Conserved Serine and Histidine Residues Are Critical for Activity of the ER-type Signal Peptidase SipW of Bacillus subtilis*

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Type I signal peptidases (SPases) are required for the removal of signal peptides from translocated proteins and, subsequently, release of the mature protein from the trans side of the membrane. Interestingly, prokaryotic (P-type) and endoplasmic reticular (ER-type) SPases are functionally equivalent, but structurally quite different, forming two distinct SPase families that share only few conserved residues. P-type SPases were, so far, exclusively identified in eubacteria and organelles, whereas ER-type SPases were found in the three kingdoms of life. Strikingly, the presence of ER-type SPases appears to be limited to sporulating Gram-positive eubacteria. The present studies were aimed at the identification of potential active site residues of the ER-type SPase SipW of Bacillus subtilis, which is required for processing of the spore-associated protein TasA. Conserved serine, histidine, and aspartic acid residues are critical for SipW activity, suggesting that the ER-type SPases employ a Ser-His-Asp catalytic triad or, alternatively, a Ser-His catalytic dyad. In contrast, the P-type SPases employ a Ser-Lys catalytic triad (Paetzel, M., Dalbey, R. E., and Strynadka, N. C. J. (1998) Nature 396, 186–190). Notably, catalytic activity of SipW was not only essential for pre-TasA processing, but also for the incorporation of mature TasA into spores.

In recent years, interesting similarities between the process of protein transport across the bacterial plasma membrane and the eukaryotic endoplasmic reticular (ER) membrane have been documented. These similarities include the direction of protein transport (i.e. export from the cytoplasm), the targeting signal (i.e. the signal peptide), the signal recognition particle, components of the translocation channel, signal peptidases (SPases), and thiol-disulfide oxidoreductases (for reviews, see Refs. 1–4). Notwithstanding these similarities, significant differences between protein transport across bacterial plasma membranes and the ER membrane do exist. First, protein transport is energized in different ways. Although bacterial protein export requires a cytosolic force-generator (i.e. SecA), and the proton-motive-force (5–7), protein import into the ER lumen is driven by the ribosome (co-translational protein transport; Refs. 8–10), or the luminal Kar2 protein (post-translational protein transport in yeast; Refs. 11 and 12). Second, as exemplified by the SPases, the similarities between components of the different protein transport machineries are rather weak (see Refs. 1 and 2).

Homologous SPases have been identified in Gram-positive and Gram-negative eubacteria, the inner membrane of yeast mitochondria, the thylakoid membrane of chloroplasts, archaean, and the ER membranes of yeast and higher eukaryotes, such as Homo sapiens (Ref. 1; see Fig. 1). These enzymes, which remove signal peptides from translocated proteins, are required for the release of the mature protein from the trans side of the membrane (for reviews, see Refs. 1 and 13). We have previously shown that SPases can be divided into two distinct subfamilies, denoted P- and ER-type SPases. P-type SPases were exclusively identified in eubacteria, mitochondria, and chloroplasts, whereas ER-type SPases were identified in eukaryotes, archaean, and four sporulating Gram-positive eubacteria, Bacillus subtilis, Bacillus anamyloliquefaciens, Bacillus anthracis, and Clostridium perfringens (Ref. 14; see Fig. 1). Recent studies have provided strong evidence that the P-type SPases employ a Ser-Lys catalytic dyad (1, 15) In contrast, the catalytic mechanism of the ER-type SPases remains to be elucidated (16).

The observation that B. subtilis contained an ER-type SPase, denoted SipW, was remarkable for two reasons. First, SipW represented the first ER-type SPase identified in a eubacterium. Second, SipW happened to be the fifth chromosomally encoded SPase of B. subtilis (14). In contrast to SipW, the other four SPases of B. subtilis, denoted SipS, SipT, SipU, and SipV, are of the P-type (14, 17, 18). Notably, SipU, SipV, and SipW are of minor importance for the processing of secretory pre-proteins, in contrast to SipS and SipT, which are critical for protein secretion and viability (14, 19). This suggested that SipU, SipV, and SipW might be required for the processing of a specific subset of the approximately 180 predicted pre-proteins of B. subtilis. The latter hypothesis was recently confirmed for SipW, which was shown to be required for the processing of the spore-associated protein TasA, displaying antibacterial activities (20). Consistent with a role in sporulation, the expression...
of the tasA gene (previously known as cotN; Ref. 14), which is located immediately downstream of the sipW gene, depended on the post-exponential growth phase-specific transcription factors SpoOA and SpoOH (20, 21). Interestingly, TasA was not only incorporated into spores, but also secreted into the growth medium. Similar to TasA, the Yqxm pre-protein, which is specified by a gene that is located immediately upstream of the sipW gene, was also processed and secreted in a SipW-dependent manner. In contrast to TasA, mature Yqxm was not incorporated into spores (22).

The present studies were aimed at the identification of potential active site residues of SipW. For this purpose, TasA was an ideal reporter because its processing is strongly dependent on the presence of the SipW protein (20, 23). Furthermore, in contrast to Yqxm, the precursor and mature forms of TasA can be readily detected in sporulating cells of B. subtilis. Finally, TasA could be used to study the importance of processing by SipW for the incorporation of this protein into spores, as neither TasA nor SipW is required for the sporulation process per se. The results show that conserved Ser, His, and Asp residues are critical for the activity of SipW, and that catalytically active SipW is required for the incorporation of mature TasA into spores.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Bacterial Strains, and Media**—Table I lists the plasmids and bacterial strains used. Tryptone/yeast extract (TY medium) containing Bacto-tryptone (1%), Bacto-yeast extract (0.5%), and NaCl (1%). When required, medium for the growth of bacterial strains used. Tryptone/yeast extract (TY medium) containing Bacto-tryptone (1%), Bacto-yeast extract (0.5%), and NaCl (1%).

| Plasmids | Relevant properties | Reference |
|----------|---------------------|-----------|
| pGDL48   | Encodes pre(A13i)-β-lactamase; replicates in E. coli and B. subtilis contains a multiple cloning site; 7.5 kb; Ap‘, Km‘ | (24) |
| pGDL41   | As pGDL48, contains the sipS gene of B. subtilis; 7.9 kb | (17) |
| pGDL40   | As pGDL48, contains a wild-type copy of the sipW gene of B. subtilis; 8.0 kb | This paper |
| pW-x     | As pGDL40, carries a mutant sipW gene (x indicates the position and type of amino acid substitution in the corresponding mutant proteins) | This paper |

| Strains   | Relevant properties | Reference |
|-----------|---------------------|-----------|
| E. coli MC1061 | F‘; araD139; Δara-leu7696; ΔlacX74; galU; galK; hsdR2; recA; mcrA; rPspL | (25) |
| B. subtilis Δ-wctasA | Derivative of B. subtilis PY79, originally referred to as AG8215; gerE36; cotE‘-Cm‘; sipW‘-Nm‘; the tasA gene is constitutively expressed | (20) |
| PM8      | Derivative of B. subtilis PY79; contains a single integrated copy of pPS490 (26), bearing a translational sshB-lacZ fusion; Cm‘ | (27) |

* kb, kilobase pair(s).

**DNA Techniques**—Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of E. coli were carried out as described by Sambrook et al. (28). Enzymes were from Roche Molecular Biochemicals. B. subtilis was transformed as described by Tjalsma et al. (14). PCR was carried out with Pwo DNA polymerase (Roche Molecular Biochemicals) as described by van Dijk et al. (29). The BLAST algorithm (30) was used for protein comparisons in GenBank[29].

To construct plasmid pGDL140, carrying the wild-type sipW gene, a PCR with the primers lbw-1 and lbw-2 (Table II) was performed using chromosomal B. subtilis 168 DNA as a template. The amplified fragment was subsequently cleaved with SalI and EcoRI, and ligated into the corresponding sites of pGDL48 (24) in such a way that sipW transcription is driven by the constitutive erythromycin promoter. Site-directed mutations were introduced into plasmid-horse copies of sipW by a two-step PCR approach (29), using lbw-1, lbw-2, and the mutagenic oligonucleotides shown in Table II. Amplified fragments were cleaved with SalI and EcoRI, and ligated into the corresponding sites of pGDL48. The resulting plasmids were named pW-x, where x indicates the position and type of amino acid substitution in the corresponding SipW mutant protein.

**Fractionation of Sporulating Cells and Trypsin Accessibility Assays**—Protoplasts were prepared from sporulating cells of B. subtilis. For this purpose, cells were resuspended in protoplast buffer (20 mM potassium phosphate, pH 7.5, 15 mM MgCl₂, 20% sucrose) and incubated for 30 min in the presence of 0.5 mg/ml lysozyme (37 °C). Next, the protoplasts were centrifuged and resuspended in fresh protoplast buffer. If desired, the protoplast-supernatant, which contains cell wall-associated proteins, was collected and used for SDS-PAGE and Western blotting. The protease accessibility of membrane-associated proteins was tested by incubating the protoplasts at 37 °C in the presence of 1 mg/ml trypsin (Sigma) for 30 min. In parallel, protoplasts were incubated without trypsin, or in the presence of trypsin and 1% Triton X-100. To isolate endospores, protoplasts were lysed by incubation for 5 min in protoplast buffer containing 1% Triton X-100. To release proteins from the spore envelope, spores were collected by centrifugation, resuspended in protoplast buffer, and incubated for 30 min in the presence of 1 mg/ml lysozyme (37 °C). Next, the samples were incubated at 37 °C in the presence of 1 mg/ml trypsin (Sigma) and 1% Triton X-100 for 30 min to test the protease sensitivity of spore-associated proteins. All trypsin digestions were terminated by the addition of 1.2 mg/ml trypsin inhibitor (Sigma), before boiling in loading buffer for SDS-PAGE.

Western blot analysis and immunodetection—To obtain SipW-specific antibodies, the pET32a plasmid (Novagen) was used to fuse part of the ER-type Signal Peptidase of B. subtilis 25103, which is located immediately downstream of the sipW gene, to a hexahistidine tag. The fusion protein was expressed in E. coli, and purified by metal affinity chromatography using the Talon resin supplied by CLONTECH. Rabbits were immunized with the fusion protein at Eurogentec.

Western blotting was performed as described by Kyhse-Andersen (31). Samples for SDS-PAGE were prepared as described by van Dijk et al. (32). After separation by SDS-PAGE, proteins were transferred to Immobilon PVDF membranes (Millipore Corp.). (Pre-)TasA, SipW, GroEL, β-lactamase, or SspB-LacZ, were visualized with specific antibodies and horseradish peroxidase anti-rabbit-IgG conjugates (Amer sham Pharmacia Biotech).

**RESULTS**

**Identification of Conserved Residues in ER-type SPases of Eukaryotes, Archaea, and Eubacteria**—Thus far, genes for 19 different ER-type SPases have been identified in Gram-positive eubacteria, archaea, and eukaryotes. This allowed a detailed comparison of the deduced amino acid sequences of these enzymes (Fig. 1), which showed that they consist of a highly conserved amino-terminal moity (Fig. 2A, indicated with ER-C) and a highly variable carboxy-terminal moiety (Fig. 2A, indicated with ER-V). In some enzymes, the variable moiety contains one or three putative membrane spanning domains (Fig. 2B). The highly conserved moiety contains one amino-terminal membrane anchor (A) and four conserved domains (B-E), which are present in all known P- and ER-type SPases (Fig. 1; see Refs. 1 and 14). Domain B contains the conserved serine residue, which has been shown to be critical for the activity of P-type SPases from E. coli (15, 44), B. subtilis (29), and mitochondria (45). Stretches of conserved amino acids resembling domain C of the P-type SPases, which seems to be involved in substrate binding (15) and catalysis (46), can be
found twice in ER-type SPases (Fig. 1, domain C and C'). Domain D of the ER-type SPases contains a strictly conserved histidine residue, replacing the strictly conserved lysine residue in domain D of the P-type SPases. Finally, domain E contains two conserved aspartic acid residues, the equivalents of which are involved in salt bridge formation in the P-type SPases (15, 47). Recent observations indicate that these salt bridges are important determinants for the stability of some

| Primer      | Sequence                                      |
|-------------|-----------------------------------------------|
| Lbw-1       | 5'-aagtcgacAGAAGGAAAGCGGGGAAGAGGATG-3'        |
| Lbw-2       | 5'-GAAGACCCGCGTACCCGACAGTACGAAACTCG-3'        |
| W-S47A      | 5'-GCAATCGGCCTCCCTACGGACACGTACGAAACTCG-3'     |
| W-H57A      | 5'-CGCAATCAGGGCTACGCGAGTAAATGACACTAAGGCGG-3'  |
| W-H57K      | 5'-CGCAATCAGGGCTACGCGAAGATGGAAATGACACTAAGGCGG-3' |
| W-R58A      | 5'-GCAATCAGGGCTACGCGAAGATGGAAATGACACTAAGGCGG-3' |
| W-K104A     | 5'-GCAATCAGGGCTACGCGAAGATGGAAATGACACTAAGGCGG-3' |
| W-D106A     | 5'-GCAATCAGGGCTACGCGAAGATGGAAATGACACTAAGGCGG-3' |
| W-D112A     | 5'-GCAATCAGGGCTACGCGAAGATGGAAATGACACTAAGGCGG-3' |

TABLE II

Oligonucleotides used for sipW mutagenesis

| Primer     | Sequence                                      |
|------------|-----------------------------------------------|
| Lbw-1      | 5'-aagtcgacAGAAGGAAAGCGGGGAAGAGGATG-3'        |
| Lbw-2      | 5'-GAAGACCCGCGTACCCGACAGTACGAAACTCG-3'        |
| W-S47A     | 5'-GCAATCGGCCTCCCTACGGACACGTACGAAACTCG-3'     |
| W-H57A     | 5'-GCAATCAGGGCTACGCGAGTAAATGACACTAAGGCGG-3'   |
| W-H57K     | 5'-GCAATCAGGGCTACGCGAAGATGGAAATGACACTAAGGCGG-3' |
| W-R58A     | 5'-GCAATCAGGGCTACGCGAAGATGGAAATGACACTAAGGCGG-3' |
| W-K104A    | 5'-GCAATCAGGGCTACGCGAAGATGGAAATGACACTAAGGCGG-3' |
| W-D106A    | 5'-GCAATCAGGGCTACGCGAAGATGGAAATGACACTAAGGCGG-3' |
| W-D112A    | 5'-GCAATCAGGGCTACGCGAAGATGGAAATGACACTAAGGCGG-3' |

Mismatches with the original sequence of sipW are indicated in lowercase letters.

Fig. 1. Conserved domains B-E in ER-type SPases from eubacteria, archaea, and eukaryotes. The comparison includes the amino acid sequences of ER-type SPases from the Gram-positive eubacteria B. subtilis (SipW (Bsu); Ref. 14), B. ankylostomatis (SipW (Ban); GenBank™ accession no. AF984597), B. anthracis (SipW (Bam); GenBank™ accession no. X864488); the archaea: A. fulgidus (Spc21 (Afu), Sec11 (Afu), Sip1 (Afu), and Sip2 (Afu); GenBank™ loci AF1657, AF1791, AF1655, and AF2078, respectively; Ref. 33), Methanobacterium thermoautotrophicum (Sip (Mth); GenBank™ locus MTH1448; Ref. 34), Methanococcus jannaschii (Sip (Mja); GenBank™ locus MJU67481; Ref. 35), and Pyrococcus horikoshii (Spc (Pho) and Sip (Pho); GenBank™ loci PH2000 and PH2056, respectively; Ref. 36); and the eukaryotes: Saccharomyces cerevisiae (Sec11p (Sce); Ref. 37), Schizosaccharomyces pombe (Sec11p (Spo); GenBank™ accession no. E1313476), Caenorhabditis elegans (Spc18 (Cel); Ref. 38), Canis familiaris (Spc18 (Cfa) and Spc21 (Cfa); Refs. 39 and 40), Rattus norvegicus (Spc18 (Rno); Ref. 41), and H. sapiens (Spc18 (Hsa); GenBank™ accession no. G3641344). Residues are printed in bold when present in at least 10 of the 19 ER-type SPases. Numbers refer to the position of the first amino acid of each boxed region (B–E) in the respective SPases. In the consensus sequence below each domain, uppercase letters indicate residues that are strictly conserved in all known ER-type SPases, and lowercase letters show identity in at least 10 sequences. A number sign (#) indicates conserved hydrophobic residues, and + indicates conserved positively charged residues. Note that domain C is duplicated in the ER-type SPases. Conserved residues of B. subtilis SipW (SipW (Bsu)), which, through site-specific mutagenesis, are replaced by alanine (A) or lysine (K), are indicated below the boxed regions. Residues required for activity (●) of SipW are indicated above the boxed regions. The consensus sequence of the domains B–E of the P-type SPases, is indicated as described by Dalbey et al. (1).
P-type SPases, such as SipS of \textit{B. subtilis} (29, 46). Interestingly, domain E of the Sec11 protein of \textit{Archeoglobus fulgidus} seems to be duplicated (Fig. 1). Taken together, these observations suggest that the conserved residues in domains B–E are required for the catalytic activity and/or stability of the ER-type SPases.

\textbf{Identification of the Potential Active Site of SipW—}To investigate whether the conserved residues of domains A–E of the ER-type SPases are required for the activity of SipW of \textit{B. subtilis}, we exploited the recent observation that SipW is of major importance for processing of the spore-associated protein TasA (20). To this purpose, a plasmid-based complementation system was developed, involving \textit{B. subtilis} strain DW-TasA, which lacks SipW ("ΔW") and constitutively produces pre-TasA ("ΔTasA"). First, \textit{B. subtilis} DW-TasA was transformed with pGDL140 ("SipW"; constitutive expression of \textit{sipW}), pGDL41 ("SipS"; 5-fold overproduction of SipS in the stationary growth phase; data not shown), or pGDL48 ("empty vector"; no SipE overproduction). Next, the effects on the processing of pre-TasA to the mature form were evaluated by growing the resulting strains in TY medium, sampling after 4, 16, and 32 h of post-exponential growth, and Western blotting of total cell and sporangial lysates. The results show that after 4 h of post-exponential growth (\(t = 4\)), only pre-TasA was present in cells of \textit{B. subtilis} DW-TasA, irrespective of the (over)production of SipS or SipW (Fig. 3A). The observation that the presence of multiple copies of \textit{sipW} did not stimulate pre-TasA processing at \(t = 4\) suggests that SipW requires an as yet unidentified partner protein that is not (yet) expressed at this time. In contrast, after 16 h of post-exponential growth (\(t = 16\)), \textit{B. subtilis} DW-TasA cells producing SipW contained both the precursor and mature forms of TasA, whereas no mature TasA was observed in \textit{B. subtilis} DW-TasA cells lacking SipW (Fig. 3A). Finally, after 32 h (\(t = 32\)), most TasA molecules in \textit{B. subtilis} DW-TasA cells producing SipW were mature. In contrast, significantly lower amounts of mature TasA were detectable in \textit{B. subtilis} DW-TasA cells lacking SipW (Fig. 3A). The SipW-independent processing of pre-TasA at \(t = 32\) was not affected by SipS overproduction, suggesting that one or more other SPases are responsible for this effect. As no SipW-independent processing of pre-TasA at \(t = 16\) was observed, this time point was selected to monitor the activity of SipW proteins with site-specific mutations.

To investigate the importance for pre-TasA processing of conserved residues in domains B–E of SipW, the residues Ser-47, His-87, Arg-88, Lys-104, Asp-106, or Asp-112 were replaced by alanine (see Fig. 1; note that the latter residue numbers correspond to the sequence of SipW of \textit{B. subtilis} (\textit{Bsu}). Alanine was chosen because it is small, and has a chemically inert side chain, minimizing conformational strain and indirect effects on catalysis. To monitor pre-TasA processing by the various SipW mutant proteins, \textit{B. subtilis} DW-TasA was transformed with plasmids encoding wild-type SipW (positive control), the SipW mutant proteins S47A, H87A, R88A, K104A, D106A, or D112A, or SipS (negative control). The resulting strains were grown in TY medium until \(t = 16\). As demonstrated by Western blotting, more than 50% of the TasA molecules in cells producing SipW were not detected in the mature form, similar to cells producing wild-type SipW.
(Fig. 3B), showing that Arg-88, Lys-104, and Asp-112 are not important for pre-TasA processing. In contrast, very low amounts of mature TasA were detectable in cells producing SipW-D106A. No mature TasA was detectable in cells producing SipW-S47A or -H87A, as in SipS-producing cells. These results show that the conserved serine and histidine residues of SipW are critical for the processing of pre-TasA, and that Asp-106 is very important for this activity. Notably, the conserved His-87 of SipW could be replaced by lysine without loss of pre-TasA processing (Fig. 3D), suggesting that a basic residue at this position is required for activity of SipW.

Unexpectedly, only the completely inactive SipW-S47A and -H87A mutant proteins reacted (weakly) with a SipW-specific antibody, whereas neither wild-type SipW nor the active SipW mutant proteins, including SipW-D106A, were detectable (Fig. 3C, upper panel). This weak interaction, or the complete absence of reactivity between SipW (mutant proteins) and SipW-specific antibodies was not due to a loss of sipW-containing plasmids, as shown by the detection of comparable amounts of the plasmid-encoded β-lactamase in all samples (Fig. 3C, lower panel). The latter observations suggest that the catalytically active forms of SipW are subject to self-cleavage, as described previously for the purified SPase of E. coli (49). In summary, these results suggest that Ser-47 and His-87 are involved in catalysis, and that Asp-106 is either involved in catalysis or in maintaining a stable conformation.

Catalytic Activity of SipW Is Required for TasA Incorporation into Spores—To investigate the importance of SipW for TasA incorporation into spores, fractionation experiments were performed (schematically presented in Fig. 4A), in which the cytosolic GroEL protein was used as a reference marker for protoplast integrity and, upon permeabilization of membranes with Triton X-100, trypsin activity. As shown with B. subtilis ΔW-CtasA (pGDL140), at t = 32, TasA has a dual localization in sporulating cells producing SipW. First, a significant fraction of the mature TasA could be released from these cells by protoplasting, showing that these molecules were present in the cell wall (Fig. 4B). Second, pre-TasA remained protoplast-associated, but was degraded when trypsin was added to the protoplasts, showing that this form of TasA accumulated at the membrane-cell wall interface. Third, significant amounts of protoplasts, showing that these molecules were present in the cell wall (Fig. 4B). Second, pre-TasA remained protoplast-associated, but was degraded when trypsin was added to the protoplasts, showing that this form of TasA accumulated at the membrane-cell wall interface. Third, significant amounts of

![Diagram](image-url)

**Fig. 4.** SipW is essential for incorporation of mature TasA into spores. To determine the localization of precursor and mature forms of TasA in sporulating cells of B. subtilis, fractionation experiments were performed as schematically presented in panel A. The cell wall (w) is indicated in gray shading. Solid lines indicate intact membranes, and dotted lines indicate membranes disrupted with Triton X-100. Intact spores were incubated with trypsin, intact cell; PP, protoplast; SP, spore; T, trypsin. B–D, cells of B. subtilis ΔW-CtasA harboring the plasmids pGDL140 (SipW; panel B) or pGDL41 (no SipW; panel C), or cells of B. subtilis PM8 (SspB-LacZ; panel D) were grown in TY medium until 32 h after the transition between exponential and post-exponential growth. Next, cells (c) were protoplasted, and protoplasts (pp) were separated from the cell wall (w) fraction (i.e., protoplast supernatant) by centrifugation. Complete cells, protoplasts, and the cell wall fraction were used for SDS-PAGE, Western blotting, and immunodetection with TasA-, GroEL-, or LacZ-specific antibodies. In addition, protoplasts were incubated for 30 min in the presence of trypsin (1 mg/ml), or trypsin plus Triton X-100 (1%). Finally, endospores (sp) were isolated from the lysed cells to investigate whether spore-associated TasA was trypsin resistant per se, or protected against trypsin by the spore envelope. Spores were incubated with lysozyme prior to the addition of trypsin plus Triton X-100. Intact and lysed spores were used for SDS-PAGE and Western blotting. The positions of GroEL (cytosolic marker), SspB-LacZ (spore-specific marker), and pre-TasA (p), mature TasA (m), and a degradation (d) product of TasA are indicated.

spore (26, 27). Thus, the fractionation of spore-associated proteins can be monitored with antibodies against LacZ. This was not possible with antibodies against GroEL, as this protein appears to be absent from spores (Fig. 4, B and C). As shown in Fig. 4D, B. subtilis PM8 showed similar fractionation patterns for TasA and GroEL as B. subtilis ΔW-CtasA (pGDL140).
mature TasA was present in protoplasts of H87A SipW mutant proteins. In contrast, trypsin-resistant not for the accumulation of mature TasA in the cell wall of required for the incorporation of mature TasA into spores, but presence of active or inactive mutant SipW proteins, or wild-type SipA accumulating in protoplasts of C from protoplasts of TasA- and GroEL-specific antibodies (cytoplasmic control). Samples were used for SDS-PAGE, Western blotting, and immunodetection with SipA- and GroEL-specific antibodies (cytoplasmic control). The positions of pre-TasA (p), mature TasA (m), and GroEL are indicated. (Fig. 4B). Furthermore, LacZ cofractionated with spores. Notably, a significant fraction of the LacZ molecules appeared to be trypsin-sensitive upon protoplast lysis, indicating that the spores were not yet mature.

To determine whether active SipW is required for the sorting of TasA to spores, fractionation experiments were performed with B. subtilis ΔW-CtsA producing SipW mutant proteins. As shown in Fig. 5, trypsin-resistant mature TasA was absent from protoplasts of B. subtilis ΔW-CtsA producing the S47A or H87A SipW mutant proteins. In contrast, trypsin-resistant mature TasA was present in protoplasts of B. subtilis ΔW-CtsA producing the H87K, R88A, K104A, D106A, or D112A SipW mutant proteins, or wild-type SipW. Irrespective of the presence of active or inactive mutant SipW proteins, the pre-TasA accumulating in protoplasts of B. subtilis ΔW-CtsA was accessible to trypsin (Fig. 5).

In summary, these observations show that active SipW is required for the incorporation of mature TasA into spores, but not for the accumulation of mature TasA in the cell wall of sporulating cells.

DISCUSSION

In the present studies, we document the importance of SipW for processing of pre-TasA and incorporation of mature TasA into spores. Translocated pre-TasA was almost exclusively processed by SipW, a process that requires three conserved residues (i.e. Ser-47, His-87, and Asp-106) of this SPase. In sporulating cells lacking active SipW, TasA did not reach the spores. Instead, TasA precursors were translocated across and remained attached to the cytoplasmic membrane, most likely with their uncleaved signal peptides. Upon prolonged growth in the absence of SipW, pre-TasA was processed by other, yet unidentified, SPases of B. subtilis. Most likely, this alternative pre-TasA processing is a result of the combined activities of SipS, SipT, SipU, and/or SipV, as the SPases of B. subtilis, including SipW, have overlapping substrate specificities (14, 18). Strikingly, mature TasA produced by alternative processing in the absence of SipW did not reach the spores, but remained associated with the cell wall. This shows that the presence of SipW is a prerequisite for the incorporation of mature TasA into spores. Interestingly, this process requires active SipW and cannot be promoted by catalytically inactive SipW molecules. It is presently not clear how SipW functions as a determinant for the incorporation of TasA into spores, or why alternatively processed mature TasA is not incorporated into spores.

As recently confirmed by the crystal structure of a catalytically active soluble fragment of the E. coli SPase I (15), also known as leader peptidase, P-type SPases employ a Ser-Lys catalytic dyad, similar to LexA-like proteases (29, 45, 50–52). The observations that the catalytic serine residue of the P-type SPases is well conserved in the ER-type SPases, and that this conserved serine residue (Ser-47) is essential for the activity of SipW, indicate that the ER-type SPases employ a serine residue for catalysis. Notably, the ER-type SPases contain a strictly conserved histidine residue instead of the catalytic lysine residue of the P-type SPases (17, 53). This conserved histidine residue (His-87) is essential for activity of SipW. Taken together, these findings suggest that Ser-47 of SipW acts as a nucleophile that attacks the carbonyl carbon of the scissile peptide bond, whereas His-87 acts as a general base by stripping the proton from the hydroxyl group of the serine side chain. A similar role has been proposed for the corresponding serine and histidine residues of Sec11p from yeast (16). The role of the strictly conserved Asp-106 residue of SipW, which is important, but not essential, for activity, is less clear. First, by analogy to the classical serine proteases, Asp-106 could form a catalytic triad together with Ser-47 and His-87, thereby serving to keep the side chain of His-87 deprotonated. Second, Asp-106 could be a determinant for the stability of SipW, as described previously for Asp-146 of SpS of B. subtilis (14, 29) and Asp-103 of Sec11p (16). The latter hypothesis is supported by the observation that both aspartic acid residues are present at equivalent positions in the conserved domain E of P- and ER-type SPases. Furthermore, the recent observation that the equivalent Asp residue in domain E of the SPase I of E. coli is not required for activity (49) suggests that this conserved Asp residue has a structural rather than a catalytic function. Thus, it is possible that ER-type SPases, such as SipW, employ a Ser-His catalytic dyad, instead of the Ser-His-Asp catalytic triad of the classical serine proteases. The latter view is strongly supported by the observation that the potential active site His-47 residue of SipW can be replaced by lysine without loss of pre-TasA processing activity. This implies that the catalytic mechanisms of P-type SPases and ER-type SPases are, at least to some extent, related.

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**FIG. 5.** Catalytically active SipW is required for TasA incorporation into spores. To determine the localization of precursor and mature forms of TasA in sporulating cells carrying mutant SipW proteins, B. subtilis ΔW-CtsA harboring the plasmids pW-S47A, pW-H87A, pW-R88A, pW-K104A, pWD106A, pWD112A, pWDL41 (no SipW; negative control), or pWDL140 (SipW; positive control) were grown in TY medium for 32 h after the transition between exponential and post-exponential growth. Subsequently, cells were protoplasted and incubated for 50 min without further additions, in the presence of trypsin (1 mg/ml), or in the presence of trypsin plus Triton X-100 (1%).
