Intramolecular Interactions between the Protease and Structural Domains Are Important for the Functions of Serine Protease Autotransporters^{∇†}

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Autotransporter (AT) is a protein secretion pathway found in Gram-negative bacteria featuring a multidomain polypeptide with a signal sequence, a passenger domain, and a translocator domain. An AT subfamily named serine protease ATs of the family *Enterobacteriaceae* (SPATEs) is characterized by the presence of a conserved serine protease motif in the passenger domain which contributes to bacterial pathogenesis. The goal of the current study is to determine the importance of the passenger domain conserved residues in the SPATE proteolytic and adhesive functions using the temperature-sensitive hemagglutinin (Tsh) protein as our model. To begin, mutations of 21 fully conserved residues in the four passenger domain conserved motifs were constructed by PCR-based site-directed mutagenesis. Seventeen mutants exhibited a wild-type secretion level; among these mutants, eight displayed reduced proteolytic activities in Tsh-specific oligopeptide and mucin cleavage assays. These eight mutants also demonstrated lower affinities to extracellular matrix proteins, collagen IV, and fibronectin. These eight conserved residues were analyzed by molecular graphics modeling to demonstrate their intramolecular interactions with the catalytic triad and other key residues. Additional mutations were made to confirm the above interactions in order to demonstrate their significance to the SPATE functions. Altogether our data suggest that certain conserved residues in the SPATE passenger domain are important for both the proteolytic and adhesive activities of SPATE by maintaining the proper protein structure via intramolecular interactions between the protease and β -helical domains. Here, we provide new insight into the structure-function relationship of the SPATEs and the functional roles of their conserved residues.

Autotransporter (AT) is a unique, two-step protein secretion pathway found in Gram-negative bacteria (9). A nascent, classical AT protein is a large polypeptide (often >100 kDa) that is composed of three domains: an N-terminal signal sequence, an internal passenger domain, and a C-terminal translocator domain (3, 9, 14). Once it is translated, the cleavable AT signal sequence mediates the translocation of the newly synthesized polypeptide across the inner membrane (IM) via Sec translocase, which is embedded in the IM. Translocation across the outer membrane (OM) depends on its C-terminal translocator domain, consisting of an α -helical linker and a β domain. After secretion, the passenger domain folds into a functional protein that can either stay on the cell surface by covalent bonding or be released into the extracellular medium by proteolysis (3, 14). Several studies have suggested that an OM protein complex (Bam) assists with insertion of the translocator domain into the OM and secretion of the passenger domain into the extracellular medium (10, 19).

Various functions of the AT proteins have been characterized to date. From a medical point of view, this pathway is

important because several of these secreted proteins are virulence factors that lead to severe health issues in both humans and animals (6, 7). They can function as adhesins, toxins, or digestive enzymes in order to facilitate invasion of the host cells during bacterial infection (6). Some AT proteins exhibit supportive functions to mediate intracellular motility, processing, or maturation of other virulence AT proteins (1, 18).

Serine protease ATs of the family *Enterobacteriaceae* (SPATEs) are a representative subfamily of the AT proteins. More than 20 SPATE members have been identified exclusively in pathogenic enteric bacterial species, including those of the genera *Escherichia*, *Shigella*, *Citrobacter*, and *Salmonella* (21). The SPATE proteins are exoproteins that exhibit virulence functions after secretion and folding of their mature passenger domains (6). The primary sequences of these proteins are highly homologous, with identities ranging from 35% to 55% (4, 6). They share a conserved serine proteolytic motif (GDSGS) in their passenger domains and an α -helical linker motif (EVNNLNKRMGD LRD) which characterize their distinctive serine protease and autoprolytic functions (4, 7). The crystal structure of the passenger domain of a SPATE protein, hemoglobin protease (Hbp) from *Escherichia coli* strains causing sepsis, was solved in 2005 (15). Due to the similarities of the sequences and structures among the SPATE members, this particular crystal structure provides useful information to facilitate investigation of the structure-function relationship of the SPATE family.

A SPATE adhesin, temperature-sensitive hemagglutinin (Tsh),

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has been studied extensively since its discovery (11, 12). Tsh was the first member of the SPATE family discovered in 1994 by Provence and Curtiss in an avian-pathogenic strain of *E. coli* (16). Its homolog has also been found in uropathogenic *E. coli* (5). Tsh has been shown to cleave different protein substrates *in vitro*, including casein, mucin, and human coagulation factor V (4, 11). Several conserved motifs within the SPATE sequence were identified using bioinformatics analysis against 20 SPATE members (21). Mutations in the conserved serine proteolytic motif and in the translocator domain of Tsh suggested that conserved residues in those regions of SPATE play critical roles in proteolytic activity and secretion, respectively (11, 12). In this study, we focused on examining the functional roles of the four conserved motifs in the Tsh passenger domain, located at the hypothetical substrate recognition and binding sites near the serine catalytic triad.

MATERIALS AND METHODS

Bioinformatics. Multiple sequence alignment of 20 SPATE proteins was performed using the ClustalW program of BioEdit software, version 7.0.5.2 (21). Molecular graphics models were generated using the coordinates of the published crystal structure of Hbp (15) (Protein Data Bank [PDB] accession number 1WXR) and the Visual Molecular Dynamics (VMD) program, version 1.8.6 (8). Three-dimensional representations of the serine proteases, chymotrypsin (PDB accession number 4CHA), trypsin (PDB accession number 1UTN), and SPATE protein Hbp were manipulated using the MBT Protein Workshop program, version 3.5.

Bacterial strains, plasmids, and growth condition. All bacterial constructs used in this study were transformed and expressed in *E. coli* K-12 XL1-Blue (Stratagene, La Jolla, CA). Plasmid pYA3418, which encodes the wild-type *tsh* gene, was cloned into plasmid pWKS30 (20) at EcoRI and BamHI restriction sites under the control of the *lac* promoter, as described previously (17). The bacteria were grown at 37°C in Luria-Bertani (LB) broth supplemented with ampicillin (100 µg/ml) or on LB agar plates supplemented with ampicillin (100 µg/ml).

Site-directed mutagenesis. Twenty-one mutants from the 21 fully conserved residues in the four conserved motifs of the Tsh passenger domain were constructed as described previously (12). A mutation of the catalytic serine residue at position 259 to an alanine (S259A) served as a negative control for the proteolytic activity. Additional mutants from three nonconserved residues in the Tsh passenger domain were constructed. The primers used for the single-base substitutions are listed in Tables S1 and S2 in the supplemental material. The PCR products were digested and purified as described previously (12). The transformants were screened for ampicillin resistance, before subsequent sequencing at the DNA sequencing laboratory at the City of Hope and Beckman Research Institute (Duarte, CA).

Cell fractionation and sample preparation. The procedures and conditions for culture preparation, protein induction, and cell harvesting were described previously (12). Supernatant fractions were collected by centrifuging the bacteria cultures at $3,214 \times g$ for 15 min at 4°C (model 5804R centrifuge; Eppendorf, Germany). The supernatant fractions were further concentrated 80 times using 100,000-molecular-weight-cutoff (MWCO) Amicon Ultra centrifugal filters (Millipore, Billerica, MA). Protein concentrations were determined using the Bio-Rad protein assay (Hercules, CA). Different cell fractions (OM and periplasm) were analyzed in order to examine the localization of Tsh in the secretion-deficient mutants. OM isolation was performed as described previously using the Sarkosyl solubilization method to remove the IM (12). To extract periplasmic fractions, harvested cell pellets were washed once in 25 mM Tris-HCl (pH 7.5), before they were resuspended in a buffer containing 10 mM Tris-HCl (pH 8.0) and 0.75 mM sucrose. The resuspensions were incubated for 30 min on ice in a solution containing 100 mM EDTA (pH 8.0) and lysozyme (4.0 mg/ml). Unbroken cells and cell debris were pelleted down by centrifugation at $13,000 \times g$ for 20 min at 4°C. The soluble periplasmic fractions were stored at -20°C until use. To verify the Tsh expression of the secretion-deficient mutants, whole-cell lysates were prepared by vortexing and boiling the bacterial cells in loading dye containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol. Acetone precipitation was performed as described previously to precipitate soluble proteins for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (12).

SDS-PAGE and immunoblot analyses. Proteins from different cell fractions were separated by SDS-PAGE and visualized by silver staining or transferred to nitrocellulose membranes (Bio-Rad) for immunoblot analysis. The membranes were then blocked with 5% (wt/vol) nonfat milk powder in Tween-Tris-buffered saline (TTBS; pH 7.5), which contains 0.242% (wt/vol) Tris, 2.924% (wt/vol) NaCl, and 0.1% (vol/vol) Tween 20, followed by incubation with primary rabbit anti-Tsh serum (1:3,000) and secondary goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibody (1:20,000). Blot development was done using a SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). The gel and blot images were captured by a DE-500 MultiImage II light cabinet (Alpha Innotech Corporation, San Leandro, CA) using FluorChem 5500 (version 4.0.1) software (Alpha Innotech Corporation).

Oligopeptide cleavage profile. An oligopeptide cleavage assay was performed as described by Dutta et al. with some modifications (4). Briefly, 20 µg of each concentrated supernatant sample was mixed with 1 mM *para*-nitroaniline (pNA)-conjugated oligopeptide Suc-Ala-Ala-Pro-Abu-pNA (Suc, succinic acid; Abu, aminobutyric acid; Calbiochem, La Jolla, CA) in morpholinepropanesulfonic acid (MOPS) buffer (pH 7.3) in a 96-well microtiter plate. LB broth and S259A with the same concentration were used as negative controls for the proteolytic activity of Tsh. The plate was incubated for 15 h at 37°C. Absorbance readings were taken at 405 nm using a Spectra max 190 instrument (Molecular Devices, Sunnyvale, CA) to detect any color development caused by the hydrolysis of the oligopeptide substrate.

Mucin cleavage profile. A mucin cleavage assay was performed as described by Dutta et al. with some modifications (4). The concentrated supernatant samples were presterilized using 0.22-µm-pore-size filters. Fifteen micrograms of wild-type and mutant concentrated supernatant samples were added into wells in LB medium containing 1.5% (wt/vol) agarose and 1% (wt/vol) mucin from bovine submaxillary glands (Sigma-Aldrich, St. Louis, MO). The medium was incubated for 24 h at 37°C. Proteinase K (5 µg) was used as a positive control for the proteolytic activity of Tsh. The S259A supernatant (15 µg) was used as a negative control. Clearing zones were visualized by incubating the incubated medium in staining solution, followed by soaking in destaining solution for 30 min at room temperature.

ELISA with ECM proteins. An enzyme-linked immunosorbent assay (ELISA) was performed as described previously with some modifications (11). An extracellular matrix (ECM) protein, either collagen IV or fibronectin, was precoated onto 96-well microtiter plates (BD Biosciences, San Jose, CA). Blocking was done in blocking solution overnight at 4°C. The plate was then washed twice with wash buffer. One microgram of each concentrated supernatant sample was added and the plate was incubated at 37°C for 2 h to initiate the binding of Tsh and the extracellular matrix protein. The plate was washed twice again, before it was incubated in a 1:3,000 dilution of primary rabbit anti-Tsh serum, diluted in diluting solution for 1.5 h at 37°C. The plate was washed three times before incubation in 1:20,000 of secondary goat anti-rabbit IgG HRP-conjugated antibody (Bio-Rad, Hercules, CA) for an hour at 37°C. To get rid of the residual antibody solution, the plate was again washed three times before development. The chromogenic substrate tetramethylbenzidine (Pierce, Rockford, IL) was added for development for 30 min at room temperature. The reaction was stopped by adding 50 µl of 1 M H₂SO₄, and the absorbance was measured at 450 nm. The wells incubated with wild type without addition of primary antibody and bovine serum albumin (BSA) were used as negative controls.

Statistical analysis. Dixon's Q test was used to identify and reject the outlier readings in all experiments. The data were analyzed using this test at a 95% confidence (Q_{95%}). In the oligopeptide cleavage assay, the wild-type reading was set equal to 100% to represent the maximum absorbance reading, and the mutant readings were normalized accordingly. In all proteolytic and adhesive analysis experiments except the mucin cleavage assay, the reactions were performed in triplicate, and the results of three independent experiments were averaged. The mucin cleavage assay was repeated five times, and the results of all trials were averaged. All means and standard deviations were calculated. All charts were plotted using Microsoft Office Excel 2003 software.

RESULTS

Multiple sequence alignment reveals five conserved motifs located in the SPATE passenger domain. Previously, multiple sequence alignment was performed on 20 members of the SPATE family in order to identify regions that are potentially critical for the secretion and virulence functions of the SPATEs (21). The alignment results revealed the presence of 10 con-

FIG. 1. Effect of mutations of the 21 conserved residues on secretion. (A) Conserved residues in the SPATE passenger domain conserved motifs. The signal sequence (SS) is represented by a black block, the passenger domain by a light gray block, and the translocator domain by a dark gray block. Residues are numbered based on the primary sequence of Tsh, starting at the first methionine in the N-terminal signal sequence. The letter x denotes a less conserved residue. Motifs are defined as conserved regions consisting of more than 3 consecutive amino acid residues. The conserved motifs were identified by aligning 20 SPATE proteins using the ClustalW program of BioEdit software, version 7.0.5.2. The conserved motifs in the signal sequence and translocator domain are not shown in this figure. (B) Secretion profiles of the 21 mutant constructs in the Tsh passenger domain. S259A is included as a negative control for Tsh proteolytic activity but has a normal secretion pattern. Each concentrated supernatant sample was resolved on 10% SDS-polyacrylamide gels and silver stained or transferred to nitrocellulose membranes for immunoblot analysis. The amount of Tsh secreted by each construct was evaluated on the basis of the level of secretion of the wild type. Four mutant constructs with different levels of secretion defect are indicated by the boxes. Top panel, silver stained gel; bottom panel, immunoblot.

served motifs in the SPATE sequence. We focused on the five conserved motifs located in the passenger domain (Fig. 1A). One of the conserved motifs (G257DSGS) in the Tsh passenger domain contains the active serine of the catalytic triad (H125, D153, and S259) of this serine protease (11, 21). This serine residue was proven to be vital for the Tsh proteolytic function (11), as a serine-to-alanine mutation at this position (S259A, in the case of Tsh) completely abolished the proteolytic activity of Tsh and several other SPATE proteins (2, 11, 13). In addition, the adhesive activity of this mutant was maintained at a lower rate than that of the wild-type protein (11). Not including the serine protease motif, the remaining conserved motifs in the passenger domain of the SPATEs are designated conserved motifs 1, 2, 3, and 4. Conserved motifs 1 (D101FS) and 2 (R158LxKxVxE) are situated in the N-terminal globular serine protease domain of the SPATE (15), whereas conserved motifs 3 (D419xLHKxGxGxL) and 4 (L441KxGxGxVxL) are situated in the β -helical stalk domain (15) (Fig. 1A; see also Fig. 4A). These four conserved motifs, which include a total of 21 conserved residues, were selected for the current mutagenesis study (Fig. 1A).

Secretion analysis of the 21 conserved mutants. Using PCR-based site-directed mutagenesis, 21 mutants were constructed by the use of single-base substitutions in order to introduce drastic changes to these conserved residues (see Table S1 in the supplemental material). In addition, a mutation of the catalytic serine at position 259 to an alanine was prepared to serve as a negative control for the proteolytic activity. Labo-

ratory *E. coli* K-12 XL1-Blue cells were transformed with the mutagenized plasmids. With the aim of ensuring that all mutant constructs secrete the same amount of Tsh protein as the wild type before examination of their effect on the SPATE functions, culture supernatant samples were resolved on a 10% SDS-polyacrylamide gel to inspect their secretion profiles. The SDS-PAGE and immunoblot analyses showed that 17 of the 21 mutants displayed the wild-type level of secretion of the 106-kDa processed form of Tsh into the culture medium (Fig. 1B). In contrast, mutations in four conserved residues (V163, K442, G444, and V448) caused significant defective phenotypes in Tsh secretion, showing multiple degradation bands of the Tsh protein (Fig. 1B). In mutants V163R and G444Y (from conserved motifs 2 and 4, respectively), a reduced amount of the 106-kDa processed Tsh and an extra Tsh band of ~90 kDa were detected. In mutant V448R (from conserved motif 4), the 106-kDa processed form of Tsh was not found in the culture supernatant; instead, the mutant Tsh broke down into smaller fragments in the culture medium that were recognized by anti-Tsh antibody. Another conserved motif 4 mutant, K442A, exhibited a severe secretion defect and did not show any processed or degraded Tsh in the supernatant. In summary, 17 Tsh mutants demonstrated the wild-type level of secretion, while 4 mutants expressed different secretion-defective phenotypes.

Mutations in four conserved residues cause significant secretion-defective phenotypes. All four secretion-defective mutants (V163R, K442A, G444Y, and V448R) showed the presence of the Tsh protein in their cell lysate samples (see Fig. S1A and S1B in the supplemental material), indicating that the Tsh proteins were expressed in all of these mutants. Because a reduced amount of processed Tsh and several Tsh fragments were observed in the culture supernatants of mutants V163R, G444Y, and V448R, the OM fractions of these mutants were evaluated to find out if any unprocessed forms of Tsh were accumulating in the OM. The 140-kDa unprocessed Tsh was not found in the OM of V163R, G444Y, or V448R (see Fig. S1A in the supplemental material), suggesting that the Tsh proteins of these three mutants were completely secreted and that the mutant proteins were degraded after they were released into the culture medium. On the other hand, the secretion by mutant K442A was abolished. Immunoblot analysis of the OM and periplasmic fractions of K442A revealed that this Tsh mutant protein accumulated in the periplasmic region (see Fig. S1B in the supplemental material). In summary, the above four mutant constructs, three of which were among the six mutant constructs from conserved motif 4, expressed different secretion-defective phenotypes.

Eight conserved residues extensively affect the cleavage of Tsh-specific substrates. In order to determine the effect of the mutations of the conserved residues on the Tsh proteolytic function, we examined the proteolytic activity of the mutants using a Tsh-specific oligopeptide assay. By split decomposition analysis, Dutta et al. reported that Tsh is closely related to the serine protease elastase in terms of the similarity in their cleavage profiles (4). An oligopeptide substrate (Suc-Ala-Ala-Pro-Abu), known as elastase substrate IV, is efficiently cleaved by both elastase and Tsh (4). In order to identify which conserved residues are critical for the serine proteolytic function of the SPATEs, the 17 mutants with a normal secretion pattern were tested to assess their cleavage activity toward this pNA-conju-

gated oligopeptide. Interestingly, all 17 mutants exhibited lower cleavage activity than the wild type (Fig. 2A). Among them, eight mutants displayed severely reduced cleavage activity (<27% of the wild-type level). These eight severely proteolysis-defective mutants are positioned in all four conserved motifs in the Tsh passenger domain. D101A (26%) and S103A (25%) are located in conserved motif 1. R158A (1%) and L159Y (16%) are located in conserved motif 2. L429Y (5%) is located in conserved motif 3; and L441Y (23%), G446Y (5%), and L450Y (16%) are located in conserved motif 4. The remaining nine mutants exhibited >27% cleavage activity. Additionally, S259A showed no cleavage activity against this oligopeptide (0%) (Fig. 2A), whereas the *E. coli* strain with the cloning vector alone demonstrated minimal oligopeptide cleavage activity that might have been due to the existence of some nonspecific endogenous proteases (data not shown).

Mucin is one of the known proteolytic substrates of Tsh (4). Another SPATE member, Pic, which is a close homologue of Tsh, has also been shown to possess mucinolytic activity (4). Both wild-type Tsh and wild-type Pic are able to degrade the mucin secreted by bovine submaxillary glands (4). These experimental findings directed us to take a look at this mucous glycoprotein as a potential target for the SPATE proteases in the progression of the host infection. In order to examine the mucinolytic effect of the mutant Tsh proteins with respect to their ability to cleave oligopeptide, concentrated supernatant samples of the eight severely proteolysis-defective mutants were added to the wells in a mucin-agarose medium. After incubation, zones of clearing were visualized using amido black, which stained the mucin blue. All eight mutants displayed smaller clearing zones than wild-type Tsh (Fig. 2B). S259A did not show any clearing zone around the well, which indicates a lack of proteolytic activity against this bovine mucin. In another assay, porcine gastric mucin was utilized in a similar way; however, wild-type Tsh and all the eight proteolysis-defective mutants did not demonstrate any mucinolytic activity against this porcine mucin (data not shown). Taken together, the eight conserved residues (D101, S103, R158, L159, L429, L441, G446, and L450) situated in the four conserved motifs in the passenger domain extensively influenced the proteolytic activity of Tsh against different substrates.

The eight proteolysis-defective mutants demonstrate lower adhesive activity against different ECM proteins. ECM proteins are potential targets for the adherence of the Tsh protein at the time of avian-pathogenic *E. coli* infection. For that reason, several ECM proteins were tested for their abilities to bind to wild-type Tsh due to their proximity to the host cells (11). Two ECM proteins, collagen IV and fibronectin, displayed dose-dependent activity for adhesion to the purified wild-type Tsh; conversely, another ECM protein, laminin, did not bind to Tsh *in vitro* (11). Both collagen IV and fibronectin were selected as the substrates for the binding assay of the current study. In order to evaluate the effect of the reduced proteolytic activity on the adhesive function and find out the relationship between these two distinct activities, we assayed the eight severely proteolysis-defective mutant constructs identified in the oligopeptide cleavage assay so as to examine their activities for adhesion to collagen IV and fibronectin. To perform the ELISA binding assay, 1 µg of concentrated supernatant samples of wild-type Tsh and the eight mutant constructs

FIG. 2. Mutations in the conserved residues of the passenger domain affect the proteolytic activity of Tsh. (A) Tsh-specific oligopeptide cleavage assay results for the 17 Tsh mutant constructs. S259A was included as a negative control for Tsh proteolytic activity. Concentrated supernatant samples of wild-type (wt) and mutant Tsh were incubated for 15 h at 37°C with 1 mM pNA-conjugated Suc-Ala-Ala-Pro-Abu in MOPS buffer (pH 7.3). The substrate cleavage level of each mutant construct is represented in percent (cleavage level of wild-type Tsh, 100%). All reactions were performed in triplicate, and the results of three independent experiments were averaged. Means and standard deviations were calculated. Arrows show the eight severely proteolysis-defective mutant constructs (<27% of the wild-type activity). (B) Mucin cleavage assay results for the eight severely proteolysis-defective Tsh mutant constructs. Fifteen micrograms of concentrated wild-type and mutant Tsh supernatant samples were added into wells in a mucin-agarose medium for incubation. Five micrograms of proteinase K (PK) was used as a positive control for the proteolytic activity. The average diameter of each clearing zone is represented in millimeters.

FIG. 3. The eight severely proteolysis-defective Tsh mutant constructs display a poorer ability to bind to extracellular matrix proteins. The binding activity of the mutant Tsh against the activities of two extracellular matrix proteins, collagen IV (A) and fibronectin (B), was quantified using ELISA. One microgram of concentrated supernatant sample was incubated in triplicate wells in a microtiter plate that was precoated with collagen IV (A) and fibronectin (B). The change in color intensity due to HRP substrate degradation enabled the quantification of the adhesive activity. Wells incubated with BSA or wild-type Tsh without primary antibody (Ab) were used as negative controls. The data were normalized on the basis of the reading of the wild-type Tsh with no primary antibody control. All reactions were performed in triplicate, and the results of three independent assays were averaged. Means and standard deviations were calculated.

was incubated in a microtiter plate that was precoated with either ECM protein. After incubation, the binding abilities of the samples were detected by anti-Tsh antibody. The adhesive activity of Tsh was quantified by the intensity of the color formed due to HRP substrate degradation. A wild-type Tsh sample that had no anti-Tsh antibody added and an unrelated protein (BSA) were used as negative controls. The eight severely proteolysis-defective mutants displayed significant reductions in their adhesive activities, ranging from 42% to 59% in the collagen IV assay and from 40% to 70% in the fibronectin assay with respect to the wild-type activity (Fig. 3A and B). S259A showed a slightly higher level of adhesive activity than the eight mutant constructs. All together, the data indicate that the mutations of the eight conserved residues critical for proteolysis and S259A moderately weakened the activity of Tsh for adhesion to the ECM proteins.

Evaluation of the mutations of the eight conserved residues critical for proteolysis. Since the sequences of Tsh and Hbp are nearly identical (>99% identity), the crystal structure of the passenger domain of Hbp has been employed to create a

three-dimensional model of the SPATE passenger domain (Fig. 4A and B). On the basis of the model, the protease region of the SPATE is restricted to one-third of the N-terminal passenger domain sequence (21). In order to further analyze the potential effect of the mutations at the eight conserved residues that cause severe reductions in the proteolytic and adhesive functions of Tsh, we utilized the VMD program (8) to study the interactions of these residues in the Tsh model. All eight conserved residues are positioned in or close to the protease domain (Fig. 4A and B). Some of these residues exhibit specific intramolecular interactions which stabilize one or more active-site residues located in the serine protease catalytic triad. These molecular interactions can be grouped into two major categories: (i) the side chains of the conserved residues in the globular serine protease domain form intramolecular interactions, such as hydrogen bonds and salt bridges, that connect the protease and β -helical domains, and thus any alterations of these conserved residues should destabilize the active site; and (ii) several conserved residues participate in forming a hydrophobic core in which the packing of their side

FIG. 4. Three-dimensional representation of the SPATE passenger domain. (A) Five conserved motifs in the SPATE passenger domain are displayed in this three-dimensional model constructed using the crystal structure of the Hbp passenger domain as a template (PDB accession number 1WXR). The conserved motifs are labeled with different colors: motif 1 (M1), cyan; motif 2 (M2), red; motif 3 (M3), orange; motif 4 (M4), magenta; and serine protease catalytic triad (CT), green. (B) Enlarged front and back views of the N-terminal globular domain (residues 53 to 308 of the nascent polypeptide) and a portion of the β -helical core, including conserved motifs 3 and 4. The positions of three nonconserved residues (T164, N353, and R493) which interact with the conserved residues are labeled with circles 1, 2, and 3, respectively. The models were manipulated using MBT Protein Workshop, version 3.5.

chains stabilizes the folded protein, and thus, mutations of the nonpolar residues to tyrosines should disturb the β conformations of the protein and, subsequently, the active site of Tsh. Among the eight conserved residues, D101, S103, and R158 belong to the first category, as they are all polar residues with different charges (D101 is negatively charged, S103 is neutral, and R158 is positively charged). On the contrary, L159, L429, L441, G446, and L450 belong to the second category, as they are all neutral and nonpolar residues. A number of key non-conserved residues were identified to be involved in the above interactions: (i) R493 forms a salt bridge with the side chain of D101 and a hydrogen bond with the side chain of S103 (Fig. 5A and B); (ii) N353 (backbone and side chain carbonyl) and G355 (backbone carbonyl) form hydrogen bonds with the side chain of R158 (Fig. 5C and D); and (iii) L162 and T164 are 7 to 17 Å away from L429, L441, G446, and L450 of conserved motifs 3 and 4. T164 in the protease domain may be important in optimizing the interface between the protease domain and the β -helical domain. Figure 5E and F exemplarily shows the

position of T164 in the globular protease domain relative to the positions of L441 and L450 in the β -helical domain.

Mutations in the three nonconserved residues in the passenger domain confirm the significance of intramolecular interactions on Tsh functions. The data from the molecular graphics modeling suggest that some residues in the passenger domain of Tsh may play a role in the stabilization of the active site in the serine protease domain. In order to demonstrate that these intramolecular interactions involving the eight critical conserved residues are important for the functions of SPATEs, we selected a nonconserved residue from each of the interactions to construct three new mutants, T164A, N353A, and R493A. All three nonconserved mutant constructs displayed the same amount of secretion of the 106-kDa processed form of Tsh as the wild type (see Fig. S2 in the supplemental material), indicating that their protein expression and secretion are undisturbed by the mutations. To assess the proteolytic activity of these mutants, an oligopeptide cleavage assay was performed as described previously (Fig. 6). R493A showed

FIG. 5. The conserved residues critical for proteolysis contribute to stabilization of the protein via intramolecular interactions. (A and B) R493 interacting with D101 and S103. (A) An overview of the SPATE is shown in a cartoon representation in green with the protease domain at the bottom left and the stalk-like β -helical domain on top of it. The region in gray (within the red box) is shown in more detail in panel B after horizontal rotation of approximately 90° toward the back to allow a better view. (B) The interactions of R493 located in the β -helical domain with

FIG. 6. Tsh-specific oligopeptide cleavage assay results for the three nonconserved mutant constructs. The assay was carried out as described in the text with mutants T164A, N353A, and R493A. S259A was included as a negative control for Tsh proteolytic activity. All reactions were performed in triplicate, and the results of three independent experiments were averaged. Means and standard deviations were calculated. The conserved mutants related to the three nonconserved mutant constructs are also included. Different molecular interactions are labeled above each group of interactions.

42% $\pm$ 10% of the wild-type activity, which is almost comparable to the activities of D101A (26% $\pm$ 4%) and S103A (25% $\pm$ 2%) within the experimental error. The activity of N353A was 46% of the wild-type activity and was therefore significantly higher than that of R158A (1%). This was expected, because the N353A mutation removes only the hydrogen bond acceptor of the side chain while leaving the backbone carbonyl hydrogen bond acceptor. T164A demonstrated 3-fold greater proteolytic activity (63%) compared to the activities of L441Y (23%) and L450Y (16%). This result suggests that threonine at the interface between the serine protease domain and the β -helical domain is less critical for proteolytic activity

than the proper packing provided by L441 and L450 within the β -helical domain.

DISCUSSION

In the present study, we investigated the functional importance of the conserved motifs in the passenger domain of the SPATEs. The mutations at eight conserved residues (D101, S103, R158, L159, L429, L441, G446, and L450) certainly influence the protease function of the SPATEs, as verified in cleavage assays utilizing the Tsh-specific substrates. The results suggest that changing the side chains and charges of these eight

residues in the protease domain are shown. The side chain of R493 forms a salt bridge with the side chain of D101 and a hydrogen bond with the side chain of S103. These residues are at the bottom of a β sheet that extends into the active site where H125 forms the catalytic triad together with D153 and S259. Amino acids are labeled at C- α . The amino acid side chains are shown as sticks, with carbon being colored gray, oxygen red, and nitrogen blue. (C and D) R158 interacting with N353 and G355. (C) An overview of the SPATE is shown in the same representation and in a very similar position as in panel A. The region in gray (within the red box) is shown in more detail in panel D after vertical rotation of approximately 45° toward the left to allow a better view. (D) The interactions of R158, located in the protease domain just 5 residues from the active-site residue D153, with N353 and G355 located in the β -helical domain are shown. The side chain of R158 forms hydrogen bonds with the side chain and backbone carbonyls of N353 and the backbone carbonyl of G355. Amino acids are labeled and represented as in panel B, except that for N353 and G355, the backbone atoms are also shown. (E and F) L441 and L450 in the β -helical domain in close proximity to T164 in the protease domain. (E) An overview of the SPATE is shown in the same representation and in a very similar position as in panel A. The region in gray (within the red box) is shown in more detail in panel F. (F) The proximity of L441 and L450, located in the β -helical domain, to the protease domain with T164, 11 residues from the active-site residue D153, is shown. The hydrophobic side chains of L441 and L450 point toward the core of the β -helical domain. Amino acids are labeled and represented as in panel B.

critical conserved residues affects the SPATE proteolytic activity, probably through the disruption of the protease tertiary structure. Even though the bovine mucin cleavage assay is nonquantitative, the results of this assay qualitatively showed slightly lower activity in all of the eight mutants than in the wild type.

The reduced abilities of all eight mutants with significantly reduced cleavage activity to bind to both collagen IV and fibronectin suggest that the conserved residues that are important for Tsh proteolytic activity also affect its adhesive function. It is possible that the protease and adhesin substrate recognition regions in Tsh share the same conserved residues and/or are located very close to each other. The eight key conserved residues that we identified may have multiple roles in forming different networks with the residues responsible for maintaining these recognition regions separately.

Using VMD software (8), two major categories of interactions were identified for the eight key conserved residues important for the SPATE functions. In the first category, the side chains of the polar and charged conserved residues (D101, S103, and R158) in the globular serine protease domain form hydrogen bonds and salt bridges to connect to the β -helical domain for stabilization of the serine protease catalytic triad. It seems likely that these interactions with other nonconserved residues in the β -helical domain hold the active and substrate recognition sites in the correct positions. As a result, disruption of any of these interactions should pose serious problems in the proteolytic and adhesive activities of the SPATEs. In the second category, the nonpolar conserved residues (L159, L429, L441, and L450) participate in stabilizing the core of the β -helical domain through hydrophobic packing of their side chains so as to support the protein's overall stability and functions.

The molecular graphics evaluation of the eight proteolysis-critical conserved residues provides us a deeper understanding of the potential intramolecular interactions between the conserved residues and the protease active site, in addition to the spatial specificity of these interactions. In conserved motif 1, mutation of D101 removes a salt bridge to R493, which is a nonconserved residue in the β helix (Fig. 5A and B). Mutation of S103 removes a hydrogen bond between the side chains of S103 and R493, which is likely to destabilize the β sheet, including H125 and D153 of the catalytic triad described in Fig. 5.

Similarly, in conserved motif 2, mutation of R158 may destabilize a β hairpin loop extending from residues 144 to 160, which is not part of the β sheet described in Fig. 5 but which is essential for maintaining the structure of the SPATE active site, including residue D153 of the catalytic triad. Normally, the side chain of R158 in the serine protease domain forms hydrogen bonds with the backbone and side chain carbonyls of N353 and the backbone carbonyl of G355 in the β -helical domain (Fig. 5C and D). These hydrogen bonds may stabilize the hairpin loop from residues 144 to 160.

Mutations L441Y and L450Y in conserved motif 4 likely disturb the hydrophobic core of the β helix. The side chains of L441 and L450 point toward the hydrophobic core and pack with the side chains of V448, F463, V466, V475, L477, and V483 of the β helix. L441 and L450 are located about 7 Å and 17 Å, respectively, from nonconserved residue T164 in the protease domain at the base of the β hairpin loop from resi-

dues 144 to 160. Possibly, disturbance of the β helix right next to T164 affects the β hairpin loop from residues 144 to 160, including D153 of the catalytic triad, and thus enzyme activity (Fig. 5E and 5F).

The evaluation of the three nonconserved residues (T164, N353, and R493) which interact with certain key conserved residues provides some explanation for the differences in the reduction of the proteolytic activity. The alterations at the two nonconserved residues (N353 and R493) led to similar proteolytic defects compared to the results for the corresponding conserved residues (R158, D101, and S103). The elimination of a hydrogen bond or a salt bridge in mutants N353A and R493A probably has a drastic effect on the Tsh proteolytic function. On the other hand, the T164A mutation resulted in a decrease of protease activity by only about one-third compared to that of the wild type, which suggests that an alanine at this position can also support the stability and enzyme activity of the protein. It would be interesting to see the result of mutating T164 to a larger and/or charged residue, such as tyrosine, arginine, or glutamate.

The mutations in four conserved residues (V163, K442, G444, and V448) caused significant defective phenotypes in the secretion of Tsh. The alterations at V163, G444, and V448 possibly cause the mutant proteins to be highly unstable; thus, they cannot fold properly and create multiple sites which enable easier access for the extracellular proteases or the protease domain of Tsh. Furthermore, the same degradation pattern observed for V163R and G444Y may be attributed to the close proximity (about 8 Å apart) of these two conserved residues (21) in the SPATE three-dimensional model (Fig. 4A and B). In contrast, the periplasmic accumulated mutant K442A protein may be structurally unstable, and the mutant protein was degraded into small Tsh fragments that were not detectable on the SDS-polyacrylamide gel. Conversely, it may exhibit abnormalities in particular steps of the OM translocation process. K442 is part of the β -helical stalk-like domain at the interface between the β -helical and protease domains, and its side chain points out of the helix toward the protease domain. This residue can interact through hydrogen bonds and/or a salt bridge with a tyrosine, a threonine, and a glutamate in the protease domain; mutation at this residue probably disrupts these stabilizing interactions. In a recent study on the SPATE structure-function relationship, an in-frame pentapeptide linker insertion at the homologous lysine residue in EspP (K429) also displayed a secretion-incompetent phenotype (2). Further investigations need to be done to elucidate the responsibility of this residue in the secretion process of the SPATEs.

Since the eight key conserved residues are located in close proximity to the protease domain, alterations at these positions can disrupt the complex interactive network, disturb the integrity of the serine protease active site, and affect enzyme activity. Our data suggest that the network of intramolecular interactions between the protease and β -helical structural domains of the SPATEs is essential for the wild-type protease function and potentially the adhesion function.

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