The N Terminus of the HasA Protein and the SecB Chaperone Cooperate in the Efficient Targeting and Secretion of HasA via the ATP-binding Cassette Transporter*

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SECRETION OF THE HasA HEMOPHORE IS MEDIATED BY A C-terminal secretion signal as part of an ATP-binding cassette (ABC) pathway in the Gram-negative bacterium Serratia marcescens. We reconstituted the HasA secretion pathway in Escherichia coli. In E. coli, this pathway required three specific secretion functions and SecB, the general chaperone of the Sec pathway that recognizes HasA. The secretion of the isolated C-terminal secretion signal was not SecB-dependent. We have previously shown that intracellular folded HasA can no longer be secreted, and we proposed a step in the secretion process before the recognition of the secretion signal. Here we show that the secretion of a functional HasA variant, lacking the first 10 N-terminal amino acids, was less efficient than that of HasA and was SecB-independent. The N terminus of HasA was required, along with SecB, for the efficient secretion of the whole protein. We have also previously shown that HasA inhibits the secretion of metalloproteases from Erwinia chrysanthemi by their specific ABC transporter. Here we show that this abortive interaction between HasA and the E. chrysanthemi metalloprotease ABC transporter required both SecB and the N terminus of HasA. N-terminal fragments of HasA displayed this abortive interaction in vitro and also interacted specifically in vitro with the ABC protein of the Prt system. SecB also interacted specifically in vitro with the ABC protein of the Prt system. Finally, the HasA variant, lacking the first 10 N-terminal amino acids did not display this abortive interaction with the Prt system. We suggest that the N-terminal domain of HasA specifically recognizes the ABC protein in a SecB-dependent fashion, facilitating functional interaction with the C-terminal secretion signal leading to efficient secretion.

In Gram-negative bacteria, proteins that cross the cell envelope are secreted into the extracellular medium via at least four different specific pathways. These pathways can be classified into two groups, according to their dependence upon the Sec pathway. Proteins that follow the Sec pathway have a signal peptide and are secreted in two steps. They are translocated to the periplasm via the Sec machinery; they then cross the outer membrane and are transported to the extracellular medium by a complex multicomponent system. Proteins using the other pathways do not have a signal peptide and are exported from the cytoplasm to the extracellular medium in one step, with no periplasmic intermediate (1).

One of these Sec-independent pathways is the ATP-binding cassette (ABC) secretion pathway (2) also referred to as the type I pathway. This pathway is used for the specific secretion of proteins of many different families, including hydrolytic enzymes, virulence factors, and hemophores. Each of these proteins is secreted via a specific apparatus, usually encoded by a series of genes organized into a single operon.

The ABC secretion apparatus forms a complex linking the inner and outer membranes and consists of three proteins: 1) an inner membrane ABC protein that hydrolyzes ATP, 2) a second inner membrane protein, the membrane fusion protein (MFP), which has a large periplasmic domain and, 3) an outer membrane protein (OMP).

Proteins secreted by the ABC pathway do not have an N-terminal signal peptide (3). Instead, they usually have an uncleaved C-terminal secretion signal located within the last 50 C-terminal amino acids responsible for directing the secretion of the protein (4). This C-terminal secretion signal is a key component of the ABC secretion pathway. It specifically recognizes the ABC protein, and this interaction is responsible for substrate specificity (5, 6). Moreover, this interaction triggers assembly of the secretion apparatus proteins into a functional complex (7, 8).

The structure of the ABC secretion system imposes specific constraints. Escherichia coli TolC, a component of several protein ABC exporters, was recently studied by crystallography and shown to form a channel across the periplasm (9). This channel has an inner diameter of 30 Angstroms. Other structural data for several protein substrates that use the TolC channel for secretion have shown that in their native form these proteins are too large to pass through this channel (10, 11).

A second constraint concerns the interaction between the C-terminal secretion signal and the ABC protein. The C-terminal position of the secretion signal implies that the exoprotein must be completely synthesized before the secretion signal can interact with the secretion apparatus. In this case, other pathways, such as complete folding, aggregation, and degradation, may compete with the post-translational secretion pathway.

Mechanisms for the regulation of protein folding have been investigated in other pathways of translocation across membranes. Some pathways, such as those responsible for translocation across the endoplasmic reticulum (12), mitochondrial import (13), and export across the bacterial cytoplasmic mem-

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1 The abbreviations used are: ABC, ATP-binding cassette; MBP, maltose-binding protein; PRT, protease secretion apparatus.
brane via the Sec pathway (14), require chaperone proteins such as Hsp70, Hsp40, or SecB. Chaperone proteins are defined by their ability to bind transiently to non-native structures in polypeptide chains. These transient interactions prevent "illegitimate" protein-protein interactions, thereby controlling the folding of proteins. For example, SecB is required for the translocation of some precursor proteins via the Sec system (15–17). SecB has two different functions in this pathway: an antifolding activity, responsible for keeping precursors in an export competent state, and a targeting activity, responsible for enhancing the interaction between the precursor and the translocon (14). Both signal peptide and SecB bind SecA, the ATPase of the translocation machinery, in a cooperative manner (14, 18).

In our laboratory, we have studied two ABC apparatuses reconstituted in E. coli as models: the Prt secretion system of Erwinia chrysanthemi (19), and the Has secretion system of Serratia marcescens (20). The Prt secretion system is involved in the secretion of E. chrysanthemi metalloproteases. The Has (heme acquisition system) secretion system is responsible for secretion of the HasA hemophore. HasA is a 188-amino acid protein that binds heme in the extracellular medium. This heme-binding protein is secreted by S. marcescens under conditions of iron limitation. It acquires heme directly or from hemoglobin and delivers it to the bacterium via a specific TonB-dependent outer membrane receptor, HasR (21).

The protease secretion (Prt) apparatus consists of the proteins PrtD (ABC protein), PrtE, and PrtF (22). The Has apparatus consists of HasD (ABC protein), HasE, and HasF, or TolC (23). TolC is the E. coli HasF homolog and complements perfectly the function of HasF in HasA secretion (24).

Previous studies on these ABC secretion systems have shown that the SecB chaperone is required for secretion of the S. marcescens hemophore, HasA, via its specific (Has) ABC transporter while the secretion of E. chrysanthemi metalloproteases via their specific ABC transporter does not require SecB. A direct interaction between SecB and HasA is responsible for this effect, and a SecB analog is also required in the natural host, S. marcescens (25). Recent experiments have shown that HasA is no longer secretion competent following accumulation in its native form in the cytoplasm (26). Thus, complete folding is not consistent with secretion via the ABC pathway. This raises the question of the conformational state of HasA during the early events of secretion by the ABC pathway. These data also suggest that a co-translational event may initiate secretion and prevent complete folding (26).

Previous studies have also shown that HasA is not secreted by the heterologous E. chrysanthemi protease secretion apparatus (Prt). However, HasA inhibits the secretion of E. chrysanthemi proteases by their own Prt transporter (23). This inhibition is due to an abortive interaction between HasA and PrtD, the E. chrysanthemi protease-specific ABC protein (5). In vitro assays have also shown a specific physical interaction between HasA and PrtD, the Prt ABC protein (7).

All these data suggest that the early interaction occurring between HasA and the ABC protein is a crucial step for the efficient secretion by the ABC transporter. We further investigated this early step by studying the determinants of the abortive interaction between HasA and PrtD as well as the HasA and SecB relationship.

In this study, we obtained evidence that the early interaction with the ABC protein involves SecB and the N-terminal domain of HasA. We also showed that the N-terminal region of HasA has an effect on its own secretion. This led us to propose a model in which SecB and the N-terminal region of HasA cooperate, targeting HasA to the ABC protein.

### Table I

| Name           | Characteristics | Oligonucleotides | Reference |
|----------------|-----------------|------------------|-----------|
| pBG518         | vector          |                  | (25)      |
| pAM238         | vector          |                  | J.P. Bouchez |
| pBAD24         | vector          |                  | (26)      |
| pRUW6          | PrtDEPBC        |                  | (27)      |
| pSYC150        | HasDE           |                  | (28)      |
| pSYC134pBG5    | HasA            |                  | (29)      |
| pHasA1–20pBG5  | HasA(11–188)    | 1/3M             | This study |
| pHasA1–10pBG5  | HasA(21–188)    | 2/3M             | This study |
| pHasA1–120pBG5 | HasA(121–188)   | 3/3M             | This study |
| pHasA1–140pBG5 | HasA(1–140)     | 4/5              | This study |
| pSYC134pAM238  | HasA            |                  | (30)      |
| pHasA11–10pAM  | HasA(11–188)    |                  | This study |
| pSYC1000       | HasA P. aeruginosa |              | (31)      |
| pFXHASA        | HasA P. fluorescens |              | (32)      |
| pHasAEpBAD     | HasA Y. pestis  |                  | (33)      |
| pHisPrtDpBAD   | His6-PrtD       | 6/7              | This study |

### EXPERIMENTAL PROCEDURES

**Strains and Growth Conditions**—E. coli MC4100 (araD139 ΔlacU169 rpsL150 relA1 rfbB501 deoC1 ptsF25 rbsB) was from our laboratory collection. MC4100secB5 with no functional SecB was kindly provided by Carol Kumamoto. Cells were grown at 30 °C in LB medium with appropriate antibiotics or at 30 °C in LB minimal medium with 0.4% glycerol as the carbon source. PAP105 (lac-pro [F traD36 pro AB lacIQ lacZAM151 to10]) was used for cloning. E. coli JP313 (MC4100Δara), used to induce the synthesis of genes under the control of ara promoters, was obtained from J. Pogliano.

**Plasmids**—All plasmids used in this study are shown in Table I, together with the proteins they encode and, for those constructed in this work, the oligonucleotides used (Table II). pRUW6 is a pACYC184 derivative encoding a functional Prt transporter, together with the two proteases, PrtB and C. pSYC150 is a pACYC184 derivative encoding the two specific Has transporter proteins, which, together with the chromosome-encoded TolC, make up the functional Has transporter. hasA11–188, hasA21–188, and hasA121–188 were constructed in the following manner: a fragment was amplified by PCR from pSYC134/ pUC (23), using a universal primer at the 3’-end and a specific primer at the 5’-end (Table II and Refs. 1 and 2); the PCR product was purified, digested with EcoRI and HindIII and ligated to pBG518 + digested with the same enzymes, yielding various plasmids encoding chimeric proteins consisting of a few amino acids encoded by the polylinker, followed by HasA sequences. The clones obtained were then sequenced to confirm their identity. The same inserts were then cloned into pAM238. The N-terminal fragment of HasA was also constructed by PCR amplification of the appropriate fragment from pSYC134/pUC, using two primers. The PCR product was digested with EcoRI and HindIII and ligated into pBG518 + digested with the same enzymes. The EcoRI site was then filled in with the Klenow fragment of DNA polymerase I and the insert sequenced. pHisPrtD was constructed in the following manner: two oligonucleotides were used to amplify a PCR product from pRUW4, a plasmid encoding PrtD (19). The purified PCR product was digested with EcoRI and HindIII and inserted into pBAD24 digested with EcoRI and HindIII; this intermediated plasmid encoding the 5’-end of prtD together with six extra His codons after the initiating Met was sequenced. A Bsl-I/HindIII fragment from pPrtBpBG5 (6) encompassing the main part of PrtD was inserted into this intermediated plasmid digested with the same enzymes, yielding pHisPrtD, under the control of the ara promoter. A SalI-HindIII fragment from pRUW4 was inserted into pHisPrtD digested with SalI and HindIII to yield pHisPrtD-DEF. This plasmid was used to test the functionality of the tagged version of PrtD, which was found to be fully functional. Isolation of plasmids, transformation of E. coli, and all DNA manipulations were done as described (29).

**Analysis of Cell Fractions**—In most cases, MC4100 and MC4100secB5 harboring recombinant plasmids were grown to late exponential growth phase at 30 °C in LB medium supplemented with the appropriate antibiotics. The culture was centrifuged at 10,000 × g for 10 min, proteins were precipitated from the supernatant by incubation with 20% trichloroacetic acid for 1 h at 4 °C, and the precipitated proteins were harvested by centrifugation, washed in 80% acetone, resuspended in sample buffer, and subjected to electrophoresis (30).

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2 C. Kumamoto, personal communication.
Cell pellets were washed once in 100 mM Tris, pH 8.0, 1 mM EDTA and directly resuspended in sample buffer. Immunodetection was carried out as previously described (23). French Press treatment at 10,000 psi was used to break open the cells resuspended in 100 mM Tris, pH 8.0, 1 mM EDTA followed by centrifugation (1 h, 50,000 × g max) to separate soluble and insoluble fractions.

**Affinity Chromatography**—The N-terminal HasA fragment (HasA-(1–140)) was prepared as follows: MC4100pHasAl1–140) cells were cultured in a volume of 1 liter at 37 °C to an A600 of 1. The N-terminal HasA fragment was found to partition between soluble and inclusion body fractions. We used inclusion bodies as the starting material because of the ease of purification from these structures. The cells were harvested, washed once with 100 mM Tris–HCl, pH 8.0, 1 mM EDTA and passed through a French press. The insoluble fraction was collected by centrifugation and treated overnight with 10% Triton, 1 mM EDTA at 37 °C. The insoluble fraction, consisting mostly of inclusion bodies, was then collected by centrifugation, treated with a minimal volume of 8 M urea for 1 h at 4 °C, and centrifuged, and the soluble fraction was collected. This fraction was diluted 1:100 in 20 mM Tris, pH 7.5, 5 mM MgCl₂, 100 mM NaCl, 20% glycerol, supplemented with protease inhibitor mixture and 0.03% n-dodecyl-β-d-maltoside, to lower the urea concentration to 80 mM. After this renaturation step, the insoluble material was eliminated by centrifugation (1 h, 25,000 × g max), and the soluble fraction was retained for further binding experiments. The C-terminal fragment of HasA containing the secretion signal was purified from cultures supplemented from MC4100pSYC150+pHasA121–188) as previously described (31).

HisPrtD was purified from membranes prepared from JP313(pHisPrtD) grown at 30 °C in LB medium to an A600 of 0.5 and induced by incubation with 0.1% arabinose for 2 h. Crude membrane preparations were obtained by passing this strain or a control strain not expressing HisPrtD through a French press and then spun down for 1 h at 50,000 × g max. These membrane preparations were solubilized for 1 h at 4 °C in 20 mM Tris, pH 7.5, 5 mM MgCl₂, 100 mM NaCl, 20% glycerol, supplemented with protease inhibitor mixture with 0.7% n-dodecyl-β-d-maltoside and subjected to centrifugation. The solubilized proteins were allowed to bind to Ni²⁺-NTA fast-flow Sepharose for 1 h, and the Sepharose was then washed three times in 20 mM Tris, pH 7.5, 5 mM MgCl₂, 100 mM NaCl, 20% glycerol, supplemented with protease inhibitor mixture and 0.03% n-dodecyl-β-d-maltoside. Soluble HasA variants or native purified SecB (gift from J.-M. Betton) were added to the beads and incubated at 4 °C for 1 h in the same buffer. The Sepharose beads were then washed once in 20 mM Tris, pH 7.5, 5 mM MgCl₂, 100 mM NaCl, 20% glycerol, supplemented with protease inhibitor mixture and 0.03% n-dodecyl-β-d-maltoside, and then again in the same buffer but with a 50% glycerol cushion. The bound fraction was eluted with 0.5 M imidazole, 0.03% laurylmaltoside, 100 mM Tris–HCl pH 7.5. The eluted fraction was then separated from the beads, solubilized in 2% SDS sample buffer and run on a 15% polyacrylamide gel, after which the proteins were transferred to a nitrocellulose membrane. Immunodetection was performed with anti-HasA and anti-SecB antibodies.

**Pulse Chase Assay**—Cells were grown at 30 °C in M9 minimal medium supplemented with glycerol as a carbon source until an A600 of 1 was reached. 35S-S-radiolabeled methionine was then added (3mCi/ml), and the cells were incubated for 1 min. Synthesis was stopped by adding kanamycin (0.1 μg/ml final concentration) and unlabeled methionine (0.2 μCi/ml final concentration). This corresponded to time 0 in the chase; an aliquot of the culture was directly mixed with 20% trichloroacetic acid to precipitate proteins, and another aliquot was centrifuged and separated into supernatant and whole cells. The same cells were collected 3 min later. All samples were subjected to an immunoprecipitation assay using polyclonal anti-HasA antibodies (diluted 1:500). Immunoprecipitated fractions were then run on a 15% polyacrylamide gel and analyzed by autoradiography, using the phosphorimager technique to quantify the data.

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### RESULTS

The N-terminal End of HasA Is Involved in the Efficiency of HasA Secretion and Its SecB Dependence—We have recently shown that folded HasA is no longer secretion competent and we have proposed that a step before the recognition of the C-terminal secretion signal was required for HasA secretion (26). We have also previously shown that whereas HasA secretion is SecB-dependent, secretion of the C-terminal secretion signal of HasA is SecB-independent (25). We could also show that SecB requirement is not limited to S. marcescens HasA but also holds true for all three known homologues, namely from *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Yersinia pestis* (Fig. 1, upper right), widening the role of SecB in HasA secretion. We thus investigated the potential role of the N-terminal region of HasA in secretion. A wild-type *E. coli* strain expressing the Has secretion function was used for pulse-chase assay. We compared the efficiency of secretion for HasA and a HasA variant lacking the first 10 N-terminal acids. Secretion efficiency was 50% lower for the HasA variant than for the entire HasA in the wild-type background (Fig. 1). Thus, the presence of the N-terminal end of HasA is required for a full HasA secretion efficiency. We assessed the binding in vivo to SecB of entire HasA and the HasA variant, using the interference with the pre-MBP processing assay developed by Bassford and co-workers (32). We found that the HasA variant bound SecB as efficiently as did entire HasA (data not shown). This suggests that the lower secretion efficiency of the HasA variant lacking the N-terminal end is not due to lower affinity for the SecB chaperone. Our results therefore suggest that the N-terminal end of HasA may itself have a positive effect on HasA secretion efficiency. This HasA variant devoid of the first 10 N-terminal amino acids also displayed levels of biological activity similar to those of the wild-type HasA. This indicates that the overall structure of this HasA variant is similar to that of HasA.

We carried out the same pulse-chase experiment with a wild-type *E. coli* strain and the isogenic secB− strain. We found that secretion efficiency was identical in the wild-type and secB− backgrounds for the HasA variant lacking the N-terminal region (Fig. 1). This suggests that SecB is not required for secretion of the HasA variant lacking the first 10 N-terminal amino acids.

The N Terminus of HasA Is Required for the Inhibition by HasA of *E. chrysanthemi* Protease Secretion—We had previously shown that intracellular HasA interacts in an abortive manner with the *E. chrysanthemi* metalloprotease transporter so as to inhibit further protease secretion and that this inhibition was at the level of the ABC protein, PrtD (5). We reasoned that this abortive interaction might be related to the initial step of secretion we proposed for HasA. We investigated further the possible role of various HasA domains in the interaction between HasA and the ABC transporter by measuring the secretion of *E. chrysanthemi* proteases in the presence of HasA fragments.
Plasmids encoding *E. chrysanthemi* proteases, the Prt transporter and the HasA variant lacking the first 10 N-terminal amino acids were coexpressed in a wild-type *E. coli* strain. Unlike the full-length HasA, this HasA variant did not inhibit protease secretion (Fig. 2). Immunodetection with whole-cell extracts showed that the amounts of entire HasA and of the HasA variant lacking 10 N-terminal amino acids in cells were similar. Furthermore both full-length HasA and the HasA variant lacking 10 N-terminal amino acids are equally soluble in the cell (Fig. 2, right). This implies that the differential aggregation properties of HasA and its variant cannot be responsible for the differences with respect to protease secretion. Thus, neither a lower concentration of the variant in cells nor a differential aggregation property is responsible for the observed protease secretion. Similar results were obtained with a variant lacking 20 amino acids at the N terminus (data not shown). Thus, the N-terminal end of HasA is required for the inhibition of *E. chrysanthemi* protease secretion strengthening its role in HasA secretion.

The N-terminal End of HasA Is Sufficient for the Inhibition of Protease Secretion—We investigated whether the N-terminal domain of HasA, lacking the secretion signal, was sufficient to inhibit protease secretion. A plasmid encoding a variant consisting of the 140 N-terminal amino acids of HasA was coexpressed with a plasmid encoding the proteases and their Prt transporter in a wild-type *E. coli* strain. Protease secretion was clearly inhibited by the 140-amino acid HasA N-terminal fragment, but to a lesser extent than by entire HasA (Fig. 3). The absolute concentrations of HasA and its variants under inhibitory conditions are not known; neither are the respective concentrations of the protease secretion functions and of the proteases. However, immunodetection with whole-cell extracts indicated that smaller amounts of this HasA N-terminal fragment than of entire HasA were present in cells (Fig. 3). The lower intracellular concentration of the 140-amino acid HasA N-terminal fragment may account for the observed residual secretion of proteases, as well as its lower solubility in the cell.

These results suggest that the N-terminal region of HasA may be responsible for the abortive interaction with the Prt transporter. They also show that the C-terminal region of HasA, containing the secretion signal, is not necessary for this abortive interaction. This suggests that the C-terminal HasA secretion signal is not the only region that interacts with the cognate ABC transporter.

SecB Is Required for the Inhibition by HasA of *E. chrysanthemi* Protease Secretion—SecB is required for HasA secretion but not for the secretion of *E. chrysanthemi* proteases and not for the HasA variant deleted of the first 10 amino acids. On the other hand HasA and not the HasA variant deleted from the first 10 amino acids interacts abortively with the Prt apparatus. We thus investigated whether SecB was required for the abortive interaction of HasA with the Prt secretion apparatus.

Genes encoding the *E. chrysanthemi* proteases, HasA and the Prt secretion functions were coexpressed in a wild-type strain and in the isogenic secB− strain. As previously shown, protease secretion was greatly reduced in the presence of HasA in the wild-type strain (see Fig. 2). In contrast, in secB− strains, similar amounts of proteases were secreted in the presence and absence of HasA, and these amounts were similar to those secreted from the wild type in the absence of HasA (Fig. 4). Therefore, HasA does not inhibit protease secretion in the absence of SecB. Immunodetection experiments with whole-cell extracts showed that the secB− strain and the wild-type strain steady-state intracellular PrtB and C levels are higher than in the wild-type background but are not affected by HasA overproduction (Fig. 4, right). These results are consistent with the observed effects on secretion. Thus, SecB is required for the abortive interaction of HasA with the *E. chrysanthemi* Prt transporter.
Our data suggest that SecB and the N-terminal end of HasA cooperate in the abortive interaction of HasA with the ABC transporter of the proteases. We therefore investigated whether the N-terminal domain of HasA was able to interact in vitro with the ABC protein.

**PrtD Interacts with the N-terminal Region of HasA and SecB**—We investigated the potential interaction between a 140-amino acid N-terminal domain of HasA and PrtD, the ABC protein of the Prt transporter. We used an *in vitro* coprecipitation assay with a functional variant of PrtD, PrtDHis6, corresponding to the Prt ABC protein with a hexahistidine tag. Solubilized PrtDHis6 was bound to nickel agarose beads and tested for binding to various HasA fragments.

The eluted fractions were analyzed by SDS-PAGE and immunodetection. We found that a 140-amino acid N-terminal domain of HasA, corresponding to the Prt ABC protein with a hexahistidine tag, bound PrtDHis6 specifically. In contrast, in this assay, a 68-amino acid C-terminal fragment of HasA, containing the HasA secretion signal, did not bind PrtDHis6 when used at a similar concentration (Fig. 5). These results suggest that there is a physical interaction between PrtD and a region located within the 140-amino acid N-terminal domain of HasA.

We carried out the same *in vitro* coprecipitation assay with purified SecB. We found that the chaperone protein SecB also bound the solubilized PrtDHis6 protein (Fig. 5). Thus, there is a direct physical interaction between SecB and the ABC protein PrtD. Our results suggest that the N-terminal region of HasA and the heterologous ABC protein PrtD interact *in vivo* and *in vitro*. Similar experiments were attempted with a hexahistidine-tagged version of HasD; unfortunately this variant is expressed at very low levels and cannot be easily purified by affinity chromatography, hindering further characterization of its interaction with potential substrates.

**DISCUSSION**

In the ABC secretion system, substrate specificity depends on interactions between the C-terminal secretion signal and the ABC protein (5, 6, 8). However, both the Prt and Has systems can interact with HasA and proteases, either productively (Has system with HasA and proteases, and Prt system with proteases) or non-productively (Prt system with HasA) (5). We used the properties of HasA in the two systems to study in more detail the functional interaction of the substrate with the ABC protein. Furthermore we have proposed that in the HasA case an initial step should occur in the secretion process (26). This led us to study the role of the N-terminal part of HasA in the secretion of HasA by its own transporter. We found that the N-terminal region of HasA is involved in secretion efficiency and renders secretion SecB-dependent. We also identified several properties of the inhibition of protease secretion by HasA overproduced in cells. (i) Similar to HasA secretion through its own transporter, this inhibition is dependent on SecB, whereas protease secretion by the Prt system is SecB-independent. (ii) In contrast to HasA secretion via its own transporter, which depends on the C-terminal secretion signal, the C-terminal part of HasA is not required for this inhibition. (iii) In the Has system with HasA and proteases, we achieved a stable soluble HasA and the heterologous ABC protein PrtD interact *in vitro*. Furthermore we have proposed that in the HasA case an initial step should occur in the secretion process (26).

These results suggest that the SecB chaperone and the N-terminal part of HasA play specific roles in the abortive recognition of the Prt ABC transporter and in the recognition of the Has transporter. This interpretation was supported by *in vitro* experiments in which a functional version of PrtD (the ABC protein of the Prt system) tagged with six histidine residues at
the N terminus, specifically recognized HasA, its N-terminal fragment and the SecB chaperone. Although the C-terminal secretion signal is strictly required for HasA secretion and can be secreted as an autonomous secretion signal, it did not seem to be involved in the inhibition of protease secretion. The C-terminal secretion signal of HasA did not interact with PrtD in our in vitro assay and, unlike the C-terminal signal of protease G, did not inhibit the ATPase activity of the purified PrtD protein, or inhibited it only very slightly, at non-physiological concentrations (6).

The dependence of protease secretion inhibition by HasA on both the SecB chaperone and the N-terminal part of HasA and the inability of the HasA-(11–188) variant to inhibit Prt secretion, despite binding to SecB as shown by pre-MBP processing interference, indicate that the N-terminal part of HasA plays a very specific role in the abortive interaction with the Prt transporter.

We demonstrated an interaction in vitro between SecB and PrtD, suggestive of a specific interaction. The in vivo abortive interaction of HasA with the Prt transporter requires both SecB and the N-terminal end of HasA, and both SecB and HasA interact in vitro with PrtD, the ABC protein of the Prt system. It is thus possible that this abortive interaction involves a ternary complex containing SecB, the N-terminal region of HasA and PrtD. In view of the SecB independence of the protease secretion, the interaction of SecB with PrtD is puzzling. It cannot be excluded that overproduction of the secretion functions in the reconstituted system could mask SecB dependence for the Prt case. Further studies will be required to assess the specificity of the SecB-ABC protein interaction.

The N-terminal end of HasA also seems to have a specific function in the secretion of HasA via its own transporter because secretion of the HasA-(11–188) variant is less efficient than that of HasA, even though the biological function of the variant is unaffected. Furthermore, under our conditions, secretion of the variant was not SecB-dependent. This strongly suggests that there is also an interaction between the N-terminal region of HasA, SecB, and the ABC protein of the Has system itself. This interaction may account for the greater secretion efficiency. Unfortunately this interaction could not be seen in the in vitro system, because HasD, the ABC protein of the Has system is much less stable than PrtD.

Thus, the C-terminal secretion signal is not the only region involved in secretion. Another region, in the N-terminal part of the protein, together with SecB, interacts with the ABC protein...

FIG. 4. Inhibition of the secretion of proteases B and C by HasA in MC4100 and MC4100secB5 strains producing a functional E. chrysanthemi Prt transporter. Left, supernatants of MC4100 (lanes 1 and 2) and MC4100secB5 (lanes 3 and 4) strains grown overnight at 37 °C in LB medium harboring pRUW6 and pBGS18+ (lanes 2 and 4) or pSYC134/pBGS (lanes 1 and 3) were analyzed by SDS-PAGE. In each case, 2 A600 equivalents were loaded in each lane. Central and right parts, immunodetection of the corresponding whole cells with anti-HasA antibodies or anti PrtB, C antibodies. 0.2 A600 nm were loaded in this case.

FIG. 5. Coprecipitation of PrtD, the ABC protein of the Prt transporter with an N-terminal fragment of HasA. Immunodetection of HasA variants after affinity purification of HisPrtD from solubilized membrane preparations from strain MC4100(pHisPrtD/pBGS) producing HisPrtD, the ABC protein of the Prt transporter (lanes 3, 4, 7, and 8), or from strain MC4100(pBGS) with a plasmid control (lanes 1, 2, 5, and 6) mixed with solubilized preparations containing either the 140-amino acid N-terminal fragment of HasA or a small C-terminal fragment. Immunodetection of SecB after affinity purification of HisPrtD from solubilized membrane preparations from strain MC4100(pHisPrtD/pBGS) producing HisPrtD (lanes 10 and 12) or from strain MC4100(pBGS) with a plasmid control (lanes 9 and 11) mixed with native purified SecB protein. Lane 13 shows the Coomassie Blue-stained gel of the purified His-PrtD protein.
and may function as a targeting element to the secretion apparatus.

SecB has two functions in the Sec system: maintaining the precursor in an unfolded conformation and targeting the precursor to SecA, the ATPase of the Sec system, which also recognizes the N-terminal signal sequence (14, 33, 34). In the Has system, SecB may also have two roles, targeting and antifolding, but the balance between antifolding and targeting activities may be different from that in the Sec system. The behavior of HasA-(11–188) resembles to some extent the SecB-independent translocation of some MBP mutants (15, 16). These MBP variants fold more slowly and bind SecB more tightly. There is, however, an important difference between these mutants and the HasA variant: the HasA variant is secreted less efficiently than the wild type whereas the MBP variants are secreted with similar efficiency to the wild type. This provides further evidence for the specific role of the N-terminal end of HasA and indicates a possible role for this part of the molecule in early targeting to the ABC protein.

We have recently shown that HasA is able to fold to its native state in the cytoplasm independently of SecB. We have also shown that folded intracellular HasA, although no longer secretion-competent even if SecB is overproduced, interacts in an abortive manner with its own transporter and inhibits the secretion of newly synthesized HasA. HasA secretion is very efficient, the time window during which HasA is competent for secretion is very short, strongly suggesting that HasA must interact early with the ABC transporter (26). The results obtained here, identifying the N-terminal part of HasA, in addition to the C-terminal secretion signal, as a key element in the secretion process, provide strong support for this interpretation and a possible underlying molecular basis. Furthermore the SecB dependence of the HasA analogs reinforces SecB function in this secretion pathway.

Our data are consistent with a model in which the N-terminal end of HasA is involved in cotranslational targeting to the ABC protein and renders HasA secretion SecB-dependent, possibly by initiating folding events incompatible with secretion via the C-terminal secretion signal. As the HasA-(11–188) variant is able to achieve a fully functional conformation, a clear interpretation of secretion independence toward SecB may be due to modifications in folding kinetics resulting in a lower probability to reach off pathway such as complete folding. However, this provides evidence for the cooperative action of the N-terminal end of HasA with SecB.

Our study also addressed the question of the number of binding sites on the ABC protein. It is possible that the N-terminal part of HasA recognizes the secretion signal binding site of the PrtD protein, hindering further access to proteases. However, as the N-terminal part of HasA plays a role in the efficiency of secretion via its own transporter and the C terminus is required for secretion, it is more likely that the HasD ABC protein has two peptide binding sites with different functions and specificities. The C-terminal secretion signal binding site would therefore be strictly required for secretion whereas the binding site for the N-terminal region would increase secretion efficiency in a SecB-dependent manner. It is unclear whether this is a general characteristic of such protein trans-

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