obtain the yeast library, the PCR product and the digested vector were transformed into yeast using lithium acetate method, as previously described[45]. A single electroporation reaction was performed using a Bio-Rad Gene Pulser System (Bio-Rad, Hercules, CA); where 12 μg of PCR product and 3 μg of digested vector were added to 300 μL of electrocompetent *Saccharomyces cerevisiae* strain EBY100 and electroporated at 2500V, 25 μF, 200 Ω. An identical electroporation reaction was also carried for a vector-only control. Library diversity was determined by plating serial dilutions of the transformation reaction in SDCAA plates, and estimated as $5x10^7$.

Combinatorial library screening

As described by Xin *et al*[32], $5x10^7$ cells can be screened per cm^2 of surface area in a cell panning experiment. Therefore, based on the dimensions of a 96-well ELISA plate, at least 4 wells are need to screen $5x10^8$ cells (10x library diversity). Accordingly, 5 wells each of a 96-well ELISA plate were coated with the following proteins, overnight at 4°C: 300 μL of 20 ng/μL lysozyme, 10% donkey serum, 10% goat serum, 10% bovine serum, or streptavidin (3 μg per well). $5x10^8$ cells were centrifuged and resuspended in 1.5 mL of PBS-BSA. 300 μL cell aliquots were transferred into each of the 5 wells. Negative selections were performed by serially incubating the cells in the wells coated with one of serum proteins or streptavidin for 1 hour each. For the positive selection, yeast cells were transferred into the lysozyme-coated wells and incubated for 1 hour, and then washed 5 times with PBS-BSA. 300 μl of SDCAA media were added to each well, and allowed to incubate at 30°C for 2 days. All selections were performed at 4°C, with slow agitation. Yeast cells that grew in each well were combined, passaged once in SDCAA, and protein expression was induced in SGCAA at 20°C. $1x10^7$ cells were labeled with 1 μM lysozyme in 100 μL of PBS-BSA for 20 minutes, at room temperature. The cells were then washed once with PBS-BSA, and labeled with 500 μL of 1:1000 dilution SA-PE in PBS-BSA, and sorted to isolate cells with high PE fluorescence using a MoFlo™ cell sorter (Beckman Coulter, Brea, CA). A total of 1 million cells were analyzed; 478 positive cells were sorted and grown in SDCAA media.

Flow cytometry analysis of enrichment for higher affinity lysozyme binders by FACS

The pools of yeast cells obtained after the panning round and after FACS were induced in SGCAA media at 20°C overnight. $5x10^6$ cells were used for each sample from each population were resuspended in 100 μL of PBS-BSA buffer containing 0, 0.25, 0.50, 1.0, 1.5, 2.5, 5.0, or 10 μM biotinylated lysozyme, and 1:100 dilution of anti-HA antibody, in PBS-BSA. The samples were allowed to equilibrate for 20 minutes at room temperature, washed once with PBS-BSA, and resuspended in 500 μL of 1:1000 dilution of SA-PE and 1:300 dilution of DAR633 in PBS-BSA, for 12 minutes on ice. Samples were washed one more time and kept on ice before analysis. 50,000 cells were analyzed with a BD Accuri™ C6 flow cytometer. The ratio of mean background-subtracted average fluorescence due to lysozyme binding, to the corresponding fluorescence due to binding of the anti-HA antibody was calculated and plotted as a function of lysozyme concentration. Three independent replicate experiments were conducted. The mean fluorescence value of the control sample containing no lysozyme was used for background subtraction.

Purification and validation of soluble lysozyme binders from yeast cell culture supernatant

Three 5 mL cultures of the pool of cells obtained after FACS were induced in SGCAA media, at 30°C for 72 hours and 250 RPM. Culture medium was centrifuged at 15,000 g for 5 minutes, and the supernatants were filtered through a 0.22 μm syringe filter. The volume of the combined supernatant was brought to 15 mL by adding 1.5 mL of 1M sodium phosphate and water, and the pH was adjusted to 7.4 using 1M sodium hydroxide. 1 mL of Ni-NTA resin was added to the pH-adjusted solution, and incubated for 1 hour, with rotation. Subsequently, the resin was recovered by centrifugation at 600g for 2 minutes, washed 3 times with PBS (pH 7.4), and resuspended in 1.5 mL of elution buffer (PBS containing 500 mM imidazole). The elution buffer was separated from the resin using a trap column, and dialyzed into PBS, pH 7.4. Purity of the elution product was analyzed by SDS-PAGE. Protein concentration was measured by BCA assay, and the yield was calculated from this measurement. Two independent purification runs were conducted.

To investigate functionality of purified lysozyme binders, streptavidin beads were functionalized with biotinylated lysozyme by overnight incubation at 4°C. Subsequently, beads were blocked with 1% BSA in PBS buffer for 3 hours. Beads were then resuspended in 100 μL PBS with 0.2% BSA and incubated with 100 μL purified lysozyme solution (final concentration 3.6 μM) for 20 minutes. After a wash with PBS-BSA, beads were incubated in 50 μL of a 1:100 dilution of anti-His Alexa-647 antibody in PBS-BSA, for 12 minutes on ice. The beads were washed once, and the fluorescence of 100,000 events was quantified using a BD Accuri™ C6 flow cytometer.

Cloning of GOx into pCT-NT-F2A and pCT-CT-F2A

Aspergillus niger spores were a kind gift from Dr. Elhanafi (Biomanufacturing Training and Education Center, North Carolina State University). Spores were incubated overnight in a 5 mL culture in YPD at 37°C. The media was filtered, and the fungus (~ 30 mg) was transferred into a microcentrifuge tube. The tube was dipped in liquid nitrogen, and the frozen cells ground with a pipette tip. Genomic DNA was extracted using a Quick-DNA™ Miniprep kit. The GOx gene was amplified by PCR using primers Pf5 and Pr5. The product

was purified and further amplified with primers Pf6 and Pr6 to introduce AvrII and BamHI restriction sites. The resulting PCR product was digested with AvrII and BamHI, and ligated into pCTCON, pCT-NT-F2A or pCT-CT-F2A digested with NheI and BamHI; AvrII and NheI result in compatible cohesive ends.

The Sso7dhFc-GOx construct was generated by cloning GOx gene into an existing pET22b(+) vector containing the Sso7dhFc-$(PT)_8$-TEV sequence. GOx was amplified by PCR using primers Pf6 and Pr7 to introduce AvrII and XhoI restriction sites to the gene. The TEV sequence was replaced with the GOx gene by digesting the plasmid with AvrII and XhoI, and ligating the PCR product. Sso7d-$(PT)_8$-Gox in pET22b(+) was then amplified by PCR using primers Pf2 and Pr6, and the PCR product was cloned into pct-NT-F2A using NheI and BamHI restriction sites.

Measurement of GOx activity

Protein expression was induced in yeast cells carrying the GOx gene in pCTCON, pCT-NT-F2A, or pCT-CT-F2A, in SGCAA media for 72 hours, at 20°C and 250 RPM. 100 μL aliquots of the supernatants were collected at 24, 48, and 72 hours after induction. The 48 and 72 hour aliquots were diluted 1:5 and 1:10, respectively, in PBS, pH 7.4. 100 μL of 2X working reagent (100 mM glucose, 0.1 mg/mL TMB substrate, 1.5 μg/mL horseradish peroxidase diluted in PBS pH 7.4) was added to the 100 μL supernatant samples and the reaction was allowed to proceed for 5 minutes at room temperature, before being quenched by 50 μL of sulfuric acid. The quenched reactions were diluted 1:4 in PBS, and 100 μL aliquots were transferred to a 96-well plate, in duplicate. The absorbance values of the solutions at 450 nm were measured using a 96-well plate reader, and used as a metric for activity of solubly secreted GOx in the supernatant. The absorbance corresponding to a 100 μL PBS sample, processed similar to the supernatant samples, was subtracted from all readings as background correction. To measure activity of GOx expressed as cell surface fusions, $5x10^6$ cells were washed once with PBS and resuspended in 100 μL PBS, prior to addition of 100 μL working reagent. Subsequent steps were the same as previously described for determining activity of solubly secreted GOx in supernatants.

Purification of Sso7dhFc-GOx and GOx

GOx and Sso7dhFc-GOx in yeast cell culture supernatant were purified by anion exchange chromatography using a BioLogic™ LP FPLC system (Bio-Rad, Hercules, CA) as follows. Protein expression was induced in 6 mL SGCAA cultures at 20°C, for yeast cells with GOx in pCT-CT-F2A, or Sso7dhFc-Gox in pCT-NT-F2A, for 72 hours. 5 mL of culture medium was collected and centrifuged, filtered through a 0.22 μm syringe filter, and combined with 5 mL of water and 5 mL of 50 mM Tris-HCl, 50 mM NaCl, pH 8 (loading buffer). The solution was loaded into a 5 mL Bio-Rad High Q column, washed with 40 mL of loading buffer, and eluted with a 12 mL step of 20% elution buffer (50 mM Tris-HCl, 2.0 M NaCl, pH 8), followed by a 30 mL linear gradient of elution buffer (30% to 50%). 3 mL fractions were collected throughout the protocol. The activity of each elution fraction was measured by transferring 100 μL of elution fraction into a 96-well plate, and then adding 100 μL of working reagent to each fraction. The reaction was allowed to proceed for 5 minutes, at room temperature, before been quenched with 50 μL of sulfuric acid. The absorbance values

at 450 nm were measured using a 96-well plate reader, and used as a metric for activity of solubly secreted GOx in the supernatant. The two fractions with highest activity were combined and dialyzed into 50 mM Tris, 300 mM NaCl, pH 7.4.

Immunoblotting analysis

For analysis of solubly secreted protein in supernatant of cells expressing Sso7d mutants, protein expression was induced by culture in SGCAA medium for 3 days at 30°C. 1 mL of media was centrifuged at 13,000 RPM, and mixed with 4 mL of ice-cold acetone. Proteins were allowed to precipitate overnight at −20 °C. The samples were then centrifuged at 10,000 g for 10 minutes, washed once with ice-cold 1:4 water: acetone solution, and centrifuged one more time. The supernatant was removed, and the pellets were allowed to dry out at room temperature, before being resuspended in 20 μL of PBS + 5% SDS. For analysis of purified Sso7dhFc-GOx, GOx, and Sso7d lysozyme binders, 200 μL of purified protein was mixed with 800 μL of ice-cold acetone, and concentrated as described earlier. 6.5 μL of precipitated sample was loaded into SDS-PAGE gels. Transfer into nitrocellulose membrane was accomplished by wet blot transfer at a constant current of 200 mA for 3 hours. The blot was blocked with 5% milk in TBST (25 mM Tris, 150 mM NaCl, 0.05% Tween-20) for 2 hours, followed by incubation with 10 mL 1:1000 chicken-anti-c-myc antibody in TBST overnight, at 4 °C. Secondary labeling was carried out for 1 hour at 4 °C using 15 mL of a 1:1000 goat-anti-chicken-HRP conjugate. Samples were detected using West-Femto substrate, according to the manufacturer's protocol. For Sso7dhFc expressed using pCT-CT-F2A-Prepro, 5 mL of cell culture supernatant was mixed with 10 mL of ice-cold acetone, incubated overnight at −20 °C, and then centrifuged and resuspended as detailed earlier. For Sso7dhFc expressed using pCT-CT-F2A-SED1SP, 15 mL of cell culture supernatant was concentrated to 200 μL using a 10K Amicon® Ultra centrifugal filter (EMD Millipore, Billerica, MA) and a 6.5 μL sample was used for loading the SDS-PAGE gel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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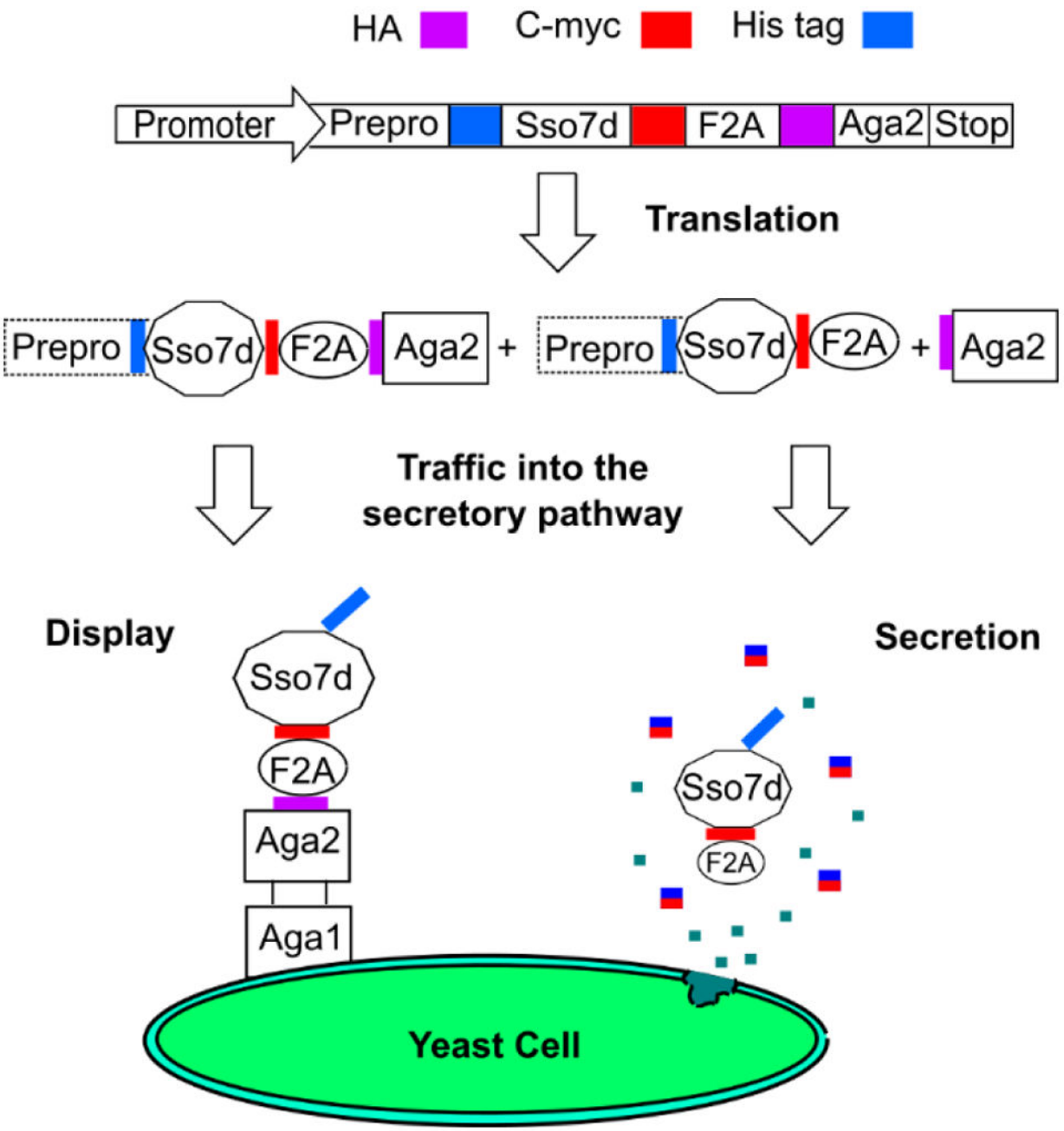

Figure 1.

F2A-based system for simultaneous secretion and surface display of proteins in *Saccharomyces cerevisiae.*

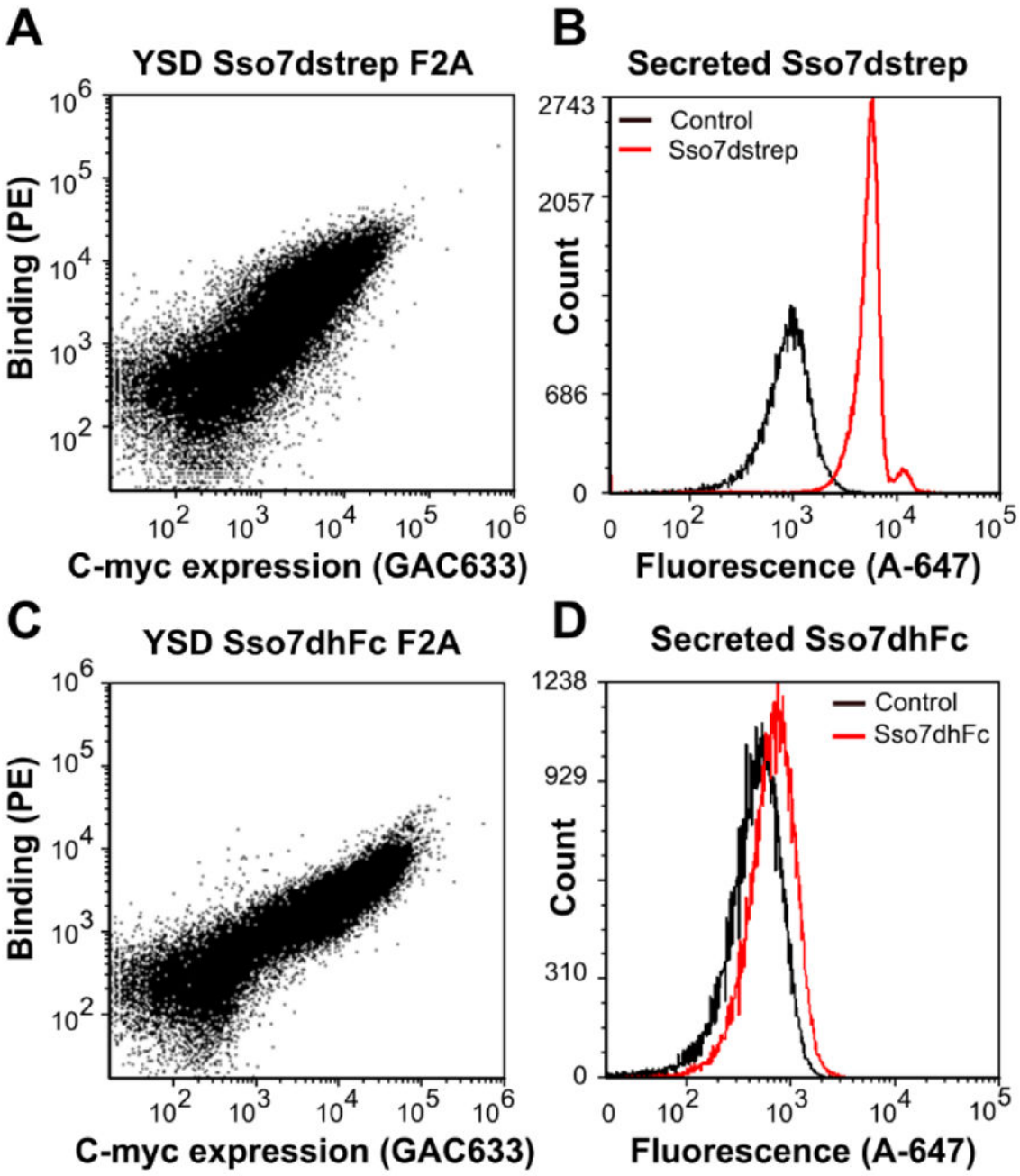

Figure 2.

Simultaneous yeast surface display and secretion of Sso7dstrep and Sso7dhFc. A) Yeast cells displaying Sso7dstrep were labeled with 90 nM streptavidin R-phycoerythrin (SA-PE) and an anti-c-myc antibody. B) Streptavidin coated beads (control) or beads incubated with supernatant from yeast cells displaying Sso7dstrep (red) were labeled with an anti-His antibody. C) Yeast cells displaying Sso7dhFc were labeled with 500 nM human IgG-biotin and an anti-c-myc antibody D) Streptavidin coated beads (control) or IgG-coated beads incubated with supernatant from yeast cells displaying Sso7hFc (red) were labeled with an anti-His antibody.

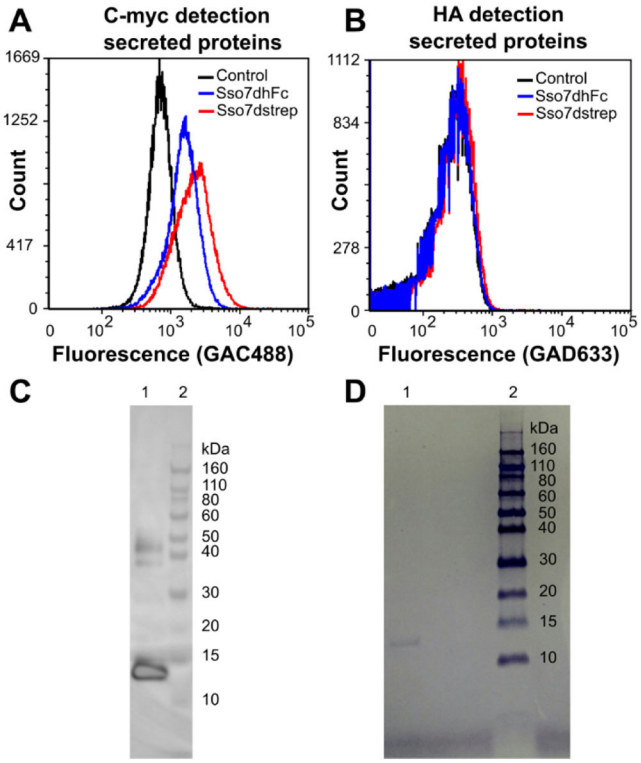

Figure 3.

Detection of solubly secreted Sso7d mutants by flow cytometry, immunoblotting, and SDS-PAGE. A) Flow cytometry analysis of solubly secreted Sso7dhFc (blue) and Sso7dstrep (red) proteins captured on His-Tag isolation beads and detected with an anti-c-myc antibody. Binding of the anti-c-myc antibody to His-Tag isolation beads (control) is shown in black. B) Flow cytometry analysis of solubly secreted Sso7dstrep (red) and Sso7dhFc (blue) mutants captured on His-Tag isolation beads and detected with an anti-HA antibody. Binding of the anti-HA antibody to His-Tag isolation beads (control) is shown in black. C) Immunoblotting analysis of supernatant from cells expressing Sso7dstrep using an anti-c-myc antibody. D) SDS-PAGE analysis of Sso7dstrep purified from cell culture supernatant using Ni-NTA beads. A single band at ~13kDa is observed. A) and B) show representative data from three independent experiments; C) and D) show representative data from two independent experiments.

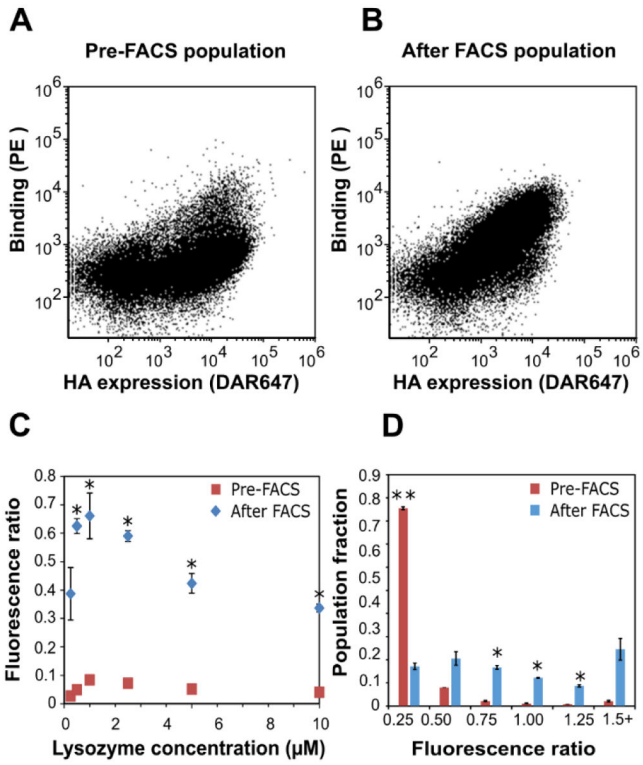

Figure 4.

Enrichment of high affinity binders from a yeast display library of Sso7d mutants incorporating the F2A peptide. A) The pool of yeast cells isolated after one round of panning (pre-FACS), or B) the pool of yeast cells after one round of FACS (after FACS), was labeled with 1 μM lysozyme and an antibody against the HA epitope tag. Representative data from three independent repeats are shown. C) Ratio of fluorescence signal due to lysozyme binding to the signal from binding of an anti-HA antibody was computed at different lysozyme concentrations for pre-FACS (red squares) and after FACS (blue diamonds) populations. Error bars correspond to standard error of the mean for three independent repeats. *$p<0.05$ for comparison with pre-FACS population at the same concentration. D) Fluorescence ratio was computed and cells were placed in one of 6 bins. The fraction of cells in each bin relative to total cells analyzed is plotted for lysozyme labeling at 1 μM (see Fig. S4 for labeling at 250 nM). The bin corresponding to all cells with fluorescence ratio 0–0.25 is denoted by 0.25, 0.25–0.5 by 0.5, and so on. All error bars indicate standard error of the mean from triplicate experiments. *$p<0.05$ for comparison with pre-FACS population in the same bin. **$p<0.05$ for comparison with post-FACS population in the same bin.

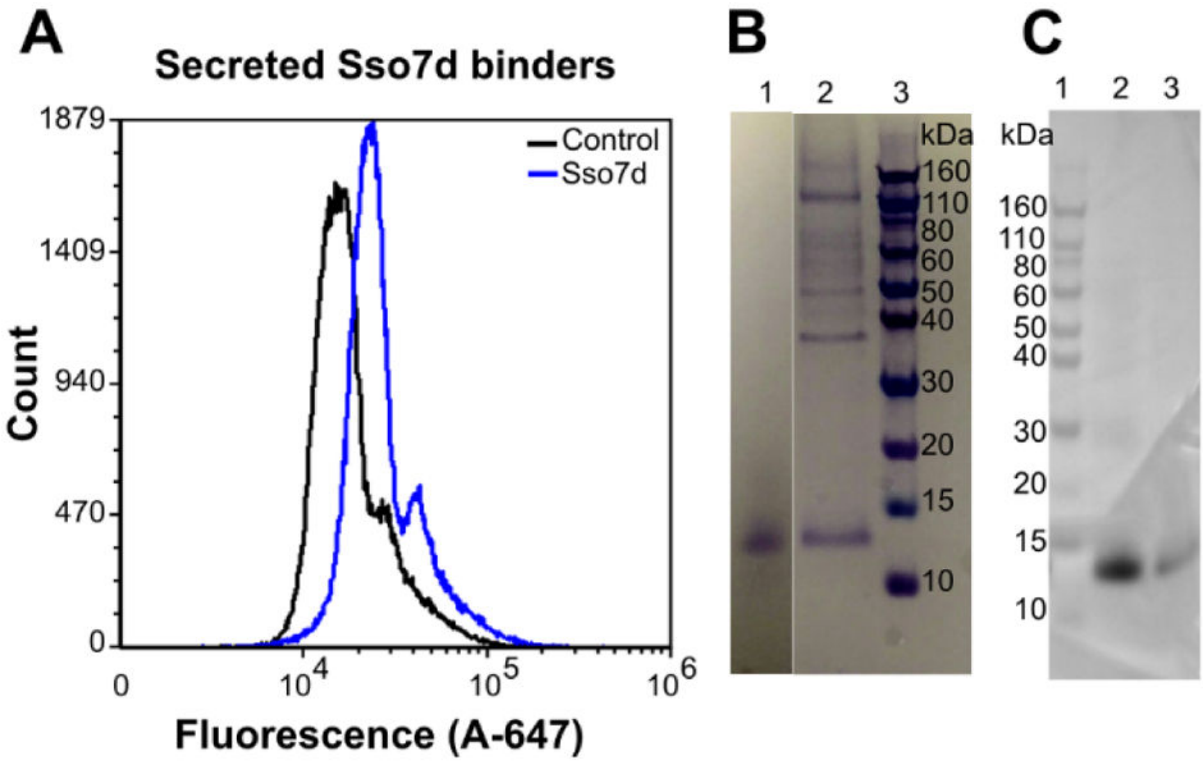

Figure 5.

Purification and characterization of a lysozyme binders solubly expressed in yeast cell culture supernatant. A) Lysozyme-coated magnetic beads were labeled with 3.6 μM Sso7d mutants that bind lysozyme, and bead-bound Sso7d proteins were detected using an anti-His antibody (blue). Control sample (black) corresponds to binding of the anti-His antibody to the lysozyme-coated beads. B) SDS-PAGE analysis of protein purified from supernatant using Ni-NTA resin (lane 1) and yeast cell culture supernatant (lane 2). A molecular weight ladder was loaded in lane 3. C) Immunoblotting analysis with an anti-c-myc antibody for concentrated supernatant (lane 2) and protein purified from supernatant using Ni-NTA resin (lane 3). A molecular weight ladder was loaded in lane 1. Representative data from two independent experiments is shown.

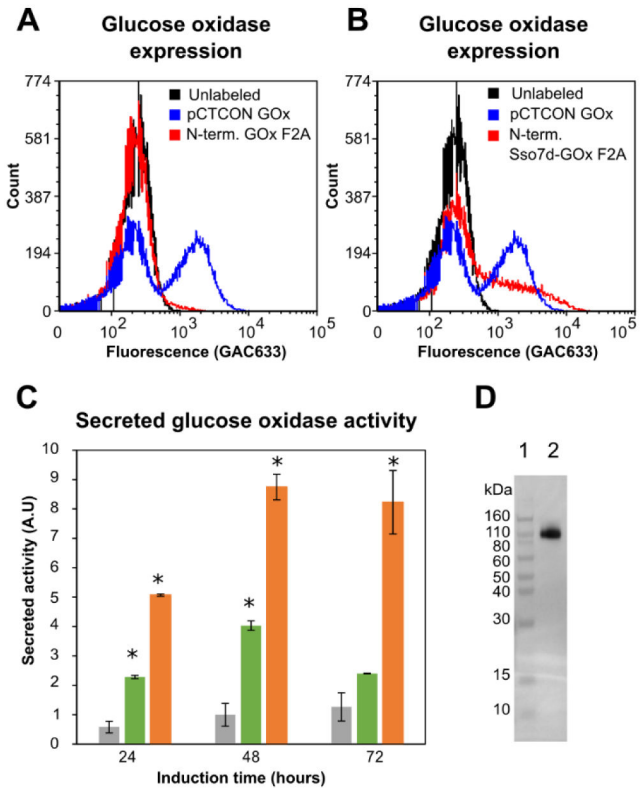

Figure 6.

Simultaneous yeast cell surface display and secretion of GOx. A) Flow cytometry analysis of unlabeled yeast cells (black), and yeast cells expressing Aga2-GOx fusions in pCTCON (blue) and GOx-F2A-Aga2 in pCT-NT-F2A (red) upon labeling with an anti-c-myc antibody. B) Flow cytometry analysis of unlabeled yeast cells (black), and yeast cells expressing Aga2-GOx fusions in pCTCON (blue) and Sso7dhFc-GOx-F2A-Aga2 in pCT-NT-F2A (red), upon labeling with an anti-c-myc antibody. C) GOx activity in supernatant after 24, 48, and 72 hours of protein induction in SGCAA medium at 20°C for GOx in pCTCON (grey), and GOx (green) and Sso7dhFc-GOx (orange) in pCT-NT-F2A. The absorbance of the quenched assay solution at 450 nm after 5 minutes of reaction is plotted as a measure of GOx activity. Error bars correspond to standard error of three independent repeats. *$p<0.05$ for comparison with GOx activity in pCTCON control. D) Immunoblotting of purified Sso7dhFc-GOx from cell culture supernatant using pCT-NT-F2A; an anti-c-myc antibody was used for analysis (lane 1). A molecular weight ladder was included in lane 2.

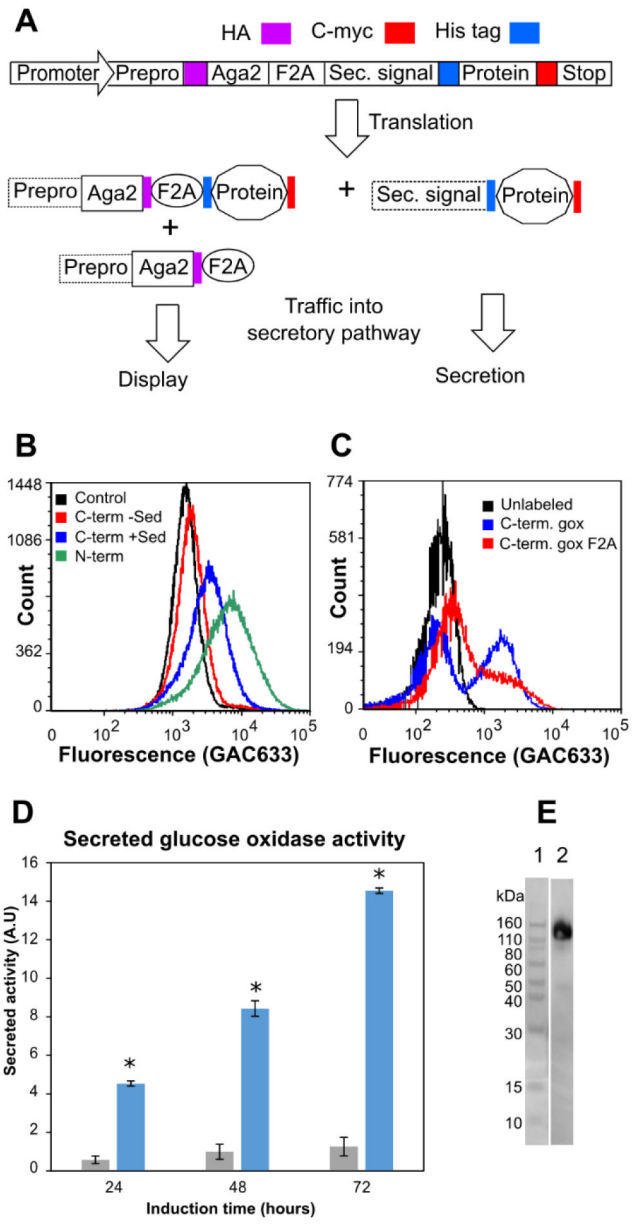

Figure 7.

Simultaneous secretion and yeast surface display of proteins expressed as C-terminal fusions to Aga2. A) Design of plasmid vector. The F2A sequence is placed after Aga2, followed by a secretory signal peptide or secretory tag (sec. tag) and the protein of interest. B) Flow cytometry analysis of solubly secreted Sso7strep from yeast cells containing pCT-CT-F2A (red), pCT-CT-F2A-SED1SP (blue), or pCT-NT-F2A (green) plasmid vectors; soluble protein was captured on His-Tag isolation beads and detected with an anti-c-myc antibody. Binding of the His-Tag isolation beads to the anti-c-myc antibody (control) is shown in black. C) Flow cytometry analysis of unlabeled yeast cells (black), and yeast cells expressing Aga2-GOx fusions in pCTCON (blue) and Aga2-F2A-GOx in pCT-CT-F2A (red), upon labeling with an anti-c-myc antibody. D) GOx activity in supernatant after 24,

48, and 72 hours of protein induction in SGCAA medium at 20°C for GOx in pCTCON (grey) and pCT-CT-F2A (blue). The absorbance of the quenched assay solution at 450 nm after 5 minutes of reaction is plotted as a measure of GOx activity. Error bars correspond to standard error of three independent repeats. *$p<0.05$ for comparison with GOx activity in pCTCON control. E) Immunoblotting of purified GOx from cell culture supernatant using pCT-CT-F2A; an anti-c-myc antibody was used for analysis (lane 2). A molecular weight ladder was included in lane 1.