NOTES

Using Superfolder Green Fluorescent Protein for Periplasmic Protein Localization Studies

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Subcellular localization studies of envelope proteins have been hampered by problems with the export of green fluorescent protein (GFP). Here we show that a superfolder variant of GFP (sfGFP) is fluorescent following Sec-mediated transport and works best when the cotranslational branch of the pathway is employed.

Use of sfGFP in the periplasm. We investigated the potential utility of a superfolder derivative of GFP (sfGFP) (17) for periplasmic protein localization studies using previously described fluorescence microscopy methods (24). The coding sequence for sfGFP (17) was synthesized by Epoch Life sciences and used to make gene fusions. All fusions were placed under the control of the wild-type lac promoter (P_{lac}). With the exception of pal-sfGFP, fusions were cloned in derivatives of CRIM vectors (12) for integration at the phage HK022 att site (attHK022) of TB28 (MG1655 ΔlacIZYA) cells (3) using the helper plasmid pTB102 as described previously (4, 12). We first studied fusions of sfGFP to the well-characterized Sec substrate MalE. Cells producing MalE-sfGFP displayed a polar fluorescent signal with some fluorescence detectable around the periphery of many cells (Fig. 1B). Polar accumulation of the exported fusion protein was expected as this was also frequently observed for Tat-exported GFP (2, 3, 20, 22). Cell fractionation and immunoblotting confirmed that while a small portion of the fusion protein was exported, the majority remained in the spheroplast fraction (Fig. 2A, lanes 2 to 4). Thus, while the export of full-length MalE-sfGFP was relatively efficient, that of SSMalE-sfGFP was largely blocked, presumably because it folds prior to transport. The difference in export efficiencies between the fusions may be due, in part, to the ability of SecB to maintain full-length MalE-sfGFP in a secretion-competent, unfolded conformation prior to export (6, 25). Also, it was shown previously that MalE and many other secretory proteins are transported by both cotranslational and posttranslational secretion pathways (14). We therefore assume that longer fusions are more likely to engage the Sec translocon before transport. The difference in export efficiencies between the fusions may be due, in part, to the ability of SecB to maintain full-length MalE-sfGFP in a secretion-competent, unfolded conformation prior to export (6, 25).

Efficient cotranslational export of sfGFP. Our results with the MalE fusions suggested that cotranslational export of sfGFP may be required to prevent it from folding in the cytoplasm and blocking export. Beckwith and coworkers have identified a subset of signal sequences that direct secretory proteins primarily through the cotranslational branch of the Sec export pathway (13, 21). The signal peptide of DsbA was one such export signal. To test whether or not SSsDsbA could direct efficient export of sfGFP to the periplasm, we constructed an SSsDsbA-sfGFP fusion (Fig. 1A) and compared its export to that of SSMalE-sfGFP. Unlike SSMalE-sfGFP, cells expressing...
the SS-DsbA-sfGFP fusion displayed a largely peripheral fluorescent signal with some polar accumulation (Fig. 1D). Subcellular fractionation indicated that the SS-DsbA-sfGFP was indeed transported efficiently (Fig. 2B, lanes 5 to 7). We conclude that fluorescent sfGFP can be efficiently transported to the periplasm provided that it is exported through a predominantly cotranslational pathway. Similar attempts to export GFPmut2 (7) using SS-DsbA did not result in a fluorescent signal (10) (data not shown). Contrary to our results, Fisher and DeLisa (10) recently reported that an SS-DsbA-sfGFP fusion accumulated in the cytoplasm and that the fraction transported to the periplasm was largely inactive (10). This discrepancy likely reflects differences in the levels of protein produced in the two studies. Fisher and DeLisa produced relatively high levels of SS-DsbA-sfGFP from a multicopy plasmid using a strong promoter (10), while our fusions were produced from a single locus in the chromosome under the control of P<sub>lac</sub>.

FIG. 1. Visualization of exported sfGFP. (A) Schematic diagram of expression constructs for the production of exported fluorescent proteins (FP). Signal peptides (SS) used for export are underlined in black below the diagram, and the gray underlines highlight the linker (L) sequence. Arrows point to sites of leader peptidase processing. RBS, ribosome-binding site; Xb, Xbal; N, NdeI; Xh, Xhol; B, BamHI; S, SalI; H, HindIII. The asterisk indicates a stop codon. (B to D) Cells of TB28 (wild type) harboring integrated expression constructs attHKTB228 (P<sub>lac</sub>::<sup>ss</sup>malE-sfGFP) (B), attHKTB262 (P<sub>lac</sub>::<sup>ss</sup>malE-sfGFP) (C), or attHKTB263 (P<sub>lac</sub>::<sup>ss</sup>dsbA-sfGFP) (D) were grown at 30°C to an optical density at 600 nm (OD<sub>600</sub>) of 0.5 to 0.7 in M9 maltose supplemented with 250 µM isopropyl-D-thiogalactopyranoside (IPTG) and visualized with GFP (panel 1) and differential interference contrast (DIC) (panel 2) optics. Bar equals 4 µm.

FIG. 2. Analysis of sfGFP export by subcellular fractionation and immunoblotting. (A) Cells of TB28 (wild type [WT]) harboring integrated expression constructs attHKTB228 (P<sub>lac</sub>::<sup>ss</sup>malE-sfGFP) (lanes 2 to 4) or attHKTB28 (P<sub>lac</sub>::<sup>ss</sup>malE-sfGFP) (lanes 5 to 7) were grown at 30°C in LB supplemented with 0.2% maltose and 500 µM IPTG. A portion of the resulting cultures was used to prepare a total cell extract (T). The remaining cells were converted to spheroplasts and pelleted by centrifugation. The resulting pellet (P) and supernatant (S) fractions, along with the total cell extract, were analyzed by SDS-PAGE and immunoblotting using antisera directed against GFP (Rockland), MalE (NEB), and FtsZ. FtsZ and native MalE served as fractionation controls for the cytoplasm and periplasm, respectively. Extract from TB28 (WT) cells without a fusion protein controlled for the specificity of the GFP antisera (lane 1). The positions of sfGFP fusion proteins and molecular mass standards (in kDa) are indicated on the right and left of the immunoblot, respectively. Stars indicate nonspecific bands. Full-length MalE is abbreviated ‘MalE. (B) Same as described for panel A except that the export of sfGFP was compared between TB28 (WT) cells producing fusions from the integrated expression constructs attHKTB262 (P<sub>lac</sub>::<sup>ss</sup>malE-sfGFP) (lanes 2 to 4) and attHKTB263 (P<sub>lac</sub>::<sup>ss</sup>dsbA-sfGFP) (lanes 5 to 7).
mCherry can be transported to the periplasm using SSMalE or SSDsbA. Based on our results with sfGFP, we wondered if the transport of RFP derivatives like mCherry also requires the use of the cotranslational pathway. We therefore constructed both SSMalE-mCherry and SSDsbA-mCherry fusions for a comparison. In contrast to sfGFP fusions, cells expressing both SSMalE-mCherry and SSDsbA-mCherry displayed a peripheral fluorescence signal consistent with proper transport to the periplasm (Fig. 3A and B). This was confirmed by cellular fractionation experiments and mCherry detection with rabbit anti-RFP antisera (catalog no. 600-401-379; Rockland Immunochemicals) (Fig. 3C). When SSMalE was used, two mCherry species present in roughly equal amounts were apparent in the immunoblots, probably corresponding to the slower-migrating precursor and faster-migrating mature form (Fig. 3C, lanes 2 to 4). Consistent with these assignments, the mature form was exclusively located in the periplasmic fraction, whereas the precursor was limited to the cytoplasmic fraction (Fig. 3C, lanes 2 to 4). Compared to that of SSMalE, the export of mCherry directed by SSDsbA appeared to be much more efficient. Only the mature form of the fusion was observed, and it was exclusively found in the periplasmic fraction (Fig. 3C, lanes 5 to 7). We conclude that mCherry can be effectively transported to

![Image](FIG. 3. mCherry is functional following post- or cotranslational Sec export. (A and B) Cells of TB28 (WT) containing the integrated expression constructs attHKTB317 (P_{lac}::SSmalE-mCherry) or attHKTU136 (P_{lac}::SSdsbA-mCherry) were grown at 30°C to an OD_{600} of 0.5 to 0.7 in M9 maltose supplemented with 100 or 50 μM IPTG, respectively, and visualized with mCherry (panel 1) and DIC (panel 2) optics. Bar equals 4 μm. (C) The same strains were grown and processed for cell fractionation and immunoblotting as described for Fig. 2A.)

FIG. 4. Localization of EnvC and Pal shown using sfGFP. (A and B) Cells of TB140(attHKTB226) [ΔenvC(P_{lac}::envC-sfGFP)] (A) or MG5/pTB223 (Δpal/P_{lac}::pal-sfGFP) (B) were grown at 30°C to an OD_{600} of 0.5 to 0.7 in M9 maltose supplemented with 0 or 10 μM IPTG and were visualized using GFP (panel 1) or DIC (panel 2) optics. Bar equals 4 μm. pTB223 is a multicopy plasmid derived from pMLB1113 (8).

Using sfGFP for protein localization studies. To test the effectiveness of sfGFP for protein localization experiments in the periplasm, we studied the localization of sfGFP fusions to EnvC and Pal, two exported proteins previously shown to be recruited to the division site using a Tat-exported GFPmut2 fusion (EnvC) and/or a monomeric RFP (EnvC and Pal) (3, 11, 24). Both the EnvC-sfGFP and Pal-sfGFP fusions were constructed to use their native export signals for transport to the periplasm. When produced in cells lacking native EnvC, the EnvC-sfGFP fusion corrected the mild chaining defect of the mutant strain (data not shown) and localized to the septum as expected from previous results (Fig. 4A) (3). Similarly, the Pal-sfGFP fusion corrected the Pal chaining and membrane blebbing phenotypes (data not shown) and displayed the expected septal localization pattern when produced in Pal cells (Fig. 4B) (11). Thus, C-terminal sfGFP fusion proteins can be used for protein localization experiments in the periplasm even when they are not specifically targeted through the signal recognition particle (SRP)-dependent cotranslational Sec export pathway using SSDsbA. As described above for the MalE-sfGFP fusion, this probably works because many secretory proteins are exported, at least in part, through a cotranslational mechanism (14).

Conclusion. We have shown that sfGFP can be used to study the subcellular localization of exported proteins. In an accompanying report (18), we use sfGFP to study the localization of the cell separation factor AmiB and perform colocalization experiments in the periplasm using both sfGFP and mCherry fusion proteins. Three recent reports also highlight the effectiveness of sfGFP for protein localization experiments in the periplasm (16, 19, 23).

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While the manuscript was in preparation, Aronson et al. (1) also reported that fluorescent sfGFP can be exported to the periplasm.

REFERENCES