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Protein Export by the Mycobacterial SecA2 System Is Determined by the Preprotein Mature Domain

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At the core of the bacterial general secretion (Sec) pathway is the SecA ATPase, which powers translocation of unfolded preproteins containing Sec signal sequences through the SecYEG membrane channel. Mycobacteria have two nonredundant SecA homologs: SecA1 and SecA2. While the essential SecA1 handles "housekeeping" export, the nonessential SecA2 exports a subset of proteins and is required for *Mycobacterium tuberculosis* virulence. Currently, it is not understood how SecA2 contributes to Sec export in mycobacteria. In this study, we focused on identifying the features of two SecA2 substrates that target them to SecA2 for export, the Ms1704 and Ms1712 lipoproteins of the model organism *Mycobacterium smegmatis*. We found that the mature domains of Ms1704 and Ms1712, not the N-terminal signal sequences, confer SecA2-dependent export. We also demonstrated that the lipid modification and the extreme N terminus of the mature protein do not impart the requirement for SecA2 in export. We further showed that the Ms1704 mature domain can be efficiently exported by the twin-arginine translocation (Tat) pathway. Because the Tat system exports only folded proteins, this result implies that SecA2 substrates can fold in the cytoplasm and suggests a putative role of SecA2 in enabling export of such proteins. Thus, the mycobacterial SecA2 system may represent another way that bacteria solve the problem of exporting proteins that can fold in the cytoplasm.

Protein export pathways exist in all bacteria and fulfill the vital role of transporting proteins synthesized in the cytoplasm to the bacterial cell envelope or extracellular environment. Many of the proteins exported by these pathways function in essential physiological processes or have important roles in bacterial pathogenesis.

There are two conserved protein export pathways found across bacteria: the general secretion (Sec) and the twin-arginine translocation (Tat) pathways (1, 2). The Sec pathway carries out the bulk of protein export, which makes Sec export an essential process in all bacteria. The core components of the Sec pathway are a cytoplasmic SecA ATPase and a membrane complex comprised of SecY, SecE, and SecG proteins (3).

Proteins bound for export by the Sec pathway are synthesized as preproteins with N-terminal signal sequences that are comprised of a positively charged N terminus, a hydrophobic core, and a polar C terminus containing a cleavage site (4). The signal sequence promotes binding of the preprotein to a cleft of SecA formed by multiple domains, including the preprotein-binding domain and C-terminal domain (5–9). SecA then targets the preprotein to SecYEG at the membrane, where the signal sequence inserts into a channel formed by SecY (10–12). SecA also contains two nucleotide-binding domains with ATPase activity. Through cycles of ATP binding and hydrolysis, coupled to conformational changes, SecA powers translocation of the preprotein across the membrane through the SecY channel (13, 14). This is a stepwise process that additionally involves multiple rounds of SecA binding, release, and rebinding to a translocating preprotein (15). Preproteins must be in an unfolded conformation to be competent for Sec export. Cytosolic chaperones and the process of translocation itself can help keep preproteins in an unfolded and translocation-competent state (16–18). After export, the signal sequence is cleaved from the preprotein by signal peptidases to yield the mature protein (19).

Proteins exported by the Tat pathway are also synthesized as preproteins containing cleavable N-terminal signal sequences (20). Tat signal sequences, however, are distinguished from Sec signal sequences by the presence of a twin-arginine motif that includes a nearly invariant pair of arginine residues (21). Tat-exported proteins are targeted to a different membrane channel comprised of the TatA, TatB, and TatC proteins (22). A major distinction between the Sec and Tat pathways is that while the Sec pathway exports proteins in an unfolded state, the Tat pathway exports only folded preproteins (23, 24).

Mycobacteria, including the human pathogen *Mycobacterium tuberculosis*, and some Gram-positive species also have a SecA2 protein export system (25, 26). SecA2 systems are defined by the presence of a second, nonredundant, homolog of the SecA ATPase. In these bacteria, SecA1 is the essential SecA protein responsible for canonical Sec export, while SecA2 is involved in exporting a subset of proteins (25, 26). In many bacterial pathogens containing SecA2 systems, such as *M. tuberculosis*, SecA2 is not essential but is required for virulence (27–32).

Some SecA2 systems are known as SecA2-SecY2 systems because they contain an extra SecY, which is presumed to function as a specialized channel (26). SecA2-SecY2 systems appear dedicated to exporting proteins that are glycosylated in the cytoplasm prior to export, such as the GspB protein of *Streptococcus gordonii* (28–30, 32). Other SecA2 systems, such as those found in mycobacteria, are known as SecA2-only systems because they lack an extra

Received 24 October 2012 **Accepted** 28 November 2012

Published ahead of print 30 November 2012

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.02032-12.

doi:10.1128/JB.02032-12

SecY or any obvious alternative membrane channel. In mycobacteria, SecA2 likely works with machinery from the general Sec pathway including SecA1 and SecYEG (33).

A past study in *Mycobacterium smegmatis* identified two proteins that require SecA2 for export to the cell wall: Ms1704 and Ms1712 (34). Both of these proteins are predicted ABC-type sugar-binding components of putative ABC transporters (35). Ms1704 and Ms1712 possess N-terminal Sec signal sequences that contain a lipobox motif [L(A/S)(G/A)C], common to exported bacterial lipoproteins (36, 37). Currently, it is not known what feature(s) makes a preprotein dependent on SecA2 for export in SecA2-only systems. In this study, we used *M. smegmatis* Ms1704 and Ms1712 to address this fundamental question about SecA2 export in mycobacteria. We tested the role of the signal sequence and mature domain in determining SecA2 dependency by swapping the signal sequences of Ms1704 and Ms1712 with those of preproteins exported by the canonical Sec pathway, independent of SecA2. These studies showed that the mature domains of Ms1704 and Ms1712, not the signal sequence, contain the information that makes these proteins SecA2 dependent. We also showed that the lipid modification and extreme N terminus of the mature domain are not sufficient to impart the requirement for SecA2 in export. Remarkably, however, when the mature domain of Ms1704 was fused to a Tat signal sequence, the protein was no longer exported in a SecA2-dependent manner; instead, Ms1704 was then exported by the Tat pathway. Because the Tat pathway is uniquely built to export folded preproteins, this suggests that SecA2 substrates may be prone to folding in the cytoplasm and that the role of SecA2 may be to facilitate export of such proteins. Our results also reinforce the differences that exist between the SecA2-SecY2 systems of other bacteria and the SecA2-dependent export system of mycobacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* DH5α was used for all DNA cloning and grown at 37°C in Luria-Bertani medium (Fisher) supplemented with 40 μg/ml kanamycin or 150 μg/ml hygromycin when appropriate. *M. smegmatis* mc^2155 (wild type) (38) or NR116 (Δ*secA2*) (33) was grown at 30°C in Mueller-Hinton medium (BD Diagnostic Systems) with 0.1% (vol/vol) Tween 80 and supplemented with 20 μg/ml kanamycin when appropriate. Because the *M. smegmatis* strains PM759 (39) and LL115 are lysine auxotrophs, they were always grown in Luria-Bertani medium (Fisher).

Construction of *M. smegmatis* Δ*blaS* Δ*secA2* mutant. Strain LL115 (*ept-1* Δ*lysA4 rpsL6* Δ*blaS1* Δ*secA2*), the *M. smegmatis* Δ*blaS* Δ*secA2* mutant, was constructed by two-step allelic exchange, as described previously (33), using the PM759 Δ*blaS* mutant strain and the pNR6 Δ*secA2* suicide plasmid. Successful deletion of *secA2* was confirmed by Southern blotting (data not shown).

Plasmid construction and *M. smegmatis* electroporation. Tables of all plasmids and oligonucleotides used in this study, as well as details of plasmid construction, can be found in the supplemental material. All cloned plasmid inserts were confirmed to be error free by DNA sequencing (Eton Biosciences, NC). Plasmids were electroporated into *M. smegmatis* as previously described (40).

Subcellular fractionation. Subcellular fractions of *M. smegmatis* cells were generated as previously described (34, 41). Briefly, whole-cell lysates (WCL) of *M. smegmatis* were generated by passing cells through a French pressure cell at 20,000 lb/in^2. Unlysed cells were removed by centrifugation at 3,000 × *g* to generate clarified whole-cell lysates. Protein concentrations of clarified lysates were determined by bicinchoninic acid (BCA) assay using a bovine serum albumin (BSA) standard (Pierce) and equalized between strains for every experiment. One milliliter of each equalized lysate was centrifuged at 27,000 × *g* for 30 min to pellet cell wall (CW) material and then at 100,000 × *g* for 2 h to separate membrane (M) and soluble (SOL) fractions. For all experiments, proteins derived from the same amounts of starting cells for each fraction were analyzed by immunoblotting.

Culture filtrate protein preparation. *M. smegmatis* was grown in Mueller-Hinton medium with Tween 80 omitted. Identical *M. smegmatis* cultures grown with Tween 80 were used to monitor bacterial growth to an optical density at 600 nm (OD_{600}) of 0.4 to 0.7 upon harvesting. Cells from the cultures where Tween 80 was omitted were separated from culture supernatants by centrifugation at 3,000 × *g* and lysed by passage through the French press to prepare WCL. Two hundred milliliters of culture supernatants was filtered through a 0.2-μm-pore-size filter to remove cell material and concentrated using a 10-kDa membrane (Amicon) in a stirred-cell concentrator (Amicon) at 4°C to a volume of approximately 10 ml. The concentrated volume was kept equivalent between strains for each experiment. Protein from 1 ml of concentrated culture supernatant was precipitated overnight at 4°C with 10% trichloroethanoic acid. Protein pellets were washed with acetone, resuspended in 25 μl of 1× SDS-PAGE buffer, and then boiled. Whole-cell lysates from the cell pellets were analyzed for protein concentration by BCA assay, and equal amounts of protein from each strain were loaded on a one-dimensional PAGE (1D-PAGE) gel. Proteins obtained from equal culture supernatant volumes between strains were also loaded on a 1D-PAGE gel.

Immunoblotting. Subcellular fraction material was combined with SDS-PAGE buffer, separated by 1D-PAGE, and transferred to a nitrocellulose membrane (Whatman). The following antibodies were used for immunoblotting: anti-hemagglutinin (HA; Covance) used at 1:10,000, anti-PhoA (Research Diagnostics International) used at 1:20,000, anti-19 kDa (provided by Douglas Young, MRC National Institute for Medical Research) used at 1:20,000, anti-BlaTEM1 (QED Biosciences) used at 1:5,000, and anti-MspA (provided by Michael Niederweis, University of Alabama at Birmingham) used at 1:20,000. For cytoplasmic and cell lysis controls, either the GroEL1 or GroEL2 chaperone was detected using anti-His (Abgent) at 1:10,000, or anti-GroEL2 (HAT5/IT-64; obtained from the World Health Organization collection) at 1:20,000. The anti-His antibody recognizes a string of endogenous histidines in GroEL1 (42). Proteins were detected using either anti-rabbit or anti-mouse secondary antibodies conjugated to either horseradish peroxidase (Bio-Rad) or alkaline phosphatase (GE Healthcare). Signal was detected using Western Lightning Chemiluminescent detection reagent (Perkin-Elmer) or enhanced chemifluorescence (ECF) reagent (GE Healthcare), as appropriate. Immunoblot signals were detected using film or a phosphorimager.

RESULTS

The mature domain of Ms1704 imparts the requirement for SecA2 in export. We set out to determine if preproteins exported by the mycobacterial SecA2 system have signal sequences that specifically target them to SecA2 for export or if their distinguishing feature resides in the mature domain. To address this question, we created a series of chimeric fusion proteins in which the signal sequence of an SecA2-dependent protein (Ms1704 or Ms1712) was swapped with the Sec signal sequence of a protein exported by the canonical Sec pathway, independent of SecA2 (Fig. 1). Export of the chimeras was assessed in the following manner. *M. smegmatis* cells expressing each chimera were lysed to generate whole-cell lysates (WCL) that were then subjected to differential ultracentrifugation to separate cell wall (CW), membrane (M), and cytosol-containing soluble (SOL) fractions. These subcellular fractions were then analyzed for localization of the fusion protein by immunoblot analysis with antibodies that recognize the mature domain or, in the case of Ms1704 and Ms1712, recognize a C-terminal HA epitope tag fused to the mature domain. To assess the

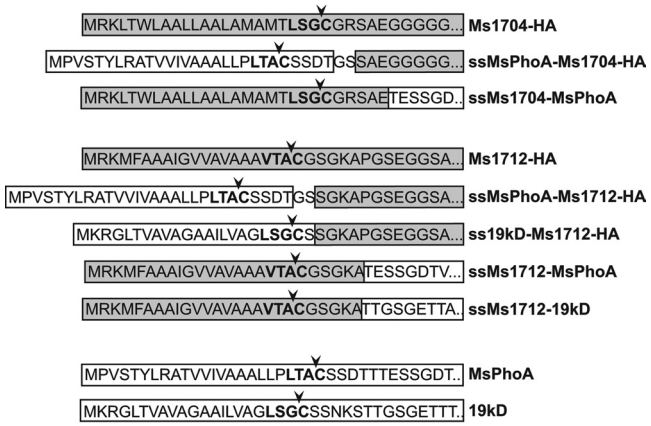

FIG 1 Schematic of signal sequence chimeras. The signal sequence regions of chimeras generated between *M. smegmatis* SecA2 substrates and SecA2-independent lipoproteins are shown. Amino acid sequences from either SecA2 substrate (Ms1704 or Ms1712) are highlighted in gray, while sequences from SecA2-independent proteins (PhoA or 19-kDa protein) are boxed in white. The predicted lipobox in each signal sequence is highlighted in bold, and the predicted cleavage site adjacent to the invariant cysteine (site of lipid modification) at the +1 position of the mature domain is noted with an arrow. Amino acids introduced from cloning are unboxed.

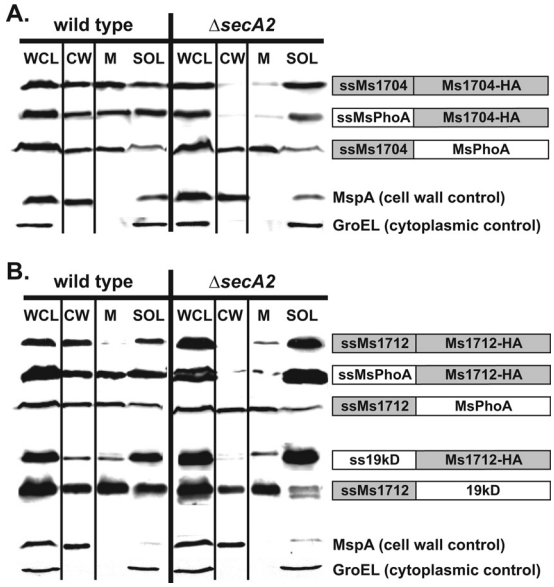

FIG 2 The mature domains of Ms1704 and Ms1712 require SecA2 for export to the cell wall. (A) Equalized whole-cell lysates (WCL) generated from wild-type and Δ*secA2* mutant *M. smegmatis* cells expressing either Ms1704-HA, ssPhoA-Ms1704-HA, or ssMs1704-PhoA were subjected to ultracentrifugation to generate subcellular fractions. Fractions were separated by SDS-PAGE, and proteins were detected with either anti-HA or anti-PhoA antibody. The total amount of cell wall (CW), membrane (M), and soluble (SOL) fractions shown is equivalent to the amount of WCL loaded. Native MspA and GroEL were detected as cell wall and cytoplasmic controls, respectively. (B) Wild-type or Δ*secA2* mutant *M. smegmatis* cells expressing either Ms1712-HA, ssPhoA-Ms1712-HA, ssMs1712-PhoA, ss19-kDa-Ms1712-HA, or ssMs1712-19-kDa were fractionated, and material was separated by SDS-PAGE. Immunoblotting was performed as described in panel A with the addition of the anti-19-kDa antibody when appropriate. The experiment shown is representative of three independent experiments.

requirement for SecA2 in protein export we specifically compared export to the cell wall between wild-type and Δ*secA2* mutant cells although all the fractions are shown on the immunoblots. Immunoblotting for a cytoplasmic GroEL chaperone and the cell wall MspA porin was used as a control for subcellular fraction integrity.

As previously reported, when expressed in wild-type *M. smegmatis*, the C-terminally HA-tagged Ms1704 protein containing its native signal sequence was exported to the cell wall (CW) (34). However, Ms1704-HA export was severely compromised when the protein was expressed in the Δ*secA2* mutant, indicating that SecA2 is required for export of this protein. In comparison, export of the MspA control protein was unchanged by deletion of *secA2*, as was also shown previously (Fig. 2A). Our first set of chimeras employed an *M. smegmatis* alkaline phosphatase (PhoA), which is exported in a SecA2-independent manner (34). In addition, *M. smegmatis* PhoA is a proven lipoprotein with a Sec signal sequence that contains a lipobox motif, as is also the case for Ms1704 and Ms1712 (43). When the signal sequence of Ms1704 was replaced with the signal sequence of *M. smegmatis* PhoA (ssPhoA), the ssPhoA-Ms1704-HA chimera was exported to the cell wall in wild-type *M. smegmatis* but was not exported to the cell wall of the Δ*secA2* mutant (Fig. 2A). These results indicated that the PhoA signal sequence can support export of the Ms1704 mature domain, but this export requires SecA2. In the converse fusion, when the Ms1704 signal sequence (ssMs1704) was fused to the mature domain of PhoA, the ssMs1704-PhoA fusion protein was efficiently exported to the cell wall in both wild-type and Δ*secA2* mutant *M. smegmatis* cells. Thus, export of the mature domain of PhoA remained SecA2 independent even though the protein contained a signal sequence from an SecA2 substrate. This set of chimeras suggested that there is something distinct about the mature domain of Ms1704 that dictates SecA2 dependency.

The mature domain of Ms1712 also imparts the requirement for SecA2 in export. To see if the above results extend to the other known *M. smegmatis* SecA2 substrate, we constructed similar chimeras with Ms1712. As previously demonstrated, Ms1712-HA containing its native signal sequence is exported to the cell wall only when SecA2 is present (34). Consistent with results using Ms1704, the Sec signal sequence from *M. smegmatis* PhoA supported export of the Ms1712 mature domain to the cell wall in wild-type *M. smegmatis*, but this export was reduced in the Δ*secA2* mutant (Fig. 2B). Further, the converse fusion with the signal sequence of Ms1712 fused to the mature domain of *M. smegmatis* PhoA (ssMs1712-PhoA) was exported equally in both the wild type and the Δ*secA2* mutant.

The *M. tuberculosis* 19-kDa lipoprotein is another protein previously shown to be exported by *M. smegmatis* in a manner independent of SecA2 (34, 44). Therefore, we also created chimeras between Ms1712 and the 19-kDa lipoprotein. In line with the results from the PhoA fusions, the Sec signal sequence of the 19-kDa protein supported export of the Ms1712 mature domain to the cell wall in wild-type *M. smegmatis* but not in the Δ*secA2* mutant. Furthermore, the signal sequence of Ms1712 could export the mature domain of the 19-kDa lipoprotein in both the wild type and cells lacking SecA2, indicating that ssMs1712-19-kDa export occurred independently of SecA2 (Fig. 2B).

The results from these signal sequence swap experiments showed that whenever a chimera contained a mature domain from an SecA2-dependent protein, the fusion protein was ex-

ported to the cell wall only in the presence of SecA2. In contrast, all chimeras containing the signal sequence of Ms1704 or Ms1712 fused to a mature domain of a SecA2-independent protein were exported equally well by the wild-type and Δ*secA2* mutant strains.

These results indicated that the mature domains of SecA2 substrates contain a feature(s) that necessitates a role for SecA2 in export and that SecA2 substrates do not contain SecA2-specific signal sequences.

SecA2-dependent export is independent of lipid modification of substrates. Because both Ms1704 and Ms1712 are lipoproteins, we considered the possibility that they have a unique lipid modification that imparts the need for SecA2 in export. To test the influence of lipid modification on SecA2-dependent export, we used site-directed mutagenesis to replace the codon for the invariant cysteine in the lipoboxes of Ms1704 and Ms1712 to one encoding an alanine [yielding Ms1704(CA) and Ms1712(CA), respectively]. This cysteine is the site of diacylglycerol attachment, which is the first step in lipid modification (36). An alanine substitution for this cysteine (CA) will prevent lipid attachment but should not prevent export because it converts the lipoprotein signal sequences of Ms1704 and Ms1712 to standard Sec signal sequences (45).

When the nonlipidated Ms1704(CA)-HA and Ms1712(CA)-HA proteins were expressed in wild-type *M. smegmatis*, we could detect the proteins in whole-cell lysates but were initially surprised to find that neither protein localized to the cell wall fraction (data not shown). For at least some mycobacterial lipoproteins, the lipid moiety is shown to be required for anchoring to the cell wall, and without lipid modification, the proteins are fully secreted (46). Indeed, both Ms1704(CA)-HA and Ms1712(CA)-HA proteins were secreted into the medium, as detected in culture filtrates (CF) from the wild-type strain. Immunoblotting of culture filtrates with anti-GroEL antibodies confirmed that the presence of Ms1704(CA)-HA and Ms1712(CA)-HA in the medium was not due to cell lysis. When culture filtrates from the wild type or the Δ*secA2* mutant expressing Ms1704(CA)-HA or Ms1712(CA)-HA were compared, we found less protein secreted in the culture filtrates obtained from the Δ*secA2* mutant (Fig. 3A). There was also evidence of Ms1712(CA)-HA accumulation in the whole-cell lysate of the Δ*secA2* mutant, which included a higher-molecular-weight band that likely represents precursor protein containing the uncleaved signal sequence. This higher-molecular-weight form is consistent with a form of Ms1712 that accumulated in the presence of globomycin, an inhibitor of the lipoprotein signal peptidase, seen in a previous study (34). These observations are indicative of an export defect in the Δ*secA2* mutant, and they showed that secretion of the nonlipidated Ms1704(CA)-HA and Ms1712(CA)-HA proteins is dependent on the presence of SecA2.

As another way of testing the importance of the lipid modification to SecA2 export, we replaced the Ms1704 signal sequence with the signal sequence of *M. tuberculosis* antigen 85B (ssAg85B), a well-known secreted protein of *M. tuberculosis* with a standard Sec signal sequence (i.e., no lipobox) (47). This chimera did not include the invariant cysteine of the Ms1704 lipobox and was therefore nonlipidated. As with the CA mutant proteins, the ssAg85B-Ms1704-HA chimera expressed by wild-type *M. smegmatis* did not localize to the cell wall but was instead secreted into the culture filtrate. In addition, secretion of the ssAg85B-Ms1704-HA protein was impaired in the Δ*secA2* mutant, which demonstrated a requirement for SecA2 in the export of this nonlipidated chimera (Fig. 3B). Taken together, these results show that SecA2-dependent export of the Ms1704 and Ms1712 mature domains is not a consequence of lipid modification.

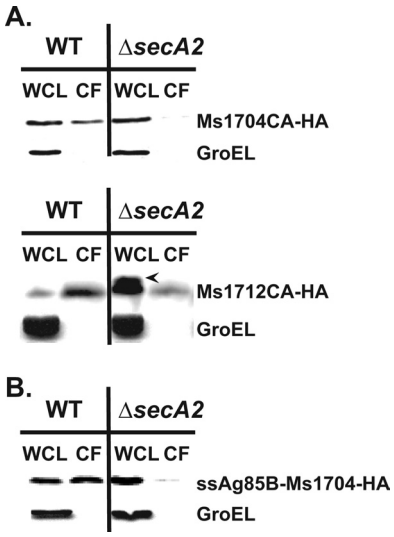

FIG 3 SecA2-dependent export occurs regardless of lipidation. (A) Culture filtrate (CF) proteins from *M. smegmatis* cells expressing either Ms1704(CA)-HA or Ms1712(CA)-HA proteins were analyzed by SDS-PAGE and immunoblotting using an anti-HA antibody. Whole-cell lysate (WCL) and CF material loaded were obtained from an equal number of wild-type (WT) or Δ*secA2 M. smegmatis* cells for each experiment. Native GroEL protein was detected as a loading and cell lysis control. Accumulation of presumptive precursor Ms1712(CA)-HA, containing an uncleaved signal sequence, in the WCL of the Δ*secA2* mutant is noted with an arrowhead. (B) Immunoblot of WCL and CF proteins from wild-type and Δ*secA2 M. smegmatis* cells expressing the nonlipidated ssAg85B-Ms1704-HA chimera. The experiments shown are representative of three independent experiments.

Export of the Ms6020 ABC-type sugar-binding lipoprotein is independent of SecA2. Ms1704 and Ms1712 share 40% amino acid similarity in their mature domains, and both are predicted to be ABC-type sugar-binding proteins. Given the signal sequence chimera results described above, it is interesting that this similarity is limited to the mature domain and does not include the signal sequences of these two proteins. We questioned whether similar structure or function of the mature domain is the reason that these proteins share an SecA2 requirement for export.

To address this possibility, we tested the role of SecA2 in the export of Ms6020, which is one of the other 19 predicted ABC-type sugar-binding proteins of *M. smegmatis* (35). Ms6020 is also a predicted lipoprotein with a lipoprotein-type Sec signal sequence (35, 37). The amino acid sequence of the Ms6020 mature domain is 48% similar to the Ms1704 mature domain and 41% similar to the Ms1712 mature domain. By expressing a C-terminal HA-tagged Ms6020 (Ms6020-HA) protein in wild-type *M. smegmatis*, we showed that the protein was, indeed, exported to the cell wall. However, Ms6020-HA was exported to the cell wall equally well in the Δ*secA2* mutant of *M. smegmatis* (Fig. 4). This result indicated that the requirement for SecA2 does not extend to all exported ABC-type sugar-binding proteins of mycobacteria.

The extreme N terminus of the Ms1704 mature domain is not required for SecA2-dependent export. Because our chimera analysis demonstrated that the mature domains of SecA2 substrates impart the requirement for SecA2, we reasoned that the

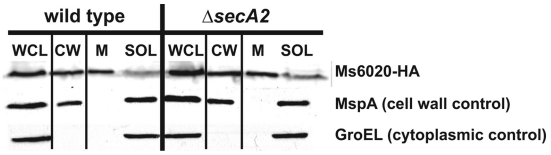

FIG 4 Ms6020-HA is exported to the cell wall independent of SecA2. Equalized whole-cell lysates (WCL) generated from wild-type and Δ*secA2 M. smegmatis* cells expressing HA-tagged Ms6020 were subjected to ultracentrifugation to generate subcellular fractions. Ms6020-HA was detected with an anti-HA antibody while native MspA and GroEL were detected as cell wall and cytoplasmic controls, respectively. The total amount of cell wall (CW), membrane (M), and soluble (SOL) material shown is equivalent to the amount of WCL loaded. Shown here is a representative of three independent experiments.

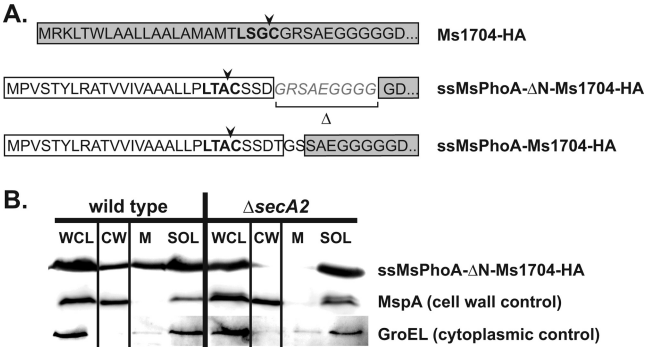

FIG 5 The extreme N terminus of the Ms1704 mature domain is not required for SecA2-mediated export. (A) The first 9 amino acids (italics) were removed from the Ms1704 mature domain, adjacent to the signal peptide cleavage site (arrowhead), to assess the contribution of this region to SecA2-mediated export. This extreme N terminus truncation was created in the ssPhoA-Ms1704-HA signal sequence chimera, where sequence derived from *M. smegmatis* PhoA is boxed in white and sequence from Ms1704 is boxed in gray. (B) Equalized whole-cell lysates (WCL) generated from wild-type and Δ*secA2 M. smegmatis* cells expressing this ssPhoA-ΔN-Ms1704-HA protein were subjected to ultracentrifugation to generate subcellular fractions. Fractions were separated by SDS-PAGE, and proteins were detected by an anti-HA antibody. The total amount of cell wall (CW), membrane (M), and soluble (SOL) fractions shown is equivalent to the amount of WCL loaded. Native MspA and GroEL were detected as cell wall and cytoplasmic controls, respectively. Shown here is a representative of three independent experiments.

mature domain may contain sequences required for SecA2 targeting. To address this possibility, we constructed versions of Ms1704 with deletions at the N terminus (ΔN-Ms1704) or C terminus of the mature domain. To focus in on the contribution of the mature domain, we made the deletions in the ssPhoA-Ms1704 chimera, which we showed above is SecA2 dependent (Fig. 2A). Unfortunately, all but one of these derivatives failed to produce enough protein for subsequent analysis, probably because the deletions yielded unstable proteins. The only construct that could be analyzed had 9 amino acids at the most extreme N terminus of the Ms1704 mature domain deleted (Fig. 5A). Export of ssPhoA-ΔN-Ms1704-HA to the cell wall was monitored in both wild-type *M. smegmatis* and the Δ*secA2* mutant (Fig. 5B). The ssPhoA-ΔN-Ms1704-HA protein was localized to the cell wall of the wild-type strain, but export was severely reduced in the Δ*secA2* mutant. This result indicated that the most extreme N terminus is not required for export by the SecA2-dependent pathway of mycobacteria. This result is interesting in light of the fact that the exported GspB protein of the *S. gordonii* SecA2-SecY2 system has a short amino acid sequence named AST (for *a*ccessory *S*ec *t*ransport) located in the extreme N terminus of its mature domain. This AST domain is required for SecA2-SecY2 targeting/export of GspB (48, 49).

Addition of a ′BlaTEM1 reporter to the C terminus of full-length Ms1704 eliminates the requirement for SecA2 in export. As another way to identify sequences in the mature domain that target the protein to the SecA2-dependent export system, we tested if SecA2 dependency could be transferred to a fused heterologous protein. For this reason, we constructed a fusion protein in which the C terminus of full-length Ms1704 was fused to the truncated β-lactamase reporter (′BlaTEM1) that lacks its native signal sequence. β-Lactam antibiotics target the bacterial cell wall; therefore the ′BlaTEM1 reporter must be exported in order to protect against these drugs. We previously showed that ′BlaTEM1 can report on export when fused to Sec-exported proteins that are expressed in a β-lactamase mutant (Δ*blaS*) of *M. smegmatis* (50). Expression of the Ms1704-′BlaTEM1 fusion protein in either an *M. smegmatis* Δ*blaS* mutant or in a Δ*blaS* Δ*secA2* double mutant resulted in β-lactam resistance (data not shown). This result indicated that Ms1704 was able to support export of the ′BlaTEM1 reporter and that this export occurred in an SecA2-independent manner. To more directly evaluate Ms1704-′BlaTEM1 export, we also localized the fusion protein to subcellular fractions by immunoblotting with anti-BlaTEM1 antibodies (Fig. 6). This analysis showed that the Ms1704-′BlaTEM1 fusion was exported to the cell wall in both wild-type and Δ*secA2 M. smegmatis* mutant strains.

While this approach also failed to reveal any evidence of SecA2 targeting signals in Ms1704, these results are significant. Unlike all the chimeras described earlier, here the presence of the mature domain of Ms1704 did not impart a requirement for SecA2 in export. This result indicated that fusion to ′BlaTEM1 influenced the feature(s) of the Ms1704 mature domain that necessitate SecA2 export. One plausible explanation is that fusion to ′BlaTEM1 altered the structure of Ms1704, enabling it now to be exported in the absence of SecA2.

Ms1704 can be exported by the Tat pathway, independent of SecA2. The SecA2-independent export of the Ms1704-′BlaTEM1 fusion suggested that a structural feature of Ms1704 may influence the need for SecA2. Because the canonical Sec pathway exports proteins that are maintained in an unfolded state, we hypothesized that SecA2 substrates may be prone to more rapid folding in the cytoplasm than strictly SecA1-dependent preproteins. To this end, we tested the ability of an SecA2 substrate to fold in the cytoplasm by engineering a signal sequence chimera, where the Tat signal sequence of *M. tuberculosis* phospholipase C (ssPlcB) was fused to the mature domain of Ms1704 (51). This strategy uses export by the twin-arginine translocation (Tat) system as a reporter for cytoplasmic folding because the Tat pathway requires preproteins to fold prior to export (24, 52). The Tat pathway has been utilized previously as an *in vivo* assay for cytoplasmic folding (53).

As seen with the nonlipidated mutants and the ssAg85B-Ms1704-HA chimera discussed above, the ssPlcB-Ms1704-HA chimera did not localize to the cell wall fraction when expressed in wild-type *M. smegmatis* but was instead secreted into the culture filtrate (Fig. 7A). This was not surprising, given the lack of a lipobox in the PlcB signal sequence. Remarkably, when ssPlcB-Ms1704-HA secretion was compared between the wild type and the Δ*secA2* mutant, the fusion was exported equally well by the

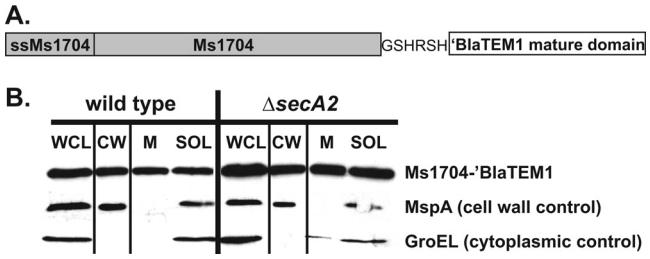

FIG 6 An Ms1704-′BlaTEM1 fusion is exported to the cell wall independently of SecA2. (A) The structure of the Ms1704-′BlaTEM1 fusion protein is shown. The signal peptide and entire Ms1704 mature domain constitute the N terminus of this fusion and are depicted in the gray rectangle. The *E. coli* ′BlaTEM1 (lacking its native signal sequence) was fused to the C terminus of Ms1704. Amino acids introduced from cloning are unboxed. (B) Equalized whole-cell lysates (WCL) generated from wild-type and Δ*secA2 M. smegmatis* cells expressing Ms1704-′BlaTEM1 were subjected to ultracentrifugation to generate subcellular fractions. The Ms1704-′BlaTEM1 fusion was detected with an anti-BlaTEM1 antibody while native MspA and GroEL were detected as cell wall and cytoplasmic controls, respectively. The total amount of cell wall (CW), membrane (M), and soluble (SOL) fractions shown is equivalent to the amount of WCL loaded. Shown is a representative of two independent experiments.

two strains. To confirm that this export was occurring through the Tat pathway, we engineered an ssPlcB-Ms1704-HA chimera where the twin arginine residues of the PlcB signal sequence were replaced with lysine residues [ssPlcB(KK)]. Such twin arginine substitutions have previously been shown to eliminate Tat export of numerous proteins (54–56). Further, we previously showed that changing the arginine residues in the PlcB signal sequence abolishes export of *M. tuberculosis* PlcB (51). The resultant protein, ssPlcB(KK)-Ms1704-HA, was not secreted into the culture supernatants of either wild-type or Δ*secA2* mutant cells (Fig. 7B). This loss of secretion demonstrated that the Tat pathway is responsible for the SecA2-independent export of ssPlcB-Ms1704-HA.

Because the Sec pathway exports proteins in an unfolded state, we expected that this Tat-dependent export of the mature domain of Ms1704 was unusual. For this reason, we similarly tested the ability of the PlcB signal sequence to direct export of the Sec signal sequence containing the 19-kDa lipoprotein using an ssPlcB-19-kDa fusion protein. When the 19-kDa protein with its native Sec signal sequence was expressed in *M. smegmatis*, the protein was detected in cell wall, membrane, and culture filtrate fractions of wild-type *M. smegmatis* (Fig. 7C and D). In contrast, although the ssPlcB-19-kDa fusion protein was detected in whole-cell lysates and in the soluble fraction of wild-type *M. smegmatis*, this chimera was not exported to the cell wall, membrane, or culture filtrate (Fig. 7C and D). Further, multiple migrating species of ssPlcB-19-kDa were observed in the whole-cell lysate and cytosol-containing soluble fractions. The larger of these species is likely unprocessed precursor, and the smaller product is a result of degradation of nonexported protein retained in the cytosol. The difference in compatibility for Tat export between the mature domains of Ms1704 and the 19-kDa protein argues for a difference in the tendency of these representative SecA2-dependent and SecA2-independent substrates to fold in the cytoplasm prior to export.

DISCUSSION

SecA2 systems are emerging as important pathways for the export of virulence factors in a set of Gram-positive pathogens and mycobacteria (26). Yet many mechanistic questions concerning SecA2 export remain, especially in organisms containing SecA2-only systems like mycobacteria. In mycobacteria, both SecA1 and SecA2 proteins are proven ATPases with significant homology to the well-studied *Escherichia coli* SecA (33, 57). However, SecA1 and SecA2 cannot substitute for one another, indicating that each has distinct functions in mycobacterial protein export, and the role of the SecA2 protein is not immediately obvious (25).

In this study, we investigated why certain preproteins require SecA2 for export even though they contain Sec signal sequences that would be expected to be compatible with the canonical SecA1. There is precedent for the amino acid composition of N-terminal signal sequences influencing the export pathway of a given protein: the posttranslational SecA pathway, the cotranslational signal recognition particle (SRP)-mediated pathway, or the Tat pathway (58–60). However, our data from the signal sequence swap experiments showed that the signal sequences of two exported *M. smegmatis* SecA2 substrates are not uniquely capable of directing preproteins to SecA2 for export. Instead, the mature domains of preproteins impart SecA2 dependency. This result, however, is not entirely unexpected because, in addition to the signal sequence, it is shown that the *E. coli* SecA interacts with the mature domains of preproteins at multiple steps during translocation (9, 11, 61, 62).

We further showed that the mature domain of an SecA2-dependent preprotein is compatible with export by the Tat pathway. The latter finding is intriguing and consistent with the possibility that a key difference between mature domains of SecA2-dependent preproteins and the larger collection of preproteins exported by the canonical SecA1/SecYEG pathway is a tendency to fold in the cytoplasm prior to export.

Comparison to SecA2-SecY2 exported proteins. While this is the first study exploring the features that define proteins exported by an SecA2-only system, we can compare our results to those published on the glycosylated preproteins exported by SecA2-SecY2 systems. In comparison to the most thoroughly studied SecA2-SecY2 substrate, the *S. gordonii* GspB protein, there are clear differences in the features that determine export by the two classes of SecA2 systems.

With GspB, the signal sequence prevents export by the canonical Sec pathway by way of three glycine residues (63). With the *M. smegmatis* Ms1704 and Ms1712 proteins, the signal sequences are required for export, as truncated derivatives of these proteins lacking the signal sequences are not exported (34). However, unlike the case with the signal sequence of GspB, our chimera analysis demonstrated that signal sequences of Ms1704 and Ms1712 do not contain features that prohibit export by the canonical Sec pathway independent of SecA2.

There are also features in the mature domain of *S. gordonii* GspB that function in preventing export by the canonical Sec pathway. The distinctive glycosylation of the GspB mature domain, which occurs prior to export, also blocks export by the canonical Sec machinery (64). A similar finding is reported for the Fap1 preprotein of the *Streptococcus parasanguinis* SecA2-SecY2 system (65). We explored the possibility that the mature domains of mycobacterial SecA2 substrates are glycosylated. However, periodic acid-Schiff staining failed to detect glycosylation of the mature domain of Ms1704 (see Fig. S1 in the supplemental material). Although this analysis cannot completely rule out glycosylation of Ms1704 as a factor in preventing export by the canonical Sec path-

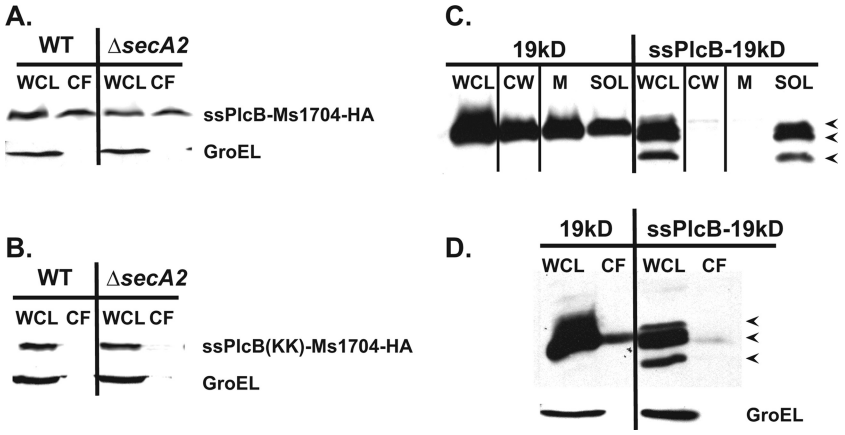

FIG 7 Ms1704 is compatible with export by the twin-arginine translocation (Tat) pathway. (A) Concentrated culture filtrate (CF) proteins from *M. smegmatis* cells expressing ssPlcB-Ms1704-HA were analyzed by SDS-PAGE and immunoblotting using an anti-HA antibody. Whole-cell lysate (WCL) and CF material loaded were obtained from an equal number of wild-type and Δ*secA2 M. smegmatis* cells for each experiment. Native GroEL was detected as a loading and cell lysis control. (B) In the signal sequence of the ssPlcB(KK)-Ms1704-HA chimera, the twin arginine residues were changed to lysines to abolish targeting to the Tat machinery. Secretion of this chimera was analyzed as described in panel A. (C) Export of the 19-kDa protein containing its native Sec signal sequence and the ssPlcB-19-kDa chimera were analyzed by SDS-PAGE and immunoblotting using an anti-19-kDa antibody. Equalized whole-cell lysates (WCL) generated from wild-type *M. smegmatis* cells expressing each protein were subjected to ultracentrifugation to generate cell wall (CW), membrane (M), and soluble (SOL) fractions. (D) WCL and culture filtrate (CF) proteins from wild-type *M. smegmatis* cells expressing either protein were also analyzed by immunoblotting. Wild-type 19-kDa protein is readily detected in both CW and CF fractions while the ssPlcB-19-kDa chimera is not exported. The ssPlcB-19-kDa chimera also exhibits three forms in the WCL, SOL, and CF fractions (indicated by arrowheads in panels C and D), likely representing precursor protein containing an uncleaved signal sequence and cytoplasmic degradation products. All immunoblots are representative of at least two independent experiments.

way, it makes this possibility seem unlikely. We also asked whether a specific lipid modification of the mature domain could be a defining feature of *M. smegmatis* SecA2 substrates, but our data from testing nonlipidated variants of Ms1704 and Ms1712 argue against this possibility.

Finally, approximately 20 amino acids at the N terminus of the GspB mature domain (the AST domain) are required for targeting this preprotein to the SecA2-SecY2 system of *S. gordonii* (48). The AST domain is predicted to adopt an alpha-helical conformation, and mutations predicted to disrupt this secondary structure reduce GspB export. There are no predicted alpha-helices within the first 20 amino acids of either the Ms1704 or Ms1712 mature domain. Further, we showed that deletion of the extreme N terminus of the Ms1704 mature domain did not affect SecA2 export. Thus, there does not appear to be an AST-like targeting element in the extreme N terminus of substrates of the mycobacterial SecA2-only system.

Model for the role of the mature domain in SecA2-dependent export in mycobacteria. How might the mature domain dictate the need for SecA2-dependent export in mycobacteria? With the mycobacterial SecA2-only system, proteins are most likely exported via the canonical SecYEG channel. In support of this model, depletion of the essential *M. smegmatis* SecA1 protein eliminates export of Ms1704, which indicates that both SecA1 and SecA2 are required for export of this SecA2 substrate (33). However, SecA1 and SecA2 are nonredundant homologs, indicating that SecA2 fulfills a unique role in promoting export in mycobacteria (25). Below we discuss three nonexclusive possibilities for how and why the preprotein mature domain may dictate a SecA2 requirement for export in mycobacteria (Fig. 8).

First, the mature domain could possess a posttranslational modification that inhibits canonical export by the SecA1/SecYEG pathway and, thereby, necessitates SecA2 function. However, as

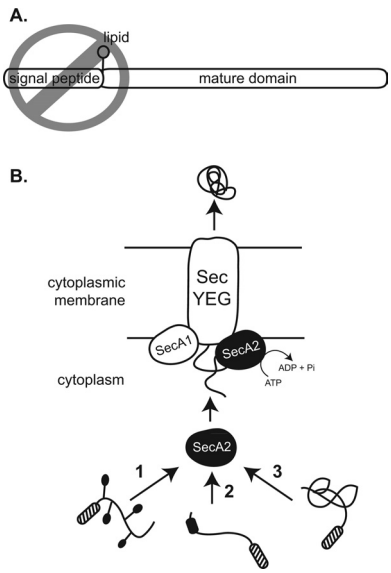

FIG 8 Models for mycobacterial SecA2 export. (A) The feature(s) that make a preprotein dependent on SecA2 for export is not contained in the N-terminal signal sequence. Instead, the mature domain of select proteins, such as Ms1704 and Ms1712, impart the requirement for SecA2. Lipidation of the mature domain is not a factor. (B) SecA2 is required for the export of certain proteins, likely through the canonical SecYEG channel. One possibility is that glycosylation or another posttranslational modification in the mature domain (depicted as attached circles) prevents export by the canonical Sec pathway and/or directs preproteins to SecA2 for export (1). A second possibility is that the mature domain contains an amino acid sequence that directs the preprotein to SecA2 and/or away from SecA1 (depicted as a thick black oval) (2). A third possibility is that the defining feature of SecA2 substrates is a tendency to fold in the cytoplasm (3). Our data are consistent with this last possibility, where SecA2 would then function in maintaining an unfolded preprotein conformation prior to or during export. The signal sequence of preproteins is depicted by the oval with diagonal lines.

described above, there is no evidence currently to support this possibility.

Second, the mature domains of SecA2 substrates may have specific targeting signals for SecA2 recognition. While we did not detect such a sequence at the extreme N terminus of the Ms1704 mature domain, we cannot rule out the possibility that SecA2 targeting signals exist elsewhere. Unfortunately, our efforts to test other regions of Ms1704 by deletion analysis or with chimeric proteins, in which we swapped regions of the SecA2-dependent Ms1704 and SecA2-independent Ms6020 proteins, were unsuccessful due to protein instability problems (data not shown). However, if an SecA2-binding sequence exists, it does not appear sufficient for SecA2-dependent export, as demonstrated by the inability of full-length Ms1704 to promote SecA2-dependent export of the fused ′BlaTEM1 protein. In fact, addition of ′BlaTEM1 to the C terminus of the Ms1704 mature domain overrode the requirement of SecA2 for export. The latter result indicates that the fusion of the ′BlaTEM1 reporter to Ms1704 influenced a property of the Ms1704 mature domain to enable export by the canonical Sec pathway in the absence of SecA2.

The third possibility is that a defining feature of the mature domain of SecA2 substrates is a propensity to fold in the cytoplasm, which would be problematic for canonical Sec export. Studies on Sec export in *E. coli* show that fast-folding preproteins, such as dihydrofolate reductase and β-galactosidase, are incompatible with export through the SecYEG channel (66, 67). Also, slower-folding variants of preproteins result in better translocation efficiency through SecYEG (23). In thinking, then, about the fusion of ′BlaTEM1 to the C terminus of full-length Ms1704, it seems that this may have abolished the requirement for SecA2 by slowing down folding of the resultant fusion protein.

In further support of this third possibility, we found that the mature domain of Ms1704 was exported by the Tat system when it was fused to a Tat signal sequence. The Tat system exports only folded proteins and will even reject Tat substrates engineered to remain unfolded (24, 53). The Tat compatibility of the Ms1704 mature domain indicates that it can fold in the cytoplasm. Tat export has been used before as a reporter for cytoplasmic folding (24, 52, 53). A limitation of this approach is that it indicates that only some, but not necessarily all, of the Ms1704 preprotein pool exists in a Tat-competent, folded state. Some preproteins exhibit a range of cytoplasmic conformations that allow export by both the Sec and Tat pathways (50). However, when we tested the SecA2-independent 19-kDa lipoprotein, it did not share the compatibility with Tat export. Interestingly, ssPlcB-Ms1704 was exported by the Tat pathway in the presence of SecA2, which suggests that any interactions between SecA2 and the preprotein do not interfere with Tat export.

Our results are consistent with a model whereby cytoplasmic folding of Ms1704 inhibits export by the canonical SecA1/SecYEG in the absence of SecA2 (Fig. 8). There is evidence in *E. coli* that SecA has some chaperone function and that the canonical Sec translocase can assist in unfolding some preproteins through the action of translocation itself (17, 68). Thus, it is possible that the mycobacterial SecA2 fulfills a chaperone-like role by keeping preproteins unfolded prior to or during export. Alternatively, SecA2 could provide the energy required to translocate such challenging substrates through SecY. It is possible that SecA2 cooperates with SecA1 to carry out such functions. This cooperation could be in the form of delivering the preprotein to SecA1 or in working with SecA1 to translocate the protein across the SecY channel. The possibility of SecA1 and SecA2 working together is supported by evidence that the canonical *E. coli* SecA can function as a dimer (69).

In *E. coli* and other Gram-negative bacteria, the SecB chaperone promotes Sec export of preproteins that fold quickly in the cytoplasm (70, 71). It should be noted that mycobacteria possess a "SecB-like" homolog, but current data suggest that this protein has a chaperone role unrelated to general Sec export (72). Interestingly, the *E. coli* ABC-type maltose-binding protein (MBP) requires the SecB chaperone in order to remain unfolded for Sec export, while export of the related ribose-binding protein (RBP) is SecB independent (73, 74). These data demonstrate that proteins with similar functions can have different folding kinetics in the cytoplasm and therefore different requirements for cytoplasmic chaperones. These differences could also explain the distinction in SecA2 dependence of Ms6020 versus the Ms1704 and Ms1712 proteins.

In *M. tuberculosis*, one of the few known SecA2-dependent exported proteins, the superoxide dismutase SodA, lacks an obvious Sec signal sequence (27). While it remains possible that the role of SecA2 in the export of *M. tuberculosis* SodA is indirect, it is interesting to discover in this study that the mature domain, not the signal sequence, harbors the feature(s) that identifies *M. smegmatis* proteins as SecA2 substrates. Thus, it is possible that SecA2 of *M. tuberculosis* facilitates the export of proteins like SodA in a manner similar to the way that *M. smegmatis* SecA2 functions with its substrates. With these ideas in mind, it is worth noting that in *Rhizobium leguminosarum* export of an SodA protein that lacks a typical Sec signal sequence was recently reported to occur in an SecA-dependent manner (75).

The data presented in this study provide a better foundation for understanding SecA2-dependent protein export. In this study, we clearly identify the preprotein mature domain as the distinguishing feature of SecA2 substrates in mycobacteria. By showing that the mature domain of a *M. smegmatis* SecA2 substrate is compatible with the Tat export pathway, this study indicates that preproteins exported by this SecA2-only system are capable of folding in the cytoplasm. Thus, acquisition of a second SecA homolog in bacteria with SecA2-only systems may represent another solution to the problem of exporting proteins that prefold in the cytoplasm prior to translocation across the cytoplasmic membrane. In addition, our results further reinforce the distinction between the two classes of SecA2 export systems: the SecA2-SecY2 and SecA2-only pathways.

ACKNOWLEDGMENTS

We thank Carol Teschke and members of the Braunstein lab for critical reading of the manuscript. We also thank Douglas Young and Michael Niederweis for providing the anti-19-kDa and MspA antibodies, respectively.

This work was supported by NIH grant AI05454.

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