Extracellular secretion of the peptide antibiotic colicin V (ColV) in *Escherichia coli* is mediated by a dedicated exporter system consisting of host TolC protein and the products of two specific genes, *cvaA* and *cvaB*, the latter being a member of the ATP binding cassette (ABC) superfamily. An amino-terminal export signal of ColV is specific for the CvaA-CvaB-TolC exporter and is processed concomitant with secretion. In this study, we attempt to characterize this processing with a secretable marker protein, ColV-1, using a newly developed *in vitro* assay. Processing is found to be dependent on both CvaA-CvaB transporters and the TolC protein and to require membrane integrity. An additional cytoplasmic soluble factor(s) is also necessary for the processing. Although the sequence of the cleavage site suggests it could be a substrate, ColV-1 cannot be processed *in vitro* by the purified leader peptidase I. Moreover, ColV-1 processing is inhibited by antipain and N-ethylmaleimide. Furthermore, the processing requires energy in the form of nucleotide hydrolysis. These results indicate that the processing of ColV-1 is specific and more complex than expected, requiring the CvaA-CvaB-TolC transporter intact in the membrane, energy, and cytosolic factors for rapid cleavage.

The localization of proteins to different cellular and extracellular compartments has been studied intensively because of its fundamental importance to all living cells. The best characterized process is the classic, signal sequence-dependent export pathway (1, 2). In Gram-negative bacteria, this pathway requires the products of several *sec* genes and precursor proteins bearing an amino-terminal signal sequence, which usually has a positively charged amino terminus and a hydrophobic core, followed by a cleavage site for signal peptidases (3).

There is, however, a group of proteins and peptides that are exported by signal sequence-independent pathways. Such substrates include yeast a factor (4), *Escherichia coli* a-hemolysin (5, 6), *Bordetella* cyclolysin (7), *Erwinia* protease (8), and an *E. coli* peptide antibiotic, microcin B17 (9). In each case, export is mediated by a dedicated export apparatus. This group is sometimes referred to as the multidrug resistance-like family because one of its best known members is the P-glycoprotein or multidrug resistance protein, which is responsible for the multidrug phenotype of tumor cells when overexpressed (10, 11). It is also referred to as the ABC1 transporter superfamily, which describes the ATP binding domain common to all members (12–17).

The bacterial ABC export system is exemplified by the secretion of α-hemolysin from *E. coli* (18, 19). The targeting signal for its secretion resides in the carboxyl-terminal 50 amino acids and is not cleaved (20–23). The transport process requires three specific envelope proteins: the ABC exporter (HlyB), accessory protein (HlyD), and outer membrane TolC protein (5, 6, 20–25). The export of peptide antibiotic colicin V (ColV) from *E. coli* also utilizes an ABC export system similar to α-hemolysin (26, 27). The structure gene for colicin antibiotic is encoded in large, low copy number virulence plasmids found in *E. coli* and other members of *Enterobacteriaceae* (28). ColV kills sensitive cells by disrupting their membrane potential (29). Four linked genes are identified in the ColV operons (26, 27): the *cva*C structural gene, the *cvi* immunity gene, and the two exporter genes *cvaA* and *cvaB*. Products of *cvaA* and *cvaB*, together with the TolC protein, function as a dedicated export system in producing cells to transport ColV through two membranes (26, 27). Although biochemical data have shown that ColV does not accumulate in the periplasm (30), the data from genetic assays indicate that a small amount of ColV appears to be present transiently in the periplasm during secretion (31).

ColV is synthesized as a 103-amino acid primary translation product (pre-ColV) which does not contain a typical Sec-dependent amino-terminal signal peptide, even though its amino-terminal 15 residues are processed concomitant with extracellular secretion (32, 33). Homology analysis reveals that the sequence of this amino-terminal signal has significant similarity to the leader peptides of nonantibiotics and some lantibiotics found in Gram-positive bacteria (32–34). The resulting 88 residues, comprising the mature colicin V peptide, has aberrant mobility on SDS-polyacrylamide gel electrophoresis (predicted *M*<sub>r</sub>, 8700; observed *M*<sub>r</sub>, 5800; Ref 32). It has been suggested that active ColV probably contains a disulfide bond between the only cysteines at positions 91 and 102 of the polypeptide (33). ColV-1 is a Tn5 ColV mutant form with the 21 carboxyl-terminal amino acids of colicin V replaced by 8 heterologous residues (32). It can be secreted efficiently into extracellular media from cells with a plasmid containing *cvaA* and *cvaB*, indicating that the carboxyl-terminal 21 amino acids of ColV are not required for secretion (32); in contrast to that of hemolysin. It loses ColV toxicity but exhibits normal mobility on SDS-polyacrylamide gel electrophoresis (predicted *M*<sub>r</sub>, 7200; 1 The abbreviations used are: ABC, ATP-binding cassette; ColV, colicin V; OG, octylglucoside; TCA, trichloroacetic acid; NEM, N-ethylmaleimide; GMP-PNP, guanylyl-imidodiphosphate.

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Observation M₃, 7300), providing a convenient tool for certain studies. In this study, we have developed an in vitro assay using CoV-1 as a marker protein to characterize the processing of CoV-1. The results show that the processing of CoV-1 in vitro requires the intact CvaA, CvaB, and TolC transporters in the membranes. Additionally, nucleotide hydrolysis and soluble factor(s) are required for the processing. Moreover, the specific inhibition in processing by antipain and NEM suggests that the peptidase responsible for CoV-1 processing might be a cysteine protease.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Media—E. coli strains and plasmids used in this study were as follows: MC4100 (ΔlacU169 araC139 rpsL150 thi-1 recA1 deoC7 fthbB5301 ptsF252 relA1), DH5α transformed with M9 medium (8.5 mM NaH₂PO₄, 100 µm thiamine, 100 µg/ml tetracycline), and with appropriate antibiotics. After cell growth for 40 min at 37°C with shaking, 0.5 mM isopropyl-1-thio-

maltose (0.1%) for 30 min. Following centrifugation, the supernatant was precipitated with ice-cold trichloroacetic acid (TCA) to a final concentration of 10%, and after centrifugation the pellet was washed

by a cell-free transcription-translation system from plasmid pXZ5 (or pXZ5-C::Tn5), which contains the precursors of the Colicin V-1, the cells were labeled for 5 min and chased with cold buffer, boiled, and subjected to gel electrophoresis. To detect the extracellular ColV-1, the cells were added either during or after protein synthesis-translation of ColV-1 was mixed with 0.1 unit of E. coli S100 to a final volume of 100 µl. S100 was prepared from S30, which was centrifuged at 100,000 rpm for 20 min in a Beckman Instruments TLA100.2 rotor. The reaction mixtures were incubated at 37°C for 15 min. Membrane vesicles were isolated by centrifugation or precipitated by 10% TCA where indicated, then dissolved in the sample buffer and submitted to SDS-polyacrylamide gel electrophoresis. Autoradiographs were quantitated by PDI image analyzing system (Protein Databases Inc., New York, NY). For the calculation of processing ratio, correction was made based on the presence of three residues of methionine in the precursor form and two in the processed form.

Removal of Nucleotides—0.3 ml of the 300,000 × g supernatant of the transcription-translation mixture was applied to a P4 polyacrylamide (Bio-Rad) column (0.5 × 40.0 cm), which had been previously equilibrated with 50 mM Tris-HCl, pH 7.6, and 1 mM diethiothreitol. Fractions (0.25 ml) were collected, and those containing CoV-1 were monitored by detection of a series of trichloroacetic acid-precipitable counts (National Diagnostics), verified by gel electrophoresis, and pooled.

Gel Electrophoresis and Immunological Assays—Regular SDS gel electrophoresis with 15% acrylamide was carried out according to the method of Laemmli (37). 15% Tricine SDS gel electrophoresis at 4°C was used for separating the precursor and processed forms of CoV-1. For immunoprecipitation, samples were diluted to 1 ml with IPB buffer (20 mM Tris-HCl, pH 8.0, 0.15 mM NaCl, 5 mM EDTA, 0.05% Triton X-100, and 0.05% SDS) and treated with premature antigen (10 µl) on ice for 30 min. Then 100 µl of 10% Sepharose-protein A suspension in IPB buffer was added for 10 min with occasional shaking. After spinning at 5,000 rpm for 2 min, the supernatant was treated with anti-CoV peptide antibody (10 µl) for 30 min, and 100 µl of 10% Sepharose-protein A suspension was added for 10 min. The resin pellet after centrifugation was washed with 1 ml of IPB buffer twice, and then proteins were eluted with 1.5 × sample buffer and boiling.

Chemicals and Reagents—Purified GroEL, GroES, DnaK, DnaJ, and GrpE were from StressGen Biotechnologies Corp. SecA, SecB, and preOmpA were laboratory stocks purified from T7-secA- and T7-secB-overproducing strains and BA15/pAM103, respectively. Nucleotides and protease inhibitors were obtained from Sigma. GMP-PNP was obtained from Boehringer Mannheim. Other chemicals were of reagent grade obtained from commercial sources.

RESULTS

CoV-1 Can Be Efficiently Processed and Secreted into the Extracellular Medium—In this study, we chose CoV-1 (Fig. 1A) as a model marker protein. The whole cvaC-1 gene was subcloned behind the T7 RNA polymerase promoter in plasmid pXZ5 (Fig. 1B). The CoV-1 precursor was uniquely labeled in the strain BL21ΔADE3/pXZ5 (Fig. 2A, lane 1). In the presence of CvaA and CvaB transporters, CoV-1 was processed (Fig. 2A, lane 2) and secreted into the extracellular medium (Fig. 2A, lanes 3 and 4). The precursor and the processed form of CoV-1 could be immunoprecipitated by CoV peptide antibody (data not shown). Their migration difference provides a convenient criterion for the identification of the processing of CoV-1. The processing was rapid and efficient (Fig. 2B). More than 50% of the T7 promoter-expressed CoV-1 precursor was processed within 1 min (Fig. 2A, lane 4).

Processing of CoV-1 Requires Soluble Factor(s) and Dedicated Transporters—As a first step to elucidate biochemically the mechanism of CoV secretion, we developed an in vitro processing assay using CoV-1 as a marker protein, transcribed and translated from plasmid pXZ5. Inverted inner membrane vesicles were isolated from MC4100/pHK11-1, which could activate the complete CoV-1 in vitro. In the preliminary experiments, this assay was carried out both co- and post-translationally; i.e., the membranes were added either during or after protein synthesis. There was not much difference between these two
processes. A very weak processed band of ColV-1, which had the same migration pattern of mature ColV-1, was detected, unless the amount of S100 from D10/pHK11-1 was increased (Fig. 3A, lanes 2–5). On the other hand, there was no processed form without addition of membranes (Fig. 3A, lane 7); therefore, the processing was not due to nonspecific proteases in the S100. However, since S100 from the D10 strain without CvaA and CvaB transporter genes also had the same enhancement effect (Fig. 3A, lane 6), the soluble factor was not specifically derived from the ColV operon.

Surprisingly, only about 20% of total processed ColV-1 was found to co-sediment with membrane vesicles, and 80% remained in the supernatant after centrifugation (data not shown). Those in the supernatant might be unable to complete the translocation in the membranes and were released after cleavage. This result was consistent with the in vivo data that some processed ColV-1 was found in cytosol (data not shown).

Since a majority of the processed form was in the supernatant, a processing assay hereafter was then carried out in total reaction mixtures precipitated by TCA without isolating membranes, unless specified.

In the transcription-translation mixture, a protein band was observed above the precursor of ColV-1, which was also processed in the assay (Fig. 3A, lanes 4–6). As shown in Fig. 3B, the precursor of ColV-1 (PColV-1) and its processed form (ColV-1) and the band above the precursor of ColV-1 (PColV-1') together with its prec-
cessed form (ColV-1') in MC4100/pHK11 membrane vesicles (lane 6) could be immunoprecipitated by ColV peptide antibodies. Therefore, this band is probably a read-through product of ColV-1, because a stem loop structure (p-independent transcription factor) lies right behind the translational stop codon of the cvaC-1 gene during the construction of the plasmid. (Indeed, a construct containing double stop codons did not yield the upper band, and the processing was similar (data not shown, but see Fig. 9).) No processed forms were observed with MC4100 membranes without the transporter (Fig. 3B, lane 4), confirming that they were all ColV derivatives and the processing was specific for the dedicated transporters.

Membrane Integrity but not Leader Peptidase I Is Required for Processing—The cleavage site for ColV is preceded by the sequence Ser-Gly-Gly, making it a potential substrate for the leader peptidase I, as noted by Path et al. (32). However, in the in vitro assay, there was no processing of ColV-1 with solubilized membranes from MC4100/pHK11-1, even in the presence of 4 μg of purified leader peptidase I (see Fig. 3C). Under the same conditions, both purified and in vitro translated proOmpA were completely processed by 0.8 μg of purified leader peptidase I or 50% processed by the leader peptidase I released from octyl glucoside (OG)- or Triton X-100-solubilized membranes (data not shown). When the membranes were treated with OG or Triton X-100 concentrations higher than 0.5 or 0.1%, respectively, the processing was completely abolished (Fig. 3D). These data indicated that membrane integrity, i.e. the transporter in the intact membrane, is required for the processing and that there might be another peptidase that processes ColV-1.

Processing of ColV-1 Is Inhibited by Antipain and N-Ethylmaleimide—To characterize the processing of ColV-1, different types of protease inhibitors were tested in the in vitro assay. Based on the nature of their active sites and mechanism of action, four mechanistic classes of protease have been recognized thus far: metalloprotease, serine protease, aspartic protease, and cysteine protease (39). Each type of protease activity can be specifically inhibited by its own inhibitors. Thus, five representative protease inhibitors were tested for their effects. Pepstatin A, phosphoramidon, and phenylmethylsulfonyl fluoride had no inhibition (Fig. 4A, lanes 3–6, 9, and 10). In contrast, antipain at 5 mM showed significant inhibition (Fig. 4A, lane 8), and NEM completely inhibited the processing of ColV-1, even at 1 mM (Fig. 4A, lane 11). When the membrane fraction and soluble factors were treated with NEM separately, the processing activity was inactivated (Fig. 4B), indicating that the membrane and soluble factors contributing to ColV-1 processing are NEM-sensitive.

The Soluble Factor(s) Is Identified as Being Protein in Nature—To characterize further the nature of the soluble factor(s) that enhanced the processing, its sensitivity to protease and to RNase was tested. As shown in Fig. 5A, proteinase K (100 μg/ml) completely inactivated the soluble factor, but not in the presence of an inhibitor, phenylmethylsulfonyl fluoride, whereas treatment with the micrococcal nuclease (500 μg/ml) had no effect. These results suggest that the soluble factor(s), which enhances the processing, is of a protein nature, and no functional RNA, if any, is accessible to RNase.

As the soluble factor(s) is not specific from ColV operons, it might be a general factor. We tested several candidates that are known to be involved in protein targeting: SecA, SecB, trigger factor, GroEL, GroES, DnaK, DnaJ, and GrpE. None of these purified proteins alone or in different combinations could enhance processing of ColV-1 (Fig. 5, B and C). To determine whether the purified factors need additional factors to function, small amounts of S100 were mixed with these purified factors. No processing enhancement was observed (Fig. 5, B, lanes 6, 11, and 13, and C, lanes 4, 6, 8, and 10). These results indicate that factors other than those tested have the enhancing activity of processing.

Processing of ColV-1 Is Dependent on CvaA-CvaB Transporter and TolC Protein—To determine whether the processing of ColV-1 requires all three gene products in the in vitro assay, membrane vesicles from strains MC4100 (cvaA cvaB tolC), MC4100/pHK11-8 (cvaA cvaB tolC'), MC4100/pHK11-4 (cvaA cvaB tolC'), and ZK796/pHK11-1 (cvaA cvaB tolC') were tested for ColV-1 processing activity. There was no processed ColV-1 with these membrane vesicles lacking any one of the intact cvaA, cvaB, and tolC gene products (Fig. 6). Under the same conditions, the membranes from strain MC4100/pHK11-1 (cvaA cvaB tolC') yielded the processed form (Fig. 6, lane 6). These results indicate that the processing is specific: both intact CvaA-CvaB transporter and TolC protein are required.

Kinetics of ColV-1 Processing—To determine the rate of processing of ColV-1 in vitro, kinetics of processing was performed in the assays with MC4100/pHK11-1 membrane vesicles. As shown in Fig. 7, processing at 37 °C was linear up to 8 min, and half of the ColV-1 was processed within 4 min, indicating that processing occurred rapidly in vitro, in a manner similar to that observed in intact cells (Fig. 2B). Processing was much less efficient but appreciable at 0 °C than at 37 °C. This result is consistent with the finding that processing is still continued at 0 °C in vivo (data not shown).

Processing of ColV-1 Requires Energy in the Form of NTP(s), and GTP Appears to Be the Most Efficient Nucleotide for This Process—It is widely accepted that ATPase activity of ABC transporters contributes energy to all active ABC protein-dependent transport. Since CvaB is a member of the ABC transporters, it was of interest to determine the energy requirement of the ColV-1 processing with our in vitro assay. Small molecules (e.g. ATP and salts), required for the transcription-translation mixtures of ColV-1, were removed by gel filtration after in vitro synthesis. Fractions containing ColV-1 were pooled and used for the assay.
1.0 mM, the efficiency of ATP was increased significantly. Surprisingly, GTP seemed to be the most efficient among all NTPs. Furthermore, there was little processing with the nonhydrolyzable GTP analogue GMP-PNP (Fig. 8), indicating that the hydrolysis of GTP was required. Addition of 1 mM ATP together with 1 mM GTP was only slightly more active than with 1 mM GTP alone (Fig. 8A, lane 11). Further titration of GTP and ATP on ColV-1 processing showed that 3 mM GTP was sufficient to maximize the observed processing, whereas ATP reached its maximum at 4 mM and was still consistently less efficient than GTP at that level (Fig. 8B). These results show that GTP is as efficient as ATP if not the most efficient nucleotide required for the processing.

CvaA-CvaB Membrane Vesicles Alone Have Little Processing Activity—It has been suggested that the CvaA-CvaB transporter could have “leader peptidase” activity (32). Moreover, CvaB has been proposed to possess protease activity (40, 41). It is thus of interest to determine whether CvaA-CvaB membrane vesicles alone have some processing activity without addition of soluble factor(s) and energy. A new construct of ColV-1, pXZ10, that contained a double stop codon and generated a single ColV-1 product (Fig. 9, lane 1) was used. With energy and S100, the product of new ColV-1 construct was processed in 15 min at 37°C as efficiently as that of the old construct (Fig. 9, lane 8). In contrast, ColV-1 precursors were not processed after incubation with CvaA−B− or CvaB− membrane vesicles, or even with CvaA-CvaB membrane vesicles without energy and factors at 37°C for 18 h (Fig. 9, lanes 2–7). Both precursor and processed forms of ColV-1 were quite stable even after overnight incubation at 37°C. When the same film was exposed longer, a faint processed form-like band is visible in Fig. 9, lane 3; however, this band is also visible, although weaker in the precursor sample (data not shown). Even if this is a real processed band, this activity is more than 6,000 times lower than that in the optimal condition. When the precursors was incubated with CvaA and CvaB membrane vesicles and energy without additional soluble factors, some processing activity (about 2%) was detected (Fig. 9, lanes 10–12). Since the in vitro synthesized ColV-1 precursor preparation contained some endogenous soluble protein (about 5% of the optimal amount), it may contribute to this residual activity. Hence, taking all of these data together, it is indicated that both energy and soluble factors are essential for efficient ColV-1 processing, and membranes containing the CvaA and CvaB transporter alone is not sufficient for the processing; presumably these findings can be extended to the processing of native ColV processing.

DISCUSSION

Amino-terminal processing appears to be the first step during the secretion of ColV. In this study, we characterized the processing of colicin V using a marker protein, ColV-1. An in

FIG. 5. Preliminary characterization of the soluble factors. A, the soluble factor is protein in nature, based on its sensitivity to proteinase K (PK). The assays were as described in Fig. 4. Lane 1, precursor alone. Treatment of S100 with proteinase K was at 0°C for 10 min. 2 mM phenylmethylsulfonyl fluoride (PMSF) was added after (lane 3) or before (lane 4) proteinase K (100 µg/ml) treatment. In lane 6, S100 was treated with micrococcal nuclease (500 µg/ml) and Ca2+ at 25°C for 30 min, and 5 mM EGTA was added after incubation. B, Purified GroEL, GroES, DnaK, DnaJ, and GrpE are not the enhancing factors. The purified proteins were tested in the assay as indicated. Lane 1, precursor of ColV-1. Lane 2, 125 µg of D10 S100 was added. C, purified SecA, SecB, and trigger factor (T.F.) do not enhance the processing. Purified proteins were tested in the assay as indicated. Lane 1, precursor of ColV-1. Lane 2, 125 µg of D10 S100 was added.

FIG. 6. Processing of ColV-1 is dependent on CvaA-CvaB transporter and TolC protein. The assay was described in Fig. 3A. Lane 1, markers for precursor and mature forms of ColV-1. Lane 2, membranes from ZK796/pHK11-1 (cvaA−cvaB−tolC−). Lane 3, membranes from MC4100/pHK11-4 (cvaA−cvaB−tolC−). Lane 4, membranes from MC4100/pHK11-8 (cvaA−cvaB−tolC−). Lane 5, membranes from strain MC4100 (wild type, cvaA cvaB). Lane 6, membranes from strain MC4100/pHK11-1 (cvaA−cvaB−tolC−). Lane 7, precursor of ColV-1.

As shown in Fig. 8A, processing was completely abolished by such nucleotide depletion (lane 1). This activity, however, could be restored when different nucleotides were added to test for the processing of ColV-1 (Fig. 8A, lanes 2–9). As expected, ATP was active in promoting the processing and more active than CTP and UTP. As the concentration was increased from 0.5 to 1.0 mM, the efficiency of ATP was increased significantly. Surprisingly, GTP seemed to be the most efficient among all NTPs. The assay was described in Fig. 4. The reaction was stopped with 10% TCA. Data are means ± S.E. (bars); n = 3.

FIG. 7. Time course of ColV-1 processing. The in vitro processing assay of ColV-1 used was the same as described in Fig. 4. The reaction was stopped with 10% TCA. Data are means ± S.E. (bars); n = 3.
Processing of Colicin V-1

Fig. 8. Processing of ColV-1 requires energy. A, small molecules in the in vitro synthesis of ColV-1 were removed by gel filtration. Fractions containing ColV-1 were pooled and used for the assay. The reaction mixture (25 µl) contained 12.5 mM Tris-HCl, pH 7.6, 0.25 mM spermidine-HCl, 2 



mM putrescine-HCl, 2 mM MgSO4, 2 mM dithiothreitol, 0.025 A280 unit of membrane vesicles, 100 



ng of S100, and 10 bars 



g of control. The reaction mixtures were incubated at 37 



C for 15 min. Data are means ± S.E. (bars); n = 3. B, titration of GTP and ATP on ColV-1 processing. The assay was as described in A. Data are means ± S.E. (bars); n = 4.

Fig. 9. CvaA-CvaB membrane vesicles alone have no processing activity. ColV-1 precursor was generated by using pXZ10 for in vitro synthesis. The in vitro processing assay was the same as described in Fig. 8. Lane 1, precursor marker. In lanes 2, 3, and 8–12, MC4100/pHK11 membrane vesicles were added. In lanes 4 and 5, MC4100 membrane vesicles were added. Lanes 10–12 were from a separate gel. Energy (ATP-regenerating system) and S100 were added as indicated. The reaction mixtures were incubated at 37 



C for the times indicated.

The findings in vitro that no ColV-1 processing was observed with CvaA-, CvaB-, or TolC- deficient membrane vesicles are also consistent with in vivo data (31). A tolC mutant abolishes the majority of extracellular ColV activity (27), however, processing in the tolC strain appears to be similar to the wild type, albeit slightly slower (31). In our in vitro assay, there is no detectable processing for ColV-1 without CvaA, CvaB, or TolC. This difference may be due to the sensitivity of the assays. The activity of isolated membrane vesicles from the tolC mutant strain may also be significantly affected by the instabil-

\[2 \text{X. Zhong and P. C. Tai, unpublished data.}\]
ity or reduced the expression of CvaA-CvaB transporters in a toC background. Furthermore, our in vitro results also indicate that processing of ColV-1 requires an intact membrane for the transporters to function, suggesting that the processing may require a specific conformation of the transporter, precursor, or peptidase for it to be active in the membranes. Since the mutants we tested are all null mutants, we cannot yet specify the exact role of each component, due to the stability effect of the whole complex in the assay. Missense mutants will be necessary to address this question.

One of the interesting findings in this study, however, is that processing of ColV-1 also requires soluble protein factor(s). It probably serves as a targeting factor, a cofactor, or chaperone for ColV-1 to interact with membrane transporters for processing and secretion. This interaction is specific and critical, since without the soluble factors, little ColV-1 is processed, although some precursors can nonspecifically bind to membrane vesicles in the assay. Many heat shock proteins function as molecular chaperones, which can maintain presecretory proteins in a translocation-competent conformation in the cytosol in the major secretion Sec system. However, we have shown that SecA, SecB, trigger factor, GroEL, GroES, DnaK, DnaJ, and GrpE are not involved in enhancing the processing of ColV-1. Preliminary fractionation of the soluble factors revealed that multiple factors are involved. It is possible that soluble factors may serve as cofactors for the peptidase to function. In this regard, it is interesting that nucleotide hydrolysis is required for the processing, and that GTP seems to be the most efficient for processing. It has been demonstrated that ATP is hydrolyzed during transport in reconstituted systems directed by the eukaryotic multidrug resistance transporter and the bacterial chaperone HisP and MalK (44-48). In a reconstituted histidine transport proteoliposome system, it was reported that GTP could replace ATP reasonably well (70%; Ref. 45), indicating that this type of ATPase has less substrate specificity. However, it does not readily explain why GTP in the ColV-1 system is a more efficient substrate. Therefore, it raises the possibility that a GTP-binding protein might be involved in the processing of ColV-1. Ffh/4.5S ribonucleoproteins contain a GTP binding domain and have greatly stimulated GTPase activity when combined with the receptor protein FtsY, and the complex has been proposed to be involved in the targeting (49, 50). The possible role of this complex in ColV-1 processing is the exact role of each component, due to the stability effect of the whole complex in the assay. Missense mutants will be necessary to address this question.

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