A Bacterial Signal Peptidase Enhances Processing of a Recombinant Single Chain Antibody Fragment in Insect Cells

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The production of an antibody single chain fragment (scFv) in insect cells was accompanied by the formation of an insoluble intracellular precursor even with the inclusion of the bee melittin signal peptide. The presence of the precursor polypeptide suggests a limitation in the processing of the signal peptide so a baculovirus containing a signal peptidase from Bacillus subtilis (SipS) was constructed for expression studies. When the wild type SipS was coexpressed with scFv, preprocessed scFv fragments were no longer detected in insect cell lysates. Conversely, coexpression of scFv alone or with an inactive mutant SipS resulted in at least 30% of the intracellular polypeptide in an unprocessed form at 3 days post infection. Production of scFv in the medium was also enhanced in the presence of SipS; however, low secretion levels indicate the presence of a post-processing bottleneck.

The use of the baculovirus expression vector system (BEVS) for the production of recombinant proteins has grown significantly in recent years (1, 2). The ability to process and perform posttranslational modifications on secreted proteins is a major strength of BEVS which has contributed to its increased use today (3, 4).

However, yields of heterologous secreted proteins expressed in insect cells may suffer due to incomplete processing and aggregation (5–9). In addition to mature length polypeptides, protein precursor polypeptides have been observed to accumulate in insect cells as well (10–13). Vernet and co-workers (14) identified insoluble aggregates containing prepropapain in Sf-9 cell lysates following recombinant baculovirus infection. Apparently, incorrect processing of the signal peptide of prepropapain resulted in limited propapain secretion and the accumulation and aggregation of a large fraction of the propapain precursor within the insect cells. A significant fraction of immunoglobulin light chain (C\textsubscript{\lambda}) expressed in insect cells was also observed to accumulate as aggregates at a higher molecular weight than the processed polypeptide chains (15). These preprocessed immunoglobulin polypeptides are believed to include the unprocessed signal peptide sequence, a hypothesis first proposed by Milstein and co-workers (16) from their observations of IgG synthesis in vitro.

The function of the signal peptide in the secretion process is to direct the newly synthesized polypeptide into the translocation machinery of the cellular membrane (17–20). Without removal of the signal peptide the polypeptide may aggregate or remain membrane bound and be prevented from further passage along the secretion pathway (21, 22). Signal peptides are sequences of amino acids found on the N-terminus of secreted polypeptides and, although there is no consensual signal sequence, sequences identified in both prokaryotes and eukaryotes share similar features (17, 18, 23). The sequences are typically 15-30 amino acids in length and are comprised of three fairly distinct regions. A short positively charged N-terminus is followed by a stretch of 10-20 hydrophobic residues and concludes with a carboxy-terminal region that contains two small residues located at the −3 and −1 positions from the cleavage site. Due to the similarity in design of signal peptides in both prokaryotic and eukaryotic cells, it is not surprising that these peptides can be recognized and processed by components located in either cell type (24–27).

One strategy to increase heterologous protein secretion in insect cells is to replace the heterologous signal peptide with an insect derived signal peptide. When the heterologous signal peptide of prepropapain was
replaced with the honeybee melittin signal peptide propapain secretion was increased five fold in Spodoptera frugiperda cells (28). However, the use of the Drosophila melanogaster a-amylase signal peptide failed to yield any propapain secretion into the cell culture medium (28). Also, signal peptides derived from insect, baculovirus, and mammalian sources did not improve secreted levels of tissue plasminogen activator (29).

A complementary strategy to replacing the signal peptide would be to enhance the signal peptide processing efficiency. During translocation the signal peptide is removed from the polypeptide on the lumenal side of the ER by a membrane bound protein known as the signal peptidase complex (18). Signal peptidases have been identified in a number of organisms including bacteria, yeast, and mammalian cells (30-32). They are comprised of one or more membrane bound units which are located in the proximity of the polypeptide translocating apparatus. Bacterial signal peptidases are monomeric and cleave the signal peptide from nascent polypeptides during transport into the periplasm or extracellular space. Eukaryotic microsomal signal peptidases contain 2 to 5 membrane anchored subunits and may be associated with the translocation machinery of the ER. Comparison of the amino acid sequences of bacterial, yeast, and mammalian signal peptidases reveal regions of conserved homology (30,33).

In this study we hypothesized that a limitation in the availability of the signal peptide complex during baculovirus infection may be a contributing factor to inefficient precursor processing. Previously, van Djil and co-workers (34) demonstrated that overexpression of a bacterial signal peptidase could improve processing of a model protein because of its propensity to form aggregates of preprocessed polypeptides when overexpressed in insect cells even with the use of the bee melittin signal peptide. The effects of signal peptidase expression on cell viability were also explored. This study demonstrates that expression of a bacterial signal peptidase can be used to enhance intracellular processing of polypeptides in insect cells.

**MATERIALS AND METHODS**

Cell culture and virus stock. Suspension cultures of Spodoptera frugiperda (SF-9) and Trichoplusia ni (BTI-TN5B1-4, High Five Invitrogen) were maintained at 27°C and rotated at 120 rpm in 250 ml shaker flasks containing 35 ml Excell 405 serum free medium (J RH Biosciences).

The recombinant baculovirus encoding the gene for a murine single chain antibody fragment (scFv) was kindly provided by J. Miller (Eli Lilly Inc.). The recombinant baculoviruses encoding the genes for Bacillus subtilis wild type and mutant signal peptidases were constructed as follows.

Baculovirus construction. Recombinant B. subtilis wild type signal peptidase I was amplified from plasmid pGDL41 (33) by PCR using Taq-Plus (Stratagene) polymerase. The forward and reverse primers used in the PCR were CAGATGAAATCAGAAATGGTCCGAAG and CCTATTTGTTTTGCGCATTTCG, respectively. The amplified gene was ligated into a linear donor vector (PCRRII) and transformed into Escherichia coli using the TA cloning kit (Invitrogen). Colonies which contained the signal peptidase insert were cultured, miniprepped and the resultant plasmid DNA was digested with Not I and Hind III restriction enzymes (BRL). The digested SipS gene fragment was purified and inserted in the pFastBac baculovirus transfer vector. Recombinant baculovirus (AcNPV-SipS) was generated using the Bac-to-Bac system by BRL. A recombinant baculovirus carrying the gene for an inactive form of B. subtilis signal peptidase I (AcNPV-SipSsa43) was similarly constructed from plasmid PMSA43 (35). However, BamH I and Xho I restriction sites were used to insert the gene into the baculovirus transfer vector. The gene encoding the mutant signal peptidase is identical to the wild type gene with the exception that serine at position 43 of the wild type was changed to alanine in the mutant.

Cell infection and harvest. Log phase High Five insect cells were seeded in a 24 well plate at 1 x 10^5 cells/well (0.5 ml). Cells were infected with 10% medium in 1.5 ml microfuge tubes and centrifuged 30 sec at 16,000 g. After removing the medium, cell pellets were incubated with 100 μl lysis buffer (1% NP-40, 50 mM tris-HCl pH 8.5, 10 mM EDTA, 150 mM NaCl), and protease inhibitors (0.5 μg/ml antipain, 0.5 μg/ml aprotinin, 15.6 μg/ml benzamidine-HCl, 0.5 μg/ml chymostatin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl [PMSF]). Clarified lysates were obtained from crude whole cell lysates by centrifugation for 15 minutes at 16,000 g and 4°C.

Immunoblot blot analysis. Whole cell and clarified lysates from an equivalent number of infected cells (approx. 5 x 10^7) were separated by SDS-PAGE (12.5 %) using reducing (60 mM Tris-HCl [pH 6.8], 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) sample buffer and then transferred onto a nitrocellulose membrane. The extracellular medium was similarly processed. However, the medium was concentrated 10 times using a Micron-3 (3000 MWCO) microconcentrator (Millipore) prior to addition of the sample buffer. The membrane was blocked 2 hours in 10% (w/v) dried milk and tris buffered saline with 0.05% Tween 20 detergent (TBS) solution. Recombinant SipS was detected by probing with rabbit anti-SipS serum (35) for 1 hr in TBST containing 2% dried milk. The anti-serum was followed by a 1 hour incubation in TBST with a horse radish peroxidase (HRP) conjugated polyclonal goat anti-rabbit IgG (Pierce). Protein bands were visualized using Lumiglo (Kirkgaard & Perry) or SuperSignal (Pierce) substrate systems.

Scanning densitometry. The films were scanned with an Apple One Scanner (Apple Computer, Inc.). The scanned films were quantified by means of the Image analysis and processing software NIH Image (version 1.61). The software permits measurement of the density of a specified area.
the gene for wild type SipS, encoding AcNPV-SipS, was harvested from Sf-9 insect cultures 4 days following transfection of the cells with plasmid DNA. Whole cell lysates from High Five insect cells infected with AcNPV-SipS or AcBB (36) were harvested after 3 days and analyzed by western blot. The immunoblot (Fig. 1) reveals the presence of two SipS immunoreactive bands at approximately 21 kDa and 24 kDa.

The higher molecular weight band was intriguing because in bacteria SipS runs as a single band at a molecular weight of 21 kDa (33). An examination of the amino acid sequence of SipS led to the identification of two possible N-glycosylation sites at asparagine (Asn) 5 and Asn 57, although glycosylation at Asn 5 is unlikely (37). N-glycosylation does not occur in bacteria, however, the bacterial SipS could be subject to post-translational modifications such as N-glycosylation following expression in a eukaryotic cell host (38-40). To determine if the heavier SipS band was due to the addition of N-linked oligosaccharides, High Five insect cells were infected with the SipS baculoviruses in the presence (1) or absence (2) of a single site serine to alanine mutation at position 43. The cells were stained with trypan blue (BRL) and counted on a hemocytometer. Cell viability was determined using trypan blue exclusion. As expected, cell viability declined for both wild type and mutant SipS baculovirus infections. However, from 2 to 4 days the viability of the cells expressing wild type SipS was from 40 to 45 percent lower than for cells expressing mutant SipS (Fig. 3). Consequently, the expression of wild type SipS in insect cells appears to have a deleterious effect on cell viability.

In vivo processing of antibody single chain fragment. Due to their importance in diagnostics, structure-function studies, and therapeutic applications, antibody and antibody fragments have been expressed in a number of organisms including insect cells (41-45). However, these polypeptides are not always properly processed and secreted from insect cells (5, 9). As a model protein we expressed an antibody scFv in insect cells using the recombinant baculovirus AcNPV-scFv. AcNPV-scFv carries the gene for a murine scFv under control of the polyhedrin promoter and does not include any potential N-glycosylation sites. To enhance processing of the scFv in insect cells the honeybee melittin signal peptide was substituted for the constitutive signal sequence. In cells infected with AcNPV-scFv two bands, representing the preprocessed (p) and processed (m) polypeptides, can be seen at approximately 34 and 32 kDa, respectively, following western blot analysis of cell lysates and extracellular medium (Fig. 4). The bands are slightly heavier than an antibody Fv fragment because the scFv contains a c-myc region for antibody recognition, hexahistidine tag for purification, and polypeptide linker chain. A substantial fraction of the intracellular protein present in the whole cell lysate is of the preprocessed form. Furthermore, only the processed form is observed in the soluble and secreted polypeptide fractions. Even with an insect derived signal sequence known to enhance protein secretion (28), a large fraction of immunoglobulin polypeptide is retained within High Five insect cells as

![Immunoblot of wild type SipS in whole cell lysates of High Five insect cells infected with a control baculovirus AcBB (BB) or AcNPV-SipS (SipS). Cells were harvested 72 hours post-infection.](image1)

**FIG. 1.** Immunoblot of wild type SipS in whole cell lysates of High Five insect cells infected with a control baculovirus AcBB (BB) or AcNPV-SipS (SipS). Cells were harvested 72 hours post infection.

**FIG. 2.** Immunoblot of wild type SipS in whole cell lysates of High Five insect cells following treatment with tunicamycin. High Five cells were infected with AcNPV-SipS in the presence (+) or absence (−) of 1 μg/ml tunicamycin for 72 hours. Tunicamycin treatment results in a downward shift in the molecular weight of the wild type SipS.
insoluble preprocessed polypeptide that is prevented from progressing through the secretory pathway.

To determine if SipS can actively process the precursor form of scFv in vivo, wild type SipS was coexpressed in High Five insect cells with scFv. As a control, the inactive mutant SipS (SipSsa43) was coexpressed with scFv as well. Van Djil and co-workers (35) demonstrated that the wild type SipS from B. subtilis was active in processing pre-β-lactamase in E. coli whereas the mutant SipS possessed negligible activity. The cells were harvested 3 days following infection with AcNPV-scFv alone, AcNPV-scFv and AcNPV-SipS, or AcNPV-scFv and AcNPV-SipSsa43, lysed, and subjected to immunoblot analysis (Fig. 5). In cells where scFv and mutant SipS (+mut) were coexpressed the processed polypeptide comprised approximately 60% of the intracellular protein. However, in cells where scFv and wild type SipS (+wt) were coexpressed the processed polypeptide represented approximately 100% of the intracellular protein, a significant increase from cells infected with AcNPV-scFv alone (70%) or coinfected with AcNPV-scFv and AcNPV-SipSsa43. Coexpression of wild type SipS also affected the levels of scFv found in the extracellular medium (Fig. 6). Densitometry analysis of multiple immunoblots revealed that the level of accumulated scFv in the extracellular medium of cells coinfected with AcNPV-scFv and AcNPV-SipS (+wt) was approximately 3.5 times higher than cells coinfected with AcNPV-scFv and AcNPV-SipSsa43 (+mut).

DISCUSSION

Expression and effects of SipS in insect cells. Western blot analysis revealed the presence of SipS in insect cells infected with a recombinant baculovirus containing the gene for a B. subtilis signal peptidase. When expressed in bacteria, SipS migrates on a SDS-PAGE gel at an approximate molecular weight of 21 kDa, however, in insect cells an additional band of approximately 24 kDa is also observed. Considering that the aa sequence of SipS reveals the presence of potential N-glycosylation sites, the 24 kDa form of SipS may be due to the addition of oligosaccharides. Tunicamycin treatment of cells during expression of SipS results in the reduction of the 24 kDa band and a subsequent increase in the 21 kDa band. The shift in MW of the 24 kDa protein to 21 kDa indicates that SipS may be glycosylated when expressed in the eukaryotic insect cell host. Oligosaccharide processing of bacterial proteins has been observed following their expression in eukaryotic hosts. (1,3-1,4)-β-
glucanase from Bacillus amyoliquefaciens and Bacillus macerans were both glycosylated when expressed in yeast (46). In addition, an endoglucanase from Clostridium thermocellum and (1,4)-β-glucanase from Bacillus subtilis were also N-glycosylated when expressed in Chinese hamster ovary cells (39, 40). Oligosaccharide processing of SipS also indicates that at least some of the bacterial SipS was transported into the ER. The lower molecular weight SipS may have also reached the insect cell ER but without any oligosaccharide processing. It is not unusual for glycoproteins overexpressed in insect cells using BEVS to be present in both glycosylated and non-glycosylated forms (47, 48). The presence of glycosylated and nonglycosylated forms may be due to incomplete oligosaccharide processing at high expression levels or the deterioration of host cell protein processing functions as a result of the baculovirus infection (49).

Both wild type and mutant SipS were expressed in insect cells; however, the wild type SipS was consistently produced at lower levels than the mutant (data not shown). The lower production level of wild type SipS is consistent with the trypan blue staining data that suggests wild type SipS has a detrimental effect on cell viability when expressed in High Five cells. The cause of the wild type SipS toxicity is not yet known. However, because the mutant SipS baculovirus is no more toxic to the insect cells than other recombinant baculoviruses tested (data not shown), the toxicity of expressed wild type SipS is probably not due to the protein's sequence or structure. The enzymatic activity present in wild type SipS may result in cleavage of factors instrumental in host cell function whereas the inactive mutant is benign.

In vivo signal peptide processing of a scFv. When expressed in High Five insect cells a large fraction of scFv was determined by Western blot analysis to be insoluble. Of this fraction, a considerable portion of the scFv polypeptide is in a precursor form. Immunoglobulin precursor light chain polypeptides and other pre-proteins including the signal peptide have also been shown to accumulate as insoluble aggregates in insect cells (10–15). As suggested in previous studies (28, 29) alteration of a eukaryotic signal peptide to an insect derived signal peptide is not sufficient of itself to overcome precursor processing limitations in insect cells. Apparently, there is an inherent bottleneck in the processing of scFv. One possible reason for the formation of insoluble precursor scFv is that, following baculovirus infection, a large fraction of synthesized scFv may undergo complete translation in the insect cell cytosol. Another possibility for precursor scFv accumulation is the lack of signal peptide processing of the polypeptide as it translocates across the ER membrane. When coexpressed with the wild type bacterial signal peptidase SipS, a significant reduction in the level of precursor polypeptide was observed in whole cell lysates when compared to coexpression of an inactive mutant SipS. Thus, it appears that overexpression of a bacterial signal peptidase can enhance signal peptide processing in the insect cell ER. This finding is compatible with previous observations which have demonstrated that bacterial signal peptidases can process eukaryotic signal peptides (25).

Wild type SipS coexpression with scFv also resulted in a 3.5 fold increase in scFv accumulation in the extracellular medium at 3 days pi. The extracellular scFv level increase may be the result of an increase in secretion or accumulation of intracellular protein from cell lysis due to SipS toxicity. The cell staining (trypan blue) analysis indicated lowered viabilities for cells expressing wild type SipS. Furthermore, even with coexpression of wild type SipS, only 25% of the total processed scFv produced during the 3 day infection period accumulates in the medium. The presence of significant levels of intracellular processed scFv suggests that scFv secretion may be limited at a post-processing step. Additional secretory bottlenecks may exist within the ER or in subsequent secretory compartments. Our research group previously reported that coexpression of the ER chaperone immunoglobulin-chain binding protein (BiP) resulted in a significant increase in secreted levels of IgG (50). BiP
assists protein assembly by preventing polypeptide aggregation during and following signal peptide cleavage. Subsequent oligosaccharide analysis suggested that IgG secretion also may be inhibited by a post-assembly bottleneck (50). Thus, multiple factors, including signal peptide processing, folding, assembly, and transport, appear to limit the secretory processing machinery in baculovirus infected insect cells (49). These limitations may be due to the deterioration of host cell factors as the viral infection progresses (10). The use of an earlier baculovirus promoter and a stable expression system have been employed in an attempt to offset the effects of host cell degradation late in the infection cycle (51–54). However, these promoters are not as strong as the polyhedrin promoter and protein expression levels are often reduced from the levels achieved with the polyhedrin promoter (51). Thus, production of recombinant secreted and membrane proteins in insect cells is a choice between enhanced protein expression or high levels of protein expression and reduced processing using strong late promoters. By coexpressing an earlier baculovirus promoter and a stable expression system have been employed in an attempt to offset processing limitations manifested in the production of recombinant secreted and membrane proteins under the control of very strong late baculovirus promoters.

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