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Green Fluorescent Protein as a protein localization and topological reporter in mycobacteria

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Abstract

The cell envelope-associated proteins of *Mycobacterium* species play critical functions in the physiology and pathogenicity of these microorganisms. Because the determination of their subcellular localization and transmembrane topology is often critical to the understanding of their function, we investigated whether the Green Fluorescent Protein (GFP) could be used as a reporter to probe protein localization and map the topology of inner membrane proteins directly in intact mycobacterial cells. To this end, two GFP-based mycobacterial reporter plasmids were engineered and their functionality validated using a variety of membrane-associated, exported and cytosolic proteins.

Keywords

Cell envelope; GFP; *Mycobacterium*; protein localization; topology; tuberculosis

The secreted and cell envelope-associated proteins of *Mycobacterium tuberculosis* play critical roles in the pathogenicity of and generation of protective immunity to the bacterium and have proven invaluable from the perspective of the development of novel vaccines and diagnostics [1]. They also play an important role in the physiology of the bacterium, participating in numerous enzymatic and transport functions such as nutrient import, the building of the mycobacterial cell envelope, cell elongation and cell division and, as such, they represent attractive targets for the development of novel therapeutic agents.

Determining the subcellular localization and topology of cell envelope-associated proteins is critical to the understanding of their biological activities and physiological functions. To this end, reporter fusions with *lacZ*, *phoA* and *blaC* have been extensively used to differentiate exported proteins from cytosolic ones and map the topology of inner membrane proteins [2-5]. *phoA* encodes an alkaline phosphatase that folds into an enzymatically active conformation only in the periplasm. β -galactosidase (β -Gal), encoded by *lacZ*, functions in

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the opposite manner of PhoA in that it is enzymatically active only when retained in the cytoplasm. BlaC is a β -lactamase whose export to the periplasm protects mycobacteria from the effect of β -lactam antibiotics [5]. While functional in mycobacteria, limitations of these reporter genes include the requirement for sample lysis and specific substrates to obtain measurement, the need to work in the background of β -lactam-susceptible mutants devoid of endogenous β -lactamase activity in the case of BlaC, and the reported toxicity of some exported β -Gal fusions [6]. Moreover, because *phoA* and *lacZ* reporter systems are more widespread in *E. coli* than in mycobacteria, *E. coli* is often used as a surrogate host to express mycobacterial gene fusions, with the caveat that the information yielded by these heterologously expressed fusions may not accurately reflect the subcellular location of the native protein domains produced in mycobacteria.

The Green Fluorescent Protein from *Aequorea victoria* (GFP) is a relatively small (27 kDa) protein that stably fluoresces as a monomer. Its stability, ease of detection in intact cells and low detection limits (< 10 ng of GFP per ml of culture; 5 ng of GFP per protein band on a standard SDS-PAGE gel) have made it a reporter of choice to probe a variety of events within living eukaryotic and prokaryotic cells. The fact that its activity can be detected directly in intact cells without requiring any specific substrates eliminates the need for time-consuming protein extraction, enzymatic reactions and immunoblots. For this reason, GFP has been used extensively to directly monitor the level of expression, folding, and stability of proteins (particularly inner membrane proteins) throughout their production, solubilization and purification from *E. coli* [7] and yeast cells [8]. The finding that GFP folds efficiently in the cytoplasm of *E. coli* but does not form an active enzyme when targeted to the periplasm [6] has further prompted applications of this protein as a transmembrane topological marker [9]. Despite its convenience as an expression and topological reporter, the use of GFP and other fluorescent proteins in mycobacteria have thus far essentially been restricted to the screening of compounds interfering with the replication of *M. tuberculosis in vitro* and inside macrophages or to monitoring gene expression and interactions between mycobacteria and host cells. Few examples exist wherein GFP was used to determine protein localization and this marker has, to the best of our knowledge, never been used to map the topology of inner membrane proteins directly in mycobacteria. The primary goal of this study was to determine whether GFP could be used as a reporter to probe the subcellular localization of proteins as well as the transmembrane topology of inner membrane proteins in intact mycobacterial cells. Two convenient expression plasmids were engineered to this end, and tested on a panel of mycobacterial transmembrane, exported and cytosolic proteins.

The pJB vector system consists of two vectors, pJB(-) and JB(+), engineered from the mycobacterial expression plasmid, pMV261 [10], and the *E. coli* expression plasmids, pWARF(+) or pWARF(-) [11]. This system allows for the mycobacterial expression under control of the *phsp60* promoter of proteins C-terminally-fused to the GFP and to a hexahistidine tag that may subsequently be easily purified from a *Mycobacterium* host. pJB(-) has the HRV 3C protease recognition site (LEVLFQ/GP), followed by GFP and a C-terminal His₈ tag. pJB(+) has the HRV 3C protease cleavage site, followed by the transmembrane segment of glycophorin A, GFP and a C-terminal His₈ tag [Fig. 1A] [11]. Gene fusions in pJB(-) and JB(+) may be generated from the same PCR fragment. Based on earlier *E. coli* studies [11], it was expected that the addition of a single transmembrane

domain from glycophorin A between the C-terminal fusion point of the protein of interest and the GFP in pJB(+) would allow membrane proteins with extracellular C-terminal fusions in mycobacteria to be converted to proteins with intracellular C-terminal fusions [Fig. 1B]. Because GFP fluoresces in the cytoplasm but not in the periplasm, a high fluorescence signal in the pJB(-) version and background fluorescence in the pJB(+) version are indicative of the C-terminal fusion of the protein being cytoplasmic. Opposite fluorescence intensities indicate, on the contrary, that the C-terminal fusion of the protein is periplasmic.

A variety of cytosolic and peripheral membrane proteins, as well as (lipo)proteins and inner membrane proteins known to require the Sec pathway for translocation across or insertion into the cytoplasmic membrane, and a protein known to be exported through the Twin-Arginine Translocation (Tat) pathway, were selected to probe the robustness of our reporter system, including (i) the cytosolic catalase-peroxidase KatG (MSMEG_6384) [12] (ii) the cytoplasmically-oriented peripheral membrane mannosyltransferase, PimB' (MSMEG_4253) [13]; (iii) a single transmembrane domain protein with a periplasmic C-terminus involved in the export of glycopeptidolipids in *M. smegmatis* (MmpS4; MSMEG_0380) [14]; (iv) a lipoprotein involved in the export of glycolipids (LprG; MSMEG_3070) [13]; (v) the membrane-associated two-component system response sensor kinase PhoR (MSMEG_5870) [13] (vi) two integral membrane polyprenyl phosphate sugar-dependent glycosyltransferases with known periplasmic catalytic sites (PimE [SMEG_5149] and EmbC [MSMEG_6387]) [13]; (vii) two integral membrane Resistance, Nodulation and Division (RND) transporters involved in the export of complex lipids (MmpL10 [Rv1183] and MmpL11 [MSMEG_0241]) [13,15]; (viii) the mycolyltransferase, FbpC (antigen 85C; MSMEG_3580), known to be secreted through the housekeeping Sec-dependent pathway [16]; and (ix) the periplasmic β -lactamase BlaC (MSMEG_2658) known to be exported through the Tat pathway [1]. All genes, either full-length or truncated, were successfully cloned into pJB(-) and JB(+) to generate the various fusions presented in Figures 2 and 3A (primer sequences are available upon request). To compare the fluorescence intensities displayed by each gene fusion in the pJB(-) and JB(+) systems, three independent pJB(-) and JB(+) *M. smegmatis* transformants for each gene were grown to log phase in 7H9 broth with 10 % ADC (albumin-dextrose-catalase) supplement (BD Biosciences) and 0.05% Tween 80, and washed with PBS prior to whole cell fluorescence measurements.

Relative fluorescence intensities confirmed the cytoplasmic location of KatG and the extra-cytoplasmic location of FbpC. They also confirmed the cytosolic location of the C-terminus of the peripheral membrane nucleotide sugar-utilizing mannosyltransferase, PimB' [Fig. 2]. That the fluorescence readout of our system was not negatively impacted by potential differences in the level of expression of the test genes in pJB(-) and JB(+) was verified by qRT-PCR for a number of prototype cytoplasmic, secreted and integral membrane proteins [Fig. S1]. Furthermore, that the potential folding of a fraction of the fusion proteins in the cytoplasm, even in the case of constructs with periplasmically-oriented GFP, did not confuse the results was verified by confirming the proper location of the fusion proteins by in-gel fluorescence [Fig. S2]. Altogether, these results thus show that the transmembrane segment of glycophorin A successfully repositions the C-terminally fused GFP of the secreted mycolyltransferase FbpC to the cytoplasm of *M. smegmatis* cells where GFP may be properly folded and fluoresce.

The computationally-predicted or experimentally-validated transmembrane topology of the membrane-associated proteins tested therein along with the point of fusions generated in pJB(-) and JB(+) are shown in Figure 3A. Fluorescence intensities confirmed the periplasmic location of the C-termini of MmpS4 and the lipoprotein LprG [13-14], the periplasmic location of the two large soluble loops of MmpL10 (which is characteristic of the topology of RND transporters), and the cytosolic localization of the C-termini of MmpL11 and the histidine kinase domain of PhoR [Fig. 3B; Fig. S2]. GFP fusions in the first soluble loop of EmbC and PimE, in the immediate vicinity of their catalytic sites [13], further confirmed the periplasmic location of the glycosyl transfer reactions catalyzed by these two polyprenyl phosphate sugar-dependent glycosyltransferases [Fig. 3 A-B].

Collectively, these results thus validate the pJB(-)/pJB(+) system not only for use with exported/secreted and cytosolic proteins but also for peripheral, integral membrane proteins and lipoproteins. The only protein wrongly predicted to localize to the cytoplasm was the β -lactamase BlaC which is exported to the periplasm through the Tat pathway. Since the Tat pathway translocates folded proteins across the inner membrane, active GFP was predictably formed in the pJB(-) *blaC* system [Fig. 2] and actually found to predominantly remain in the cytosolic fraction [Fig. S2]. Few proteins, however, are exported through this pathway in mycobacteria and they may be identified by the presence of a characteristic Tat signal peptide [1]. Another noticeable limitation of this GFP-based reporter system has to do with the length of the soluble C-terminal domain of proteins. While results unambiguously pointed to the cytosolic or periplasmic localization of the C-terminal domains of MmpL11, MmpS4 and PhoR, which all display greater than 118 amino acid-long soluble C-termini, relative fluorescence intensities were unreliable with PimE which harbors a short (approximately 12 amino acid-long) soluble C-terminal domain. The lack of statistically significant difference in fluorescence intensities between the pJB(-) and pJB(+) fusions in this case may be due to the impaired folding or activity of GFP when located too close to the surface of the inner membrane. This issue may, however, easily be addressed by increasing the length of the C-terminal fusion upstream of the *gfp* gene. Despite these predictable shortcomings, the reporter system described herein presents numerous advantages including the facts that (i) the same PCR fragment is used to generate GFP-fusions under control of the same promoter in pJB(-) and pJB(+), (ii) the activity measurements are based on GFP fluorescence and thus extremely sensitive, (iii) they are performed on intact cells without any requirement for cell lysis and specific substrates, and (iv) GFP-fusions are produced in their native mycobacterial environment instead of *E. coli* which may facilitate the expression of GC-rich mycobacterial genes and increase the accuracy of topology mapping. We note that, similar to the situation in *E. coli* [9], some fusion proteins with a cytoplasmic C-terminus expressed in *M. smegmatis* tend to undergo proteolysis releasing active GFP into the cytoplasm (e.g., PimB'; see Fig. S2). It is thus critical that fluorescence comparisons between pJB(-) and pJB(+) systems be carried out on whole cells rather than on isolated membranes.

Like other GFP-based reporter systems, the pJB(-)/pJB(+) system may further be used to monitor gene expression in mycobacteria and study the interactions of mycobacteria with host cells. Importantly, as efforts to optimize the expression and purification of recombinant proteins from mycobacteria for biological activity and crystallography studies continue, GFP

fusions generated in pJB(-)/pJB(+) may be used to screen in a relatively high-throughput fashion the best conditions for the production of properly folded proteins and their subsequent purification from mycobacteria, without the need for laborious and time-consuming immunoblotting. This is particularly important for membrane proteins whose expression levels are usually low, immunoblotting technically challenging, and whose purification often requires the screening of numerous detergents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. The pJB(-) and pJB(+) expression plasmids and principle of the GFP fusions generated in this reporter system

(A) Relevant features of the pJB(-) and pJB(+) plasmids showing the localization of the *hsp60* promoter, the multiple cloning sites, the region encoding the transmembrane (TM) segment of glycoprotein A (GpA), and the *gfp* gene.

(B) Principle of the GFP reporter fusions generated in the pJB(-)/pJB(+) system. pJB(-) fuses GFP to the C-terminus of a protein. GFP fluoresces if the C-terminus is in the cytoplasm. pJB(+) fuses glycoprotein A and GFP to the C-terminus of a protein. Periplasmically-oriented C-termini from Sec-dependent inner membrane and exported/secreted proteins are reoriented to the cytoplasmic side of the inner membrane by the single transmembrane domain of glycoprotein A leading to GFP fluorescence.

Figure 2. Subcellular localization of the C-termini of KatG, FbpC, PimB' and BlaC as predicted by GFP fusions

Inset: Expected subcellular localization of KatG, PimB', FbpC and BlaC. The full-length *katG*, *pimB'*, *fbpC* and *blaC* genes were cloned in pJB(-) and JB(+) and the resulting plasmids introduced in *M. smegmatis* mc²155 by electroporation. Cultures of transformants grown to log phase ($OD_{600nm} = 0.8-1$) were washed twice with PBS and resuspended in 100 ml of the same buffer prior to being transferred to black 96-well plates with transparent bottoms (Corning) for whole cell fluorescence measurements. Fluorescence was determined using a 2030 MultiLabel Reader Victor X5 plate reader (Perkin Elmer) at excitation and emission wavelengths of 485 nm and 535 nm, respectively. The fluorescence value of each sample was normalized to the OD_{600nm} of the culture. Shown on the graph are the average fluorescence values $\pm$ standard deviations (error bars) measured in triplicate on three independent transformants for each GFP fusion. The pJB(-)/JB(+) reporter system confirms the cytoplasmic location of KatG and PimB', and the extra-cytoplasmic location of FbpC but, as expected, wrongly points to the cytoplasmic location of BlaC which is exported as a folded protein through the Tat pathway. Statistical comparisons (Student's *t* test) between pJB(-) and JB(+) transformants: ** $p < 0.050$, * $p < 0.010$.

Figure 3. Topology of selected domains of membrane-associated proteins in *M. smegmatis*

(A) Topology of MmpS4, EmbC, PhoR, LprG, PimE, MmpL10 and MmpL11 as predicted by MEMSAT-3. Topology models were generated using TOPO2. The different points of fusion with GFP generated in pJB(-) and pJB(+) are indicated by arrows. The demonstrated periplasmic catalytic residues of PimE and EmbC are highlighted.

(B) Different C-terminal fusions between *mmpS4*, *embC*, *pimE*, *lprG*, *phoR*, *mmpL10*, *mmpL11* and *gfp* were generated in pJB(-) and JB(+). Fluorescence intensities represent the average and standard deviations of three independent transformants and were normalized to the OD_{600nm} of the cultures as described in Fig. 2. Statistical comparisons (Student *t* test) between pJB(-) and JB(+) transformants: ** *p* < 0.050, * *p* < 0.010.