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Functional Characterization of Bacterial Signal Peptide OmpA in a Baculovirus-Mediated Expression System

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ABSTRACT. **Signal sequences are evolutionarily conserved and are often functionally interchangeable between prokaryotes and eukaryotes. However, we have found that the bacterial signal peptide, OmpA, functions incompletely in insect cells. Upon baculovirus-mediated expression of chloramphenicol acetyltransferase (CAT) in insect cells, OmpA signal peptide led to the cytosolic accumulation of the CAT molecules in an aglycosylated, signal-peptide cleaved form, in addition to the secretion of the glycosylated CAT. When green fluorescent protein (GFP) was used as another reporter, the GFP molecules expressed from the OmpA-GFP construct was distributed primarily in the cytosol as aggresome-like structures. These results together suggest that, subsequent to the cleavage of OmpA signal peptide in the ER, some of the processed proteins are returned to the cytoplasm. Since the prototypical insect signal peptide, melittin, did not result in this ER-to-cytosol dislocation of the reporter proteins, we proposed a model explaining the dislocation process in insect cells, apparently selective to the OmpA-directed secretory pathway bypassing the co-translational transport.**

Key words: aggresome/baculovirus/ER dislocation/post-translational transport/signal peptide

In eukaryotic cells, the transport of secretory proteins with amino-terminal signal peptide begins with their translocation across the endoplasmic reticulum (ER) membrane, when signal peptides are cleaved off by signal peptidase, located at the lumenal face of the ER membrane (Martoglio and Dobberstein, 1998). The independent ability of signal peptides to direct nascent proteins to the secretory pathway is often capitalized in recombinant protein production to direct the latter to the secretion route. The baculovirus/insect cell expression system is one of the most powerful methods commonly used for the production of recombinant proteins (Smith *et al.*, 1983; O'Reilly *et al.*, 1992). As in the other expression systems, baculovirus vectors with secretory signal sequence have been developed to simplify purification of the expression product(s) (Tessier *et al.*, 1991; O'Reilly *et al.*, 1992). The earlier explorations of optimal signal peptides for application in the baculovirus expression system examined diverse signal peptides, derived not only from insects but also from several heterologous organisms, including bacteria (Bustos *et al.*, 1988; Iacono-Connors *et al.*, 1990; Jarvis *et al.*, 1993; Mroczkowski *et al.*, 1994). These signal peptides are usually processed normally in the baculovirus expression system, though low secretion efficiency has frequently been observed, independent of the signal sequence used (Jarvis *et al.*, 1993).

OmpA, an *E. coli* outer membrane protein, is synthesized as a precursor (pro-OmpA) with a prototypical signal peptide and is translocated across the bacterial inner membrane. The translocation process across the bacterial

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Abbreviations: CAT, chloramphenicol acetyltransferase; ER, endoplasmic reticulum; ERAD, ER-associated protein degradation; GFP, green fluorescent protein; hpi, hours-postinfection; MCS, multi cloning site; MOI, multiplicity of infection; MT, microtubule; MTOC, centrosome/microtubule-organizing center; PAGE, polyacrylamide gel electrophoresis; PNGase, peptide:N-glycosidase; SRP, signal recognition particle.

cytoplasmic membrane or the eukaryotic ER membrane can be either co-translational or post-translational. In the co-translational pathway, translocation occurs as the polypeptide chain is synthesized on membrane-bound ribosomes. In the post-translational pathway, however, the nascent polypeptide synthesis is completed on soluble ribosomes, before the polypeptide is translocated across the membrane (Walter and Johnson, 1994; Rapoport *et al.*, 1996). Although co-translational transport pathway in higher eukaryotes is the principal targeting route for secretory proteins, it has been shown in baker's yeast and the dog that pro-OmpA can be translocated into the ER via post-translational transport pathway (Sanz and Meyer, 1988; Matlack *et al.*, 1999), as in *E. coli* (SecA/SecB-dependent pathway) (Koch *et al.*, 1999; Scotti *et al.*, 1999). In both bacteria and yeast, the signal peptides of presecretory proteins thus specify the transport pathway (i.e., co- or post-translational pathway) (Ng *et al.*, 1996; Lee and Bernstein, 2001). Although various signal peptides are known to function in the baculovirus expression system, it is not known whether and how OmpA would function in insect cells.

In this work, we investigated the functioning of OmpA signal peptide in High Five insect cells using recombinant baculoviruses expressing the two common reporter genes, chloramphenicol acetyltransferase (CAT) and green fluorescent protein (GFP), each fused to the OmpA signal sequence. High Five cell lines infected with these baculoviruses expressed both reporters well and secreted a portion of each reporter protein population, but accumulated the remainder to the cytoplasm as mature proteins. We report the results here and propose a model predicting the possible mechanism by which OmpA signal peptide directs protein secretion in insect cells.

Materials and Methods

Preparation of recombinant viruses

Recombinant baculoviruses were generated using the Bac-to-Bac system (Invitrogen). Recombinant pFastBac1 plasmids were constructed as follows. To obtain pFastBac-nss-CAT and pFastBac-nss-GFP, coding sequences of CAT and GFP from pMAMneo-CAT (Clontech) and pEGFP-1 (Clontech), respectively, were subcloned into the MCS of pFastBac1. DNA sequence encoding the immunoglobulin light chain in pFastBac-OmpA-light-chain (that was constructed from the pFastBac1 and an inserted DNA fragment of a recombinant plasmid, pARA XT CHLOR TT [Barbas III *et al.*, 1991]) was replaced either with CAT or with GFP-coding sequence, to produce pFastBac-OmpA-CAT or pFastBac-OmpA-GFP. Similarly, pFastBac-mel-CAT and pFastBac-mel-GFP were constructed from pFastBac-mel-heavy-chain (that was created by subcloning the heavy chain-encoding DNA fragment of pARA XT CHLOR TT [Barbas III *et al.*, 1991] into pFastBac1 and by replacement of melittin-coding sequence of pMBac [Stratagene]) with CAT- or GFP-coding sequence. pFastBac-OmpA-CAT-AΔ1E, pFastBac-mel-CAT-AΔ1E, pFastBac-ns-CAT and pFastBac-ns-GFP were generated from the above constructs using the Transformer Site-Directed Mutagenesis kit (Clontech). Recombinant bacmids were prepared according to manufacturer's instructions using the transfer vectors as above. Sf9 cells were transfected with the recombinant bacmid DNA.

Production of recombinant proteins

High Five cells (Invitrogen), maintained in the insect cell culture medium, SF900-II (Invitrogen), were infected with a test virus preparation at an MOI of 5 or 10. After a 1-h adsorption period, the viral inoculum was replaced with fresh medium containing tunicamycin (5 μg/ml) as and when needed. The infected cells were incubated at 27°C. At 24 or 48 hours-postinfection (hpi), the culture medium and the infected cells were collected separately. The cells were washed with Tris-saline (50 mM Tris-HCl, pH 7.5, 140 mM NaCl) and lysed with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% beta mercaptoethanol, 10% glycerol) for SDS-PAGE/immunoblotting analysis. The culture medium was concentrated up to ~100× using Microcon-10 (Amicon), and appropriate volumes of the concentrate were analyzed by SDS-PAGE/immunoblotting, after boiling with SDS sample-buffer. For treatment with *N*-glycanase (PNGase) and endoglycosidase H, NP40 (final conc. 2%) was added to the cell lyastes in the SDS sample buffer and the sample mixtures were then incubated with *N*-glycanase (1 U [Roche]) or endoglycosidase H (1/200 U [Roche]) at 37°C for ~20 h. Following the addition of SDS (final conc. 3%), the enzyme-treated samples were analyzed by SDS-PAGE/immunoblotting.

Subcellular fractionation

High Five cells, infected with recombinant viruses as above, were collected at 24 and 48 hpi and homogenized using a micro-homogenizer. The homogenates were separated by low speed centrifugation at 8,000×*g* for 10 min, and the supernatants were centrifuged again at 100,000×*g* for 1 h. The supernatant obtained by this high speed-centrifugation was defined as the soluble fraction and analyzed by SDS-PAGE/immunoblotting.

Gel electrophoresis and immunoblotting

SDS-PAGE was performed according to Laemmli (1970). Proteins were transferred onto PVDF membranes using a semi-dry apparatus, and the membrane was incubated with anti-CAT (5 Prime-3 Prime) or anti-GFP (Molecular Probes) antibodies and developed with the ECL detection kit (Amersham). To quantify the immunoreactive bands, the treated membranes were scanned using Molecular Imager GS250 (Bio-Rad) and analyzed using the Molecular Analyst software (Bio-Rad).

Confocal microscopy

To visualize fluorescence of viable cells expressing GFP, the cells were seeded in glass-bottom dishes and were infected with the GFP-expressing viruses. At 24 or 48 hpi, the infected cells were

analyzed using a confocal microscope, equipped with an argon-krypton laser (Leica TCS-NT). Green fluorescence was detected at λ>515 nm, following excitation by 488 nm Ar laser. For treatment with nocodazole, the infected cells were cultured throughout the experimental period in medium containing the drug (10 μg/ml).

Pulse-chase analysis

High Five cells were infected with recombinant viruses, as above. At 23 hpi, the culture medium was replaced with methionine-free medium, with tunicamycin (5 μg/ml) included as and when needed. After 1-h deprivation period, the infected cells were incubated with [^{35}S]methionin/cysteine (500 μCi met eq./ml) for 5 min and were lysed directly with SDS-saline (50 mM Tris-HCl, pH 7.5, 1% SDS, 140 mM NaCl), or following incubation for 120 min with the chase-medium including methionine (0.5 mg/ml) and cysteine (0.5 mg/ml), and tunicamycin (5 μg/ml), when required. After boiling for 3 min, the resultant cell lysates were mixed successively with Triton X-100 (2%) and anti-GFP (Molecular Probes) or anti-CAT (5 Prime-3 Prime) antibodies and incubated for 1 h at room temperature. Then, protein A-agarose beads (quarter volume) were added to the mixture and incubated for another 1 hour, with occasional agitation. After the protein A-beads were washed, twice with Triton-saline (50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 140 mM NaCl) and twice with Tris-saline, the immunoprecipitates were finally collected into SDS sample-buffer and subjected to SDS-PAGE. The [^{35}S]-labeled polypeptides in dried polyacrylamide gels were detected using a PhosphoImager (BAS 2500, Fuji Photo Film Co.) or by fluorography.

Results

Insect cells expressing OmpA-CAT show intracellular accumulation of CAT in both glycosylated and aglycosylated forms

In this study, we investigated the functional mechanism of OmpA signal peptide in the baculovirus expression system utilizing two well-characterized reporter genes, CAT and GFP. The reporter genes were expressed in fusion with OmpA signal sequence (OmpA-CAT and OmpA-GFP) or, as a control, with melittin, a prototypical insect signal sequence (Mroczkowski *et al.*, 1994) (mel-CAT and mel-GFP), under the control of polyhedrin promoter (Fig. 1A).

As the first step, cultured insect cells were infected with recombinant viruses, each expressing the signal sequence-CAT construct, and the infected cells as well as the culture medium were collected separately at various time points and analyzed by SDS-PAGE/immunoblotting. The overall expression level of each CAT construct was four times as high at 48 hpi as at 24 hpi, but both constructs displayed decreasing secretion efficiency with increasing time after infection, regardless of the signal peptide used (see below). The secretion efficiency of each protein, assessed as the ratio of secreted to total product, was 33% and 17% at 24 hpi, and 12% and 6% at 48 hpi for OmpA-CAT and mel-CAT, respectively. As indicated by the relatively low (<3%) efficiency of unmodified CAT (nss-CAT, with neither signal sequence nor spacer [Fig. 1A]), it is clear that the observed low efficiency of secretion of OmpA-CAT and mel-CAT was not a result of non-specific leakage from insect cells due to viral infection. Rather, these data suggest that while a minor fraction of the expression products was actively secreted, a larger fraction was retained intracellularly. The progressively decreasing efficiency of overall secretion for heterologous proteins has been demonstrated to be intrinsic to the baculovirus/insect cell system (Jarvis *et al.*, 1993).

Although CAT intrinsically is a bacterial cytoplasmic protein, since the primary sequence of native CAT has one potential N-glycosylation site (Asn34), we were interested in finding out if the glycosylation site would be recognized by insect cells and if there is a correlation between secretion and glycosylation status, viz., to determine whether the secreted, the intracellular, or both fractions of CAT were glycosylated. To this end, we examined the effect of tunicamycin, an inhibitor of N-linked glycosylation, on CAT expression. The culture medium of the untreated control cell line, irrespective of the signal peptide used, showed a single 30-kDa band at 24 hpi (Fig. 1B, lanes 1 and 3, band d) as well as at 48 hpi (data not shown). However, tunicamycin-treated cell lines at 24 and 48 hpi showed a 27-kDa position (band b) instead of at 30-kDa (lanes 2 and 4). This indicated that the 30- and 27-kDa bands were the glycosylated and aglycosylated versions of CAT, respectively, and that the secreted CAT was glycosylated.

A profile similar to that seen with the culture media was obtained with each cell lysate at 24 hpi, except that a 27-kDa band, in addition to the 30-kDa band, was detected in cells expressing OmpA-CAT in the absence of tunicamycin treatment (Fig. 1B, lane 7). Detection of a 30-kDa band (band d) in the cells expressing mel-CAT in the absence of tunicamycin (lane 9) indicated that most of the CAT that was retained intracellularly, akin to the secreted CAT, was glycosylated. However, since both 30- and 27-kDa bands were simultaneously detected in the cells expressing OmpA-CAT in the absence of tunicamycin (lane 7), but only 27-kDa band was seen in its presence (lane 8), a significant proportion of intracellular CAT seems to have remained unglycosylated or undergone deglycosylation. In the unmodified control, CAT (nss-CAT) migrated at the position of Mr. ~25 kDa (band a), as expected by its predicted molecular mass (lanes 11 and 12). The observed difference in molecular mass between the unmodified CAT (25 kDa) and the 27-kDa CAT was consistent with the estimated size of the spacer region (Fig. 1A, and see below). To confirm the glycosylation status of 30- and 27-kDa bands, we further performed experiments in which infected cell lysates were treated with *N*-glycanase (PNGase) or endoglycosidase H, instead of tunicamycin. As expected, both treatments promoted a shift of 30-kDa band to 27-kDa position

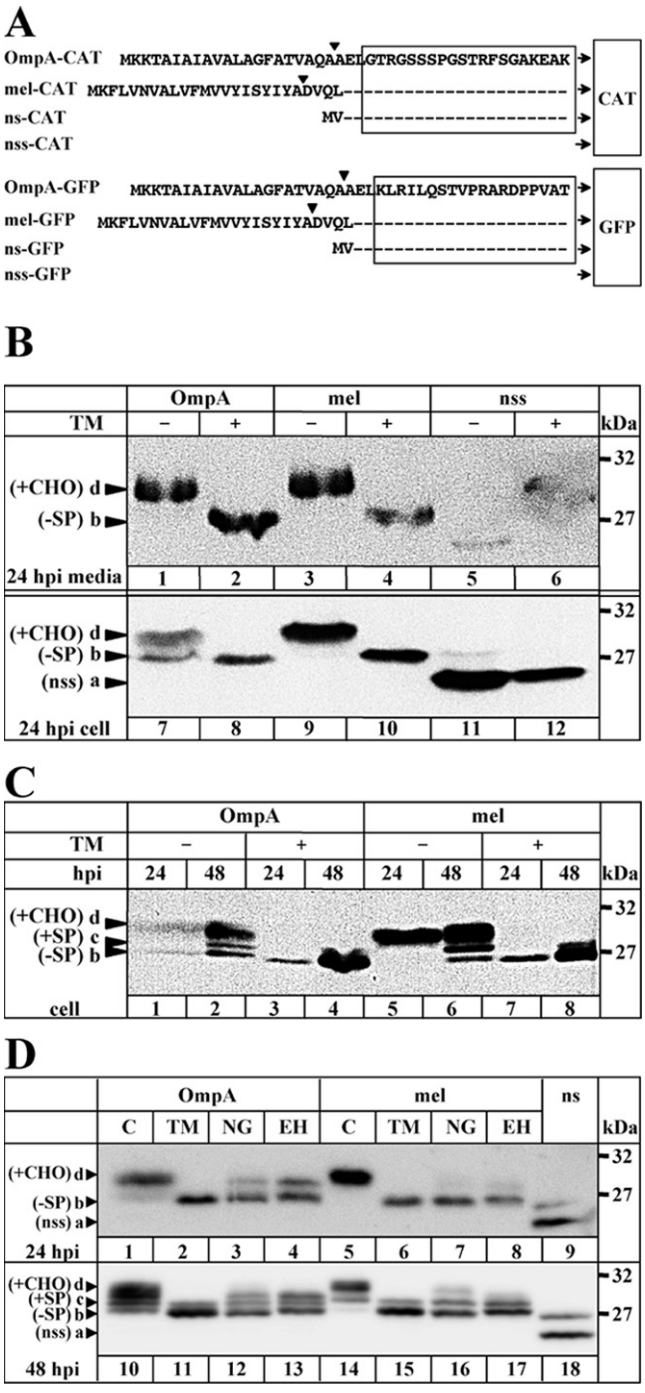


Fig. 1.

(Fig. 1D, lanes 3, 4, 7 and 8), revealing that the 30- and 27-kDa bands were the glycosylated and aglycosylated versions, respectively, of the CAT molecules. Glycosylated CAT molecules (band d) often showed inconsistent mobility in immunoblots (e.g. Fig. 1B, lane 7). Since the band pattern of nascent glycosylated CAT molecules shown in a pulse-labeling experiment without chase time was sharper than that with chase time (see Fig. 5B), the modification of their oligosaccharide seemed to partly contribute to their heterogeneity.

Aglycosylated CAT produced by insect cells expressing OmpA-CAT is mature

At 48 hpi, in addition to the 30-kDa and 27-kDa bands, a minor band of ~28 kDa (band c) was detectable in lysates of all the cells (each expressing OmpA-CAT or mel-CAT) irrespective of whether or not tunicamycin was present (Fig. 1C lanes 2, 4, 6 and 8 and 1D lanes 10, 11, 14 and 15). The 28-kDa band (band c), by inference from its late appearance after infection (Fig. 1C and D) and its increased Mr. size, appears to be aglycosylated, immature CAT, since signal peptides tend to remain increasingly uncleaved with increasing time after infection. To determine the status of signal peptide cleavage of the two aglycosylated forms, 27-kDa and 28-kDa CAT (bands b and c), we generated two types of recombinant baculoviruses: one, expressing CAT with "uncleavable" signal peptide (AΔ1E), and the other expressing CAT with the spacer region but without signal peptide (ns-CAT) (Fig. 1A).

To block signal peptide cleavage, the Ala residue at –1 position (immediately in front of the cleavage site) in each

Fig. 1. Expression of CAT in insect cells. (A) Amino acid sequences of the amino-terminal ends of the reporter proteins analyzed. Identical amino acid sequences in spacer regions generated from the recombinant plasmids are boxed. Arrowheads mark the cleavage sites of native signal-peptidase. (B) High Five cells were infected with the recombinant baculoviruses expressing CAT constructs with OmpA (OmpA) or melittin (mel) signal sequence and were cultured in the presence (+) or absence (–) of tunicamycin (TM, 5 μg/ml). The culture media at 24 hpi (lanes 1–6) and the infected cells at 24 hpi (lanes 7–12) were collected and analyzed by SDS-PAGE/immunoblotting. Baculovirus with the sequence coding for unmodified CAT (nss; neither signal sequence nor spacer) was included as control. The positions of different forms of CAT, i.e., unmodified (a), signal peptide-cleaved but aglycosylated (b) and glycosylated (d) are indicated on the left. (C) To facilitate efficient comparison between 24-hpi cells (24) and 48-hpi cells (48), infected with baculoviruses expressing OmpA-CAT or mel-CAT, both sets of samples were loaded on the same gel. The positions of different forms of CAT, i.e., signal peptide-cleaved but aglycosylated (b), signal peptide-uncleaved (c) and glycosylated (d) are indicated on the left. (D) High Five cells infected with the recombinant baculoviruses, each expressing the indicated CAT construct (ns: no signal sequence but with spacer) were cultured in the absence or presence of tunicamycin (TM, 5 μg/ml) and were collected at 24 hpi (lanes 1–9) and 48 hpi (lanes 10–18). The collected samples were untreated (C) or treated with *N*-glycanase (NG) or endoglycosidase H (EH), followed by SDS-PAGE/immunoblotting. The markings, a–d, on the left, indicate various forms of CAT, as described above.

construct (OmpA-CAT and mel-CAT) was replaced with Glu (OmpA-CAT-AΔ1E and mel-CAT-AΔ1E, respectively), resulting in violation of the (–3, –1) rule (von Heijne, 1985). In the absence of tunicamycin, the cells expressing OmpA-CAT-AΔ1E showed mainly 28-kDa band (Fig. 2, band c), irrespective of the harvesting time, 24 hpi or 48 hpi (lanes 1 and 7). In the same immunoblots exposed for longer time, 30-kDa band (band d), in addition to the 28-kDa band, was observed (data not shown). The cells treated with tunicamycin, however, showed only a single band of 28-kDa both at 24 hpi and 48 hpi (lanes 2 and 8). The untreated control cells expressing mel-CAT-AΔ1E, on the other hand, showed a single 30-kDa band, while tunicamycin-treated cells showed a single 28-kDa band at both 24 hpi and 48 hpi (lanes 13, 14, 19 and 20). This result clearly suggests that the 28-kDa band (band c) corresponds to aglycosylated CAT with intact signal peptide.

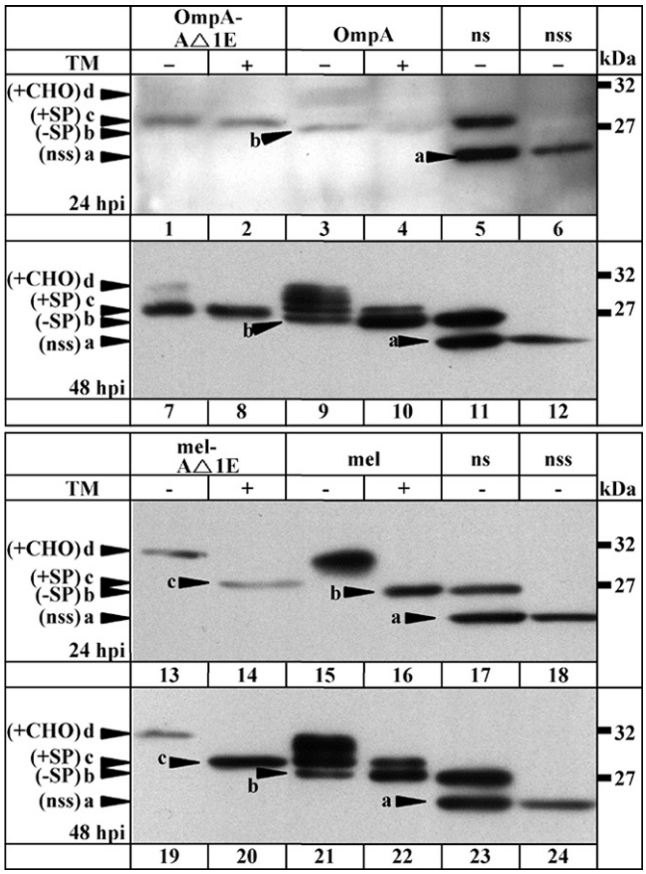


Fig. 2. Determination of the signal peptide-cleavage status of CAT. High Five cells were infected with the recombinant baculoviruses, each expressing a different type of CAT construct as in Fig. 1A (AΔ1E indicates CAT with uncleavable signal peptide due to the replacement of Ala with Glu at –1 position). The infected cells were cultured in the presence (+) or absence (–) of tunicamycin (TM, 5 μg/ml) and were collected at 24 or 48 hpi for SDS-PAGE/immunoblotting. The markings, a–d, on the left, indicate various forms of CAT, as described in Fig. 1 legend.

On the contrary, ns-CAT (the construct with the spacer region but no signal peptide) yielded two bands of 25- and 27-kDa (Fig. 2, lanes 5, 11, 17 and 23, bands a and b), regardless of whether or not tunicamycin was present (data not shown). Since the 25-kDa band co-migrated with nss-CAT and the initiation AUG codon in the ns-CAT construct does not obey Kozak's rule (Kozak, 1984), we suspected the 25-kDa protein may be an alternative translational product generated by the recognition of the downstream AUG codon in native CAT sequence. As ns-CAT migrated at the position of 27-kDa (band b), but not 28-kDa (band c), this result supports the conclusion that the two bands of 27- and 28-kDa (bands b and c) represent a mature aglycosylated CAT and its precursor with uncleaved signal peptide, respectively. Thus, the presence of the 27-kDa band in OmpA-CAT-expressing cells at 24 hpi, in contrast to cells expressing mel-CAT, demonstrates that a significant population of OmpA-CAT remains unglycosylated or undergoes deglycosylation, in spite of the signal peptide being cleaved off by a signal peptidase located in the ER.

Insect cells expressing OmpA-CAT but not those expressing mel-CAT accumulate aglycosylated mature CAT in the cytosol

The proteins that are misfolded or unassembled in the ER are rapidly degraded. This ER-associated protein degradation (ERAD) is mediated by a cytosolic proteolytic machinery, the proteasome (for reviews, see Kopito, 1997; Brodsky and McCracken, 1997; Plemper and Wolf, 1999). Overexpression of ERAD substrates often leads to their accumulation in the cytosol or on the cytoplasmic face of the ER membrane, where they are deglycosylated (Johnston *et al.*, 1998). In other words, such dislocated proteins appear to be mature, but aglycosylated. To test whether insect cells also have these OmpA-directed CAT accumulations (i.e., aglycosylated, mature CAT) in the cytosol, we subjected soluble fractions of OmpA-CAT-expressing cells to immunoblotting with anti-CAT antibody. The baculovirus-infected cells were homogenized in a microtube and centrifuged at 8,000×*g* for 10 min. The resulting supernatant was then centrifuged at 100,000×*g* for one hour, and the supernatant of this high-speed centrifugation was used as the soluble fraction. In a preliminary experiment by our method, a membrane fraction including microsomes seemed to be recovered predominantly in the precipitation of the low-speed centrifugation but not of the high-speed one (data not shown). Nevertheless, over 90% of the soluble, cytosolic fraction was recovered in the supernatant of the high-speed centrifugation (Fig. 3A). Therefore, we compared the supernatant of the high-speed centrifugation with the corresponding total cell lysate to demonstrate the cytosolic recovery of CAT molecules plainly (Fig. 3B).

CAT recovered at 24 hpi in the soluble fraction of the cells expressing OmpA-CAT, but for a tiny fraction, was

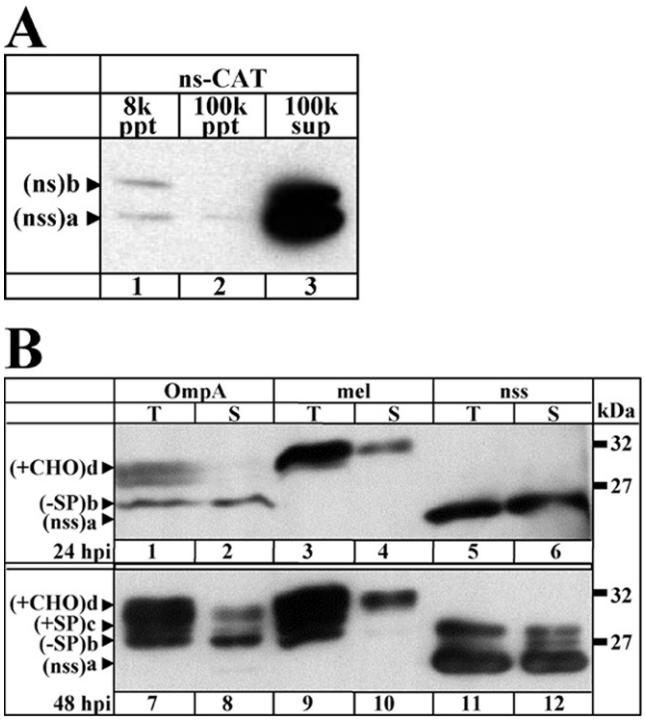


Fig. 3. Accumulation of CAT in soluble fraction of insect cells. (A) High Five cells infected with the recombinant baculovirus expressing ns-CAT (the construct with the spacer region but no signal peptide) were harvested at 24 hpi and homogenized in a microtube. The homogenate was centrifuged at 8,000×*g* for 10 min and the resulting precipitation was recovered as the low-speed precipitation fraction (8 k ppt). Its supernatant was again centrifuged at 100,000×*g* for one hour and the precipitation and the supernatant of this high-speed centrifugation were collected separately and used as the high-speed precipitation fraction (100 k ppt) and its supernatant fraction (100 k sup) respectively. These fractions were analyzed by SDS-PAGE/immunoblotting. (B) High Five cells were infected with the recombinant baculoviruses each expressing a CAT construct with OmpA (OmpA) or melittin (mel) signal sequence. At 24 or 48 hpi, the infected cells were collected and applied to SDS-PAGE/immunoblotting in the form of total cell lysate without subcellular fractionation (T) or as soluble fraction following high-speed centrifugation (S). Baculovirus with the sequence coding for unmodified CAT (nss: neither signal sequence nor spacer) was included as control. The markings, a–d, on the left, indicate various forms of CAT, as described in Fig. 1 legend.

aglycosylated (compare bands d and b in lanes 2 and 1 in Fig. 3B). This result suggests that aglycosylated, mature CAT accumulates mainly in the cytosol at 24 hpi. Although some proportion of glycosylated form of each CAT (band d) was also recovered in the soluble fraction (lanes 2 and 4), we could not determine whether it was an artifact or a result of viral infection or other physiological phenomena. In the control experiment, where CAT was expressed without any signal peptide (nss-CAT, band a), most of the expression product was recovered in the soluble fraction, as expected (compare lane 6 with lane 5).

At 48 hpi, as seen above (Figs. 1 and 2), aglycosylated, mature CAT was detectable in the cells expressing mel-CAT as well as in the cells expressing OmpA-CAT (Fig. 3B, lanes 7 and 9, band b). However, this form of CAT was not detectable in the soluble fraction of the cells expressing mel-CAT (Fig. 3B, lane 10, band b), suggesting that a aglycosylated, mature CAT that was specifically generated at a late stage of viral infection accumulated possibly in the ER but not in the cytosol. By contrast, a large amount of aglycosylated, mature CAT (band b) was still recovered at 48 hpi in the soluble fraction of cells expressing OmpA-CAT (lane 8) as well as in the cells at 24 hpi, suggesting that a significant proportion of aglycosylated, mature OmpA-CAT was stably distributed in the cytosol at a late stage of viral infection. Thus, cytosolic accumulation of aglycosylated, mature CAT seems to be selective to OmpA-CAT. Taken together with the fact that cleavage of signal peptide occurs in the lumen of ER, these results suggest that CAT expressed by OmpA-CAT, but not mel-CAT, is dislocated from the ER to the cytosol as a aglycosylated, mature protein.

GFP produced by insect cells expressing OmpA-GFP is also localized in the cytosol as aggresome-like structures

To examine whether cytosolic distribution of the OmpA signal peptide-directed, mature proteins is a phenomenon exclusive to CAT, a glycoprotein, we generated recombinant baculoviruses, each expressing a reporter protein without glycosylation sites, GFP, in fusion with each of the signal peptides, OmpA and melittin (Fig. 1A). Before embarking on microscopic analysis, we performed immunoblotting of the lysates of the insect cells expressing GFP. As shown in Fig. 4A, a single band of ~30-kDa (band b) was observed at 24 hpi (lanes 1 and 2), while a minor band of ~32 kDa (band c), in addition to the 30-kDa band, was detected at 48 hpi (lanes 5 and 6) in the cells expressing OmpA-GFP and mel-GFP. The ns-GFP construct (with the spacer region but no signal peptide) produced two bands, of ~28- and 30-kDa (lanes 3 and 7), the former of which co-migrated with nss-GFP (with neither signal peptide nor spacer) (lanes 4 and 8), as in the case of CAT. Since GFP has no N-glycosylation sites, this result suggests that the 30-kDa band (band b) at 24 hpi corresponds to mature GFP. Appearance of a 32-kDa band at 48 hpi, on the contrary, suggests the possibility that the signal peptidase activity in the ER may become insufficient or inefficient with increasing time after infection.

Panels B-I in Fig. 4 show typical confocal microscopic fluorescent images of GFP-fusions. At 24 hpi, fluorescence from GFP was detectable selectively in the perinuclear region of the cell expressing mel-GFP, but not in the nucleus (panel C). However, a significant level of nuclear fluorescence as well as localized bright perinuclear fluorescence was observed in the cells expressing OmpA-GFP (panel B). In the control cells expressing ns-GFP (with the spacer region but no signal peptide) and nss-GFP (with

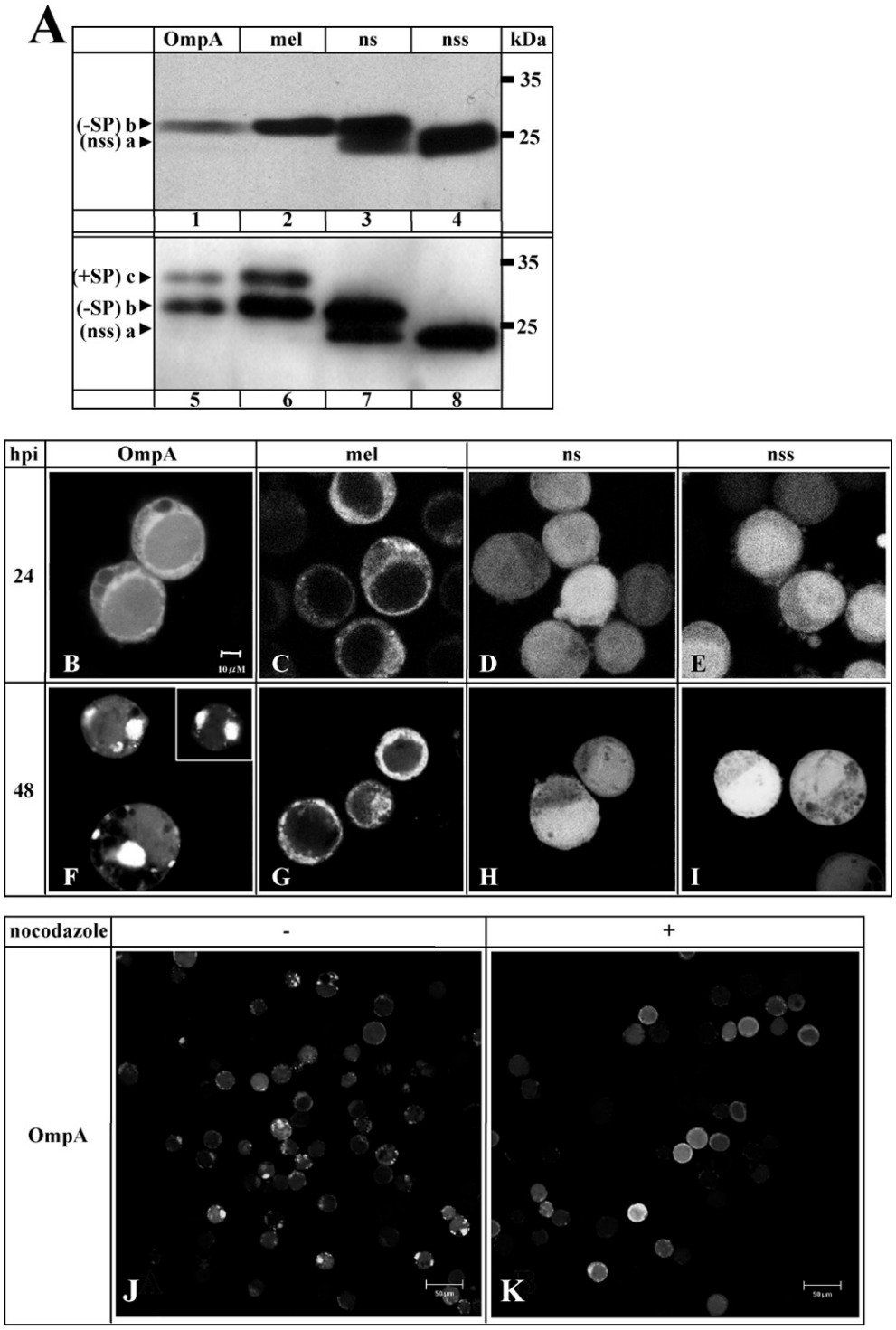


Fig. 4. Expression of GFP in insect cells. (A) High Five cells were infected with the recombinant baculoviruses, each expressing a different GFP construct as shown in Fig. 1A. At 24 or 48 hpi, the infected cells were collected and analyzed by SDS-PAGE/immunoblotting. The positions of unmodified (a), signal peptide-cleaved (b), and signal peptide-uncleaved (c) GFPs are indicated on the left. (B–I) At 24 (B–E) and 48 (F–I) hpi, High Five cells, infected with recombinant viruses, each expressing OmpA- (B and F), mel- (C and G), ns- (D and H) or nss- (E and I) GFP, were analyzed by confocal microscopy. Bar, 10 μm. (J and K) High Five cells were infected with the recombinant baculoviruses expressing OmpA-GFP and were cultured in the absence (J) or presence (K) of nocodazole (10 μg/ml). At 48 hpi, the infected cells were analyzed by confocal microscopy. Bar, 50 μm.

neither signal peptide nor spacer), fluorescence was distributed in the nucleus as well as cytoplasm (panels D and E), since in mammalian cells GFP can pass through the nuclear pore by passive transport (reviewed in Gerdes and Kaether, 1996). Combined with the immunoblotting data, this result suggests that a significant proportion of GFP in the cells expressing OmpA-GFP was exported to the cytosol, in spite of the signal peptide being cleaved off. This observation supports and extends the conclusion that a considerable population of OmpA signal peptide-directed proteins is dislocated from the ER back to the cytosol, irrespective of whether or not the protein intrinsically is a glycoprotein.

At 48 hpi, while the intensity of fluorescence increased markedly, its distribution remained essentially unchanged in cells expressing mel-GFP (Fig. 4, panel G). However, fluorescence distribution in the cells expressing OmpA-GFP showed a strikingly different pattern, where a small number (usually, one or two) of large bright patches were observed in the cytoplasm with relatively diminished levels of nuclear fluorescence (panel F).

Previously, Johnston *et al.* (1998) have reported a novel intracellular structure in the course of the investigation of the ERAD mechanism. They found that when the capacity of the proteasome is exceeded either by overexpression of ERAD substrates or by inhibition of the proteasome activity, the substrates exported to the cytosol, instead of being degraded, are delivered to a distinct pericentriolar structure, known as the aggresome. Aggresomes form at the centrosome/microtubule (MT)-organizing center (MTOC) (Wigley *et al.*, 1999; García-Mata *et al.*, 1999). The accumulation sites of GFP in the cells (48 hpi) expressing OmpA-GFP in juxtanuclear position as well as the aggresome, and the symmetric accumulation of GFP at both sides of the cells (e.g. inset in Fig. 4F) suggest potential arrest of infected cells at the mitotic phase, where MTOC should be symmetrically distributed between the two sides. The similarities between aggresome and the GFP-accumulating structure in cells expressing OmpA-GFP with respect to their fluorescent images and behavior (dislocation from the ER to the cytosol), suggest that GFP possibly accumulates in aggresomes. We explored this possibility by examining the effect of a MT-disrupting reagent, nocodazole, on the accumulation pattern of GFP in cells expressing OmpA-GFP. As can be seen in Fig. 4 J and K, although aggresome-like structures were observed in control cells without nocodazole-treatment (panel J), fluorescence distribution was relatively dispersed through the nucleus as well as cytoplasm in nocodazole-treated cells (panel K). This inhibitory effect of nocodazole suggests that cytosolic GFP may actually be transported into aggresome, possibly by retrograde motors in MT.

Pulse-labeling without chase shows transient immature precursors in insect cells expressing OmpA-fusions

As demonstrated earlier, OmpA- and mel- constructs show sharply contrasting behavior in insect cells. Hence, to identify the distinct pathways involved in the generation of such contrasting behavior, we analyzed the cells expressing each construct using pulse-chase analysis. Insect cells infected with the recombinant viruses were pulse-labeled at 24 hpi with [^{35}S]methionine/cysteine for 5 min and chased subsequently in the presence of excess amino acids for 120 min, followed by immunoprecipitation with anti-GFP or anti-CAT antibodies.

In the cells expressing mel-GFP, signal-peptide immature precursors could not be detected by pulse-labeling even for 5 min without chase time, as well as with chase time allowed for 120 min (Fig. 5A, lanes 3 and 4), indicating that cleavage of the signal peptide occurred with little or no lag following the polypeptide synthesis, presumably in a co-translational fashion. In OmpA-GFP-expressing cells (lanes 1 and 2), on the contrary, in addition to mature GFP (band b), an immature precursor (band c) was clearly evident upon pulse-labeling, but the latter disappeared or was considerably diminished after a 120-min chase (lane 2). These results reveal that at least a portion of GFP population in the cells expressing OmpA-GFP is processed post-translationally and that the translocation process of OmpA-GFP is distinctively different from the 'obligate' co-translational translocation observed in cells expressing mel-GFP. In the pulse-chase analysis of CAT, we obtained a similar result with tunicamycin-treatment (Fig. 5B, lanes 2, 4, 6 and 8). In its absence, aglycosylated precursors were observed in OmpA-CAT-expressing cells (lane 5, band c) but not in mel-CAT-expressing cells, where co-translationally glycosylated CAT was predominant (lane 1, band d). Taken together, these results suggest that OmpA signal peptide-directed proteins likely are being translocated across the ER membrane via a 'contingent' post-translational pathway. Previous reports have noted that pro-OmpA can be translocated into yeast or canine microsome post-translationally (Sanz and Meyer, 1988; Matlack *et al.*, 1999).

Discussion

Owing to their evolutionarily conserved homology, signal peptides are often functionally interchangeable even between prokaryotes and eukaryotes (von Heijne, 1985; Pugsley, 1989). Several studies have shown signal peptides of diverse origins to be capable of efficiently directing proteins into the ER followed by proper maturation in baculovirus-infected insect cells (O'Reilly *et al.*, 1992). In this study, we found that bacterial signal peptide, OmpA, can be completely cleaved in baculovirus-infected cells by 24 hpi (Figs. 1B [lanes 7 and 8] and 4A [lane 1]) and, afterward, can be done with the same efficiency as signal peptides of

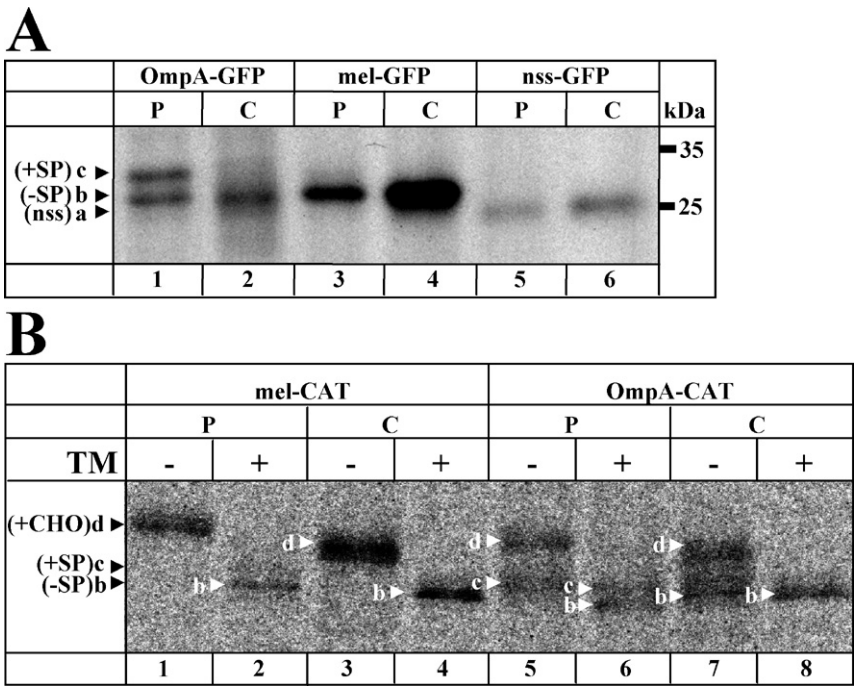


Fig. 5. Pulse-labeling of insect cells infected with recombinant viruses. High Five cells were infected with the recombinant baculoviruses, each expressing the indicated GFP construct (A) or CAT construct (B). After 24-h incubation period in the absence (A and B [–]) or presence (B [+]) of tunicamycin (TM, 5 μg/ml), the infected cells were pulse-labeled with [^{35}S]methionine/cysteine for 5 min (P) and chased for 120 min (C) in the absence (A and B [–]) or presence (B [+]) of tunicamycin. The labeled cells were lysed and used for immunoprecipitation with anti-GFP or anti-CAT antibody. The markings, a–c, on the left of panel A, indicate different forms of GFP as described in Fig. 4 legend and b–d, on the left of panel B, indicate different forms of CAT as described in Fig. 1 legend.

insect origin (Figs. 1C [compare lanes 2 and 6] and 4A [compare lanes 5 and 6]). Surprisingly, however, we also found that, following maturation in the ER, recombinant proteins fused to OmpA signal peptide are returned to the cytosol in a significant proportion as aglycosylated proteins.

OmpA signal peptide-directed proteins are structurally indistinguishable (at least with respect to primary structure) from those with melittin signal peptides after their maturation in the ER. Since signal peptide cleavage is thought to be an early event taking place during the translocation of the preprotein across the ER membrane (Helenius, 1994), structural differences between OmpA fusions and melittin fusions might be hardly distinguishable by cellular machinery in the ER, if they should be subjected to an identical translocation pathway. Therefore, the fact that OmpA fusions, but not melittin fusions, were dislocated from the ER (i.e., occurrence of distinct behavior between OmpA fusions and melittin fusions) led us to speculate that an alternative anterograde transport pathway existed for OmpA fusions.

In vitro experiments using the microsomes of yeast or dog pancreas have shown that proOmpA can be translocated into the ER via post-translational pathway (Sanz and Meyer, 1988; Matlack *et al.*, 1999). Although relatively few proteins translocate via post-translational pathway in higher eukaryotes, the components of its machinery, homologs of sec62 and sec63, have been identified in the ER of various mammals including dog pancreas microsomes (Meyer *et al.*, 2000; Tyedmers *et al.*, 2000). Yeast studies have also demonstrated that signal peptides not only specify whether a protein is to be retained in the cytoplasm or is secreted, but also determine the transport pathway utilized, i.e., whether the nascent protein may enter the co-translational pathway or the post-translational pathway (Ng *et al.*, 1996). These data raise the tantalizing possibility that OmpA signal peptide could serve as a determinant for post-translational transport in eukaryotes. The results of our pulse-chase study support this hypothesis (Fig. 5). Non-detectability of the pre-secretory precursors of mel-fusions by short pulse labeling suggests co-translational processing of the signal peptide(s). On the other hand, processing of OmpA signal peptide, at least in part, appears to be post-translational. These data support the hypothesis that, while the melittin signal peptide follows the co-translational pathway, the OmpA signal peptide follows the post-translational pathway. Alternatively, whereas a considerable proportion of proteins in cells expressing OmpA-constructs are post-tanslationally translocated (and dislocated to the cytosol), the

others are co-translationally translocated and are subsequently secreted or retained in the ER. Although this possibility could not be ruled out currently, the secretory efficiency of GFP is quite different between OmpA-GFP and mel-GFP (56% and 10% at 24 hpi, and 30% and <3% at 48 hpi, for OmpA-GFP and mel-GFP, respectively). An identical (co-translational) pathway for both secreted OmpA- and mel-constructs cannot explain the observed relative secretory efficiency of GFP in the cells expressing OmpA-GFP. It rather implies the existence of distinct retention mechanisms in each transport pathway, that is, a continuity of the diverse pathways beyond the ER membrane.

Ng *et al.* (1996), after analyzing SRP (signal recognition particle) recognition patterns of signal peptides in the yeast, have proposed that the degree of hydrophobicity of a signal peptide could be the determinant of, and consequently can be used as a guide for predicting, the protein transport pathway selected by that signal peptide. According to their hypothesis, a signal sequence with a more hydrophobic core would opt for the co-translational pathway for transport of the target protein into the ER. Therefore, to verify the significance of the hydrophobic degree of OmpA signal peptide in selecting its transport pathway, we generated several recombinant baculoviruses expressing GFP in fusion with mutated OmpA signal peptides. Each mutation comprised several substitutions (from one to six) of leucine for alanine. These replacements, however, did not lead to the changing of the pathway from "OmpA" to "mel", suggesting that the degree of hydrophobicity of a signal peptide did not always contribute to the selection of the protein transport pathway in insect cells (Kawasaki *et al.* unpublished data).

Yeast post-translational pathway is highly efficient and dislocation of its substrates is selective for specific proteins, such as mutated proteins (Plemper and Wolf, 1999). In insect cells, however, the dislocation of substrates for the post-translational pathway seems to be independent of their structural specificity. Rather, any substrates for "post"-pathway could be dislocated from the ER back to the cytosol in insect cells. Therefore, the dislocation (or incomplete translocation) of presecretory proteins in insect "post"-pathway may be due to its rather inefficient translocon relative to yeast "post-translational" translocon (Sec complex). While the "co"-pathway in higher eukaryotes appears to have evolved as a main route for proteins crossing the ER membrane, the "post"-pathway could be regarded as a supplementary or salvage route, or even as a vestigial pathway.

Post-translational translocation of proteins in OmpA constructs could help dissect possible mechanisms of their specific dislocation. Accordingly, we propose a model based on the assumption of back-and-forth sliding movements of protein in OmpA constructs in the translocon (the 'back-sliding' model in Fig. 6). In this model, post-translational translocation across the ER membrane in insect cells is an inefficient process, where some of the translocating intermediates move into the ER lumen, while others slip backward to the cytosol after the cleavage of their signal-peptides. The back-sliding of translocating intermediates in post-translational transport has been demonstrated using a reconstituted system (Matlack *et al.*, 1999). Cytosolic HSP70, an abundant molecular chaperon, may be a key molecule facilitating the dislocation mechanisms, as it has been postulated to be involved not only in post-translational

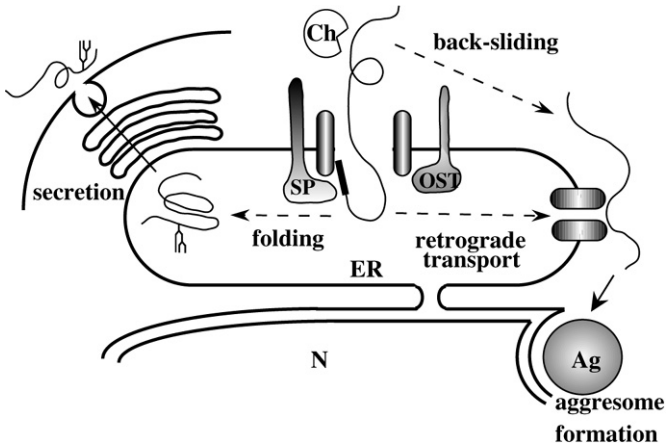


Fig. 6. A model depicting possible intracellular fates of OmpA signal peptide-directed proteins. In the initial step of the model, OmpA signal peptide, post-translationally recognized by the insect cell translocon, would initiate translocation of the reporter protein across the ER membrane. This results in the maturation and proper folding of the recombinant protein in the lumen of the ER. The properly folded proteins would then exit from the ER and be secreted. The proteins that are inefficiently translocated or otherwise marked for elimination from the ER/secretory pathway, following initial processing in the ER, are dislocated by one of two plausible mechanisms from the ER to the cytosol to form aggresomes. One dislocation mechanism envisaged is the backsliding of translocating intermediates before completion of import into the ER, while the other involves retrograde transport at the completion of import into the ER. OST: oligosaccharyltransferase; SP: signal peptidase; Ch: molecular chaperon. See text for further details.

anterograde translocation (Gething and Sambrook, 1992), but also in retrograde translocation (Fisher *et al.*, 1997). In another model (the 'retrograde-transport' model, Fig. 6), based on the involvement of the ER quality control system, the substrates for post-translational pathway are temporarily transported into the ER lumen, but they are recognized by the quality control system for as yet unknown reasons and are marked for ER dislocation. Since most substrates for ER dislocation have been reported to be misfolded or unassembled proteins (Plemper and Wolf, 1999), the folding process of translocated proteins may be less efficient in the insect post-translational transport pathway.

While the validation of our model awaits further studies, the results reported in this study provide an important first insight into the mechanism of post-translational transport in the secretory pathway and its evolution in insects and other higher eukaryotes. Further, our finding that proteins dislocated via "post"-translational pathway are efficiently segregated into aggresomes reveals the importance of this system in elucidating potential mechanisms of aggresome formation, with important implications to cellular homeostasis and pathogenesis (Johnston *et al.*, 1998).

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