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The Levels of Endoplasmic Reticulum Proteins and ATP Affect Folding and Secretion of Selective Proteins

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Abstract. Proteins transiting the endoplasmic reticulum (ER) interact with a number of lumenal proteins, such as the glucose regulated proteins (GRPs), that either facilitate or prohibit protein folding and transport out of the ER compartment. We compared the relative amounts of mRNA encoding lumenal ER proteins in cells that secrete high levels of protein to those that do not secrete significant levels of protein. One of these proteins, GRP78, is thought to act as a chaperone to assist protein folding. We evaluated the effect of altered GRP78 expression on the secretion efficiency of heterologous proteins expressed in CHO cells. The secretion efficiency of proteins detected in significant association with GRP78 was reduced when GRP78 levels were overexpressed and improved when GRP78 levels were reduced. The results suggest that GRP78 does not act in a positive manner to promote protein folding and/or secretion. In addition, proteins that interact with GRP78 displayed a unique high requirement for intracellular ATP for secretion. Expression of firefly luciferase in the lumen of the ER detected ATP in the ER lumen of intact cells as monitored by light emission. Since luciferase light emission is proportional to ATP concentration, the amount of light emission may provide an approach to study the effect of altered ER intralumenal ATP on protein folding and secretion.

Introduction

The compartmentalization of mammalian cells requires that polypeptides contain information required for their transport and subsequent localization to specific organelles where they function. Proteins that transit the secretory apparatus in mammalian cells are first cotranslationally translocated into the lumen of the endoplasmic reticulum (ER). Insertion into the lumen of the ER is usually directed by the presence of a hydrophobic signal peptide. The ER is the site of initial processing events that are crucial for proper folding of the nascent polypeptide. These processing events include signal peptide cleavage,[1] addition of core N-linked oligosaccharides at consensus recognition sites,[2] and disulfide bond formation mediated by protein disulfide isomerase.[3] Attaining an appropriate conformation is essential for a protein to be efficiently transported from the ER to the Golgi complex (GC).[4] For most proteins, transport from the ER to the GC is the rate limiting step for secretion.[5,6] This transport step requires ATP.[7] Retention in the ER and eventual degradation is the fate of many secretion incompetent proteins.[8]

Purified denatured proteins can fold and attain their native conformation *in vitro*,[9] although this process is slow. In cells protein folding is rapid and it is likely that intracellular protein folding is assisted by cellular factors such as molecular chaperones, members of the glucose regulated protein (GRP) family,[10] protein disulfide isomerase, and peptidyl-prolyl cis-trans-isomerase. Most data has accumulated for the role of PDI in assisting protein folding *in vitro* and *in vivo*.[11,12] Members of the GRP family include GRP78, GRP94, ERp72,[13] and calnexin or p88.[14] ERp72 shares amino acid homology with PDI at regions which constitute the active sites of PDI.[15] GRPs are constitutively expressed in all eukaryotic cells and are induced to high levels by a variety of physiological stresses that result in the accumulation of misfolded or unglycosylated proteins in the ER.[10]

GRP78 has been extensively studied. GRP78 is also known as immunoglobulin binding protein BiP.[16,17] Expression of GRP78-associated proteins such as aberrantly folded proteins or unassembled protein subunits within the ER induces GRP78 expression.[10,18,19] GRP78 stably associates with some secretion incompetent proteins that fail to be efficiently secreted.[20–24] In many cases, associated proteins are improperly glycosylated, incompletely assembled, or misfolded. GRP78 also associates with some apparently normal proteins that are destined for secretion.[20,25–28] GRP78 displays ATP binding[29]

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and peptide-dependent ATP hydrolysis activities[30] and *in vitro* release from a complex with GRP78 requires the hydrolysis of ATP.[16,30] Previous studies indicate that GRP78 binds specific sequences in denatured proteins.[31] The optimal peptide size for stimulating the GRP78 ATPase activity is a 7-mer.[30] To date, two studies have reported on the amino acid requirements for peptide binding to GRP78 and both indicate that aliphatic amino acids are enriched in BiP binding peptides.[31,32] However, despite intensive investigation, the role of GRP78 in protein folding is unknown, although two, not mutually exclusive, hypotheses have been put forward for GRP78 function. One hypothesis is that GRP78 assists protein folding by maintaining proteins in a conformation where they are folding competent.[33,34] This model is supported by the transient association with polypeptides destined for secretion and an ATP dependence for proper folding, disulfide bond formation, and secretion.[7,35,36] In contrast, other studies[37–40] suggest that GRP78 acts as a retention mechanism for quality control to prevent aberrantly folded proteins from exiting the secretory pathway. Despite many efforts to date, except for one example in yeast,[41] there is no direct demonstration that GRP78 binding to protein substrates actually catalyses protein folding.[12] In this report we have characterized the levels of GRP expression in different cell types and in CHO cells that express heterologous secreted proteins at different levels. In addition, we summarize results on the effect of altered GRP78 expression on protein secretion. We conclude that high levels of intracellular ATP are uniquely required for GRP78 associated proteins to be released from GRP78 and secreted. Finally, firefly luciferase was expressed in the lumen of the ER to directly monitor the presence of ATP in that compartment.

Materials and methods

Northern blot analysis

Quantitation of specific mRNAs in different cell lines was performed by Northern blot hybridization using specific probes as previously described.[19]

Construction of luciferase expression vector and cell line development

Mutagenesis of the firefly luciferase cDNA in the vector pSV232AL-A 5′ (obtained from *S. Subramani*) was accomplished by polymerase chain reaction (PCR) utilizing specific oligonucleotide primers. First, a BglII restriction site was added to the amino-terminus in place of the initiator methionine codon. Sequences coding for KDEL followed by two termination codons and a SalI restriction site were placed at the carboxy-terminus. A BglII-SalI restriction fragment encoding luciferase containing carboxy-terminal KDEL was isolated. The prepro-leader peptide encoding sequence of tissue plasminogen activatory (tPA) was isolated as an NdeI-BglII fragment from a tPA expression vector pMT2pc.tpa4. Other expression vector sequences were obtained on a NdeI-SalI fragment derived from pED.[43] A three way ligation of these fragments resulted in the plasmid pERluc. Transcription from the adenovirus major late promoter results in synthesis of a dicistronic mRNA. Translation of the 3′ proximal dihydrofolate reductase (DHFR) gene is under control of the encephelomyelocarditis virus (EMCV) leader sequence that allows internal initiation of protein synthesis.[43] pERluc was introduced into the DHFR deficient Chinese hamster ovary (CHO) cell line, DUKX-B11, by electroporation. Cells were initially selected for growth in the absence of nucleosides and amplified by selection for growth in increasing concentrations of methotrexate.[44] Cells grown in 10 μM methotrexate were analysed for luciferase expression by Western immunoblot analysis and luciferase activity assay as described below and subjected to single cell cloning to obtain the cell line designated ERlucA.

Luciferase assays

To assay cell extracts, 10 cm dishes of ERlucA cells and parental CHO cells were washed 3× with phosphate buffered saline (PBS) and cells harvested by scraping in 1 ml of Extraction buffer (100 mM potassium phosphate, pH 7·8, 1 mM DTT). Cells were pelleted and resuspended in 0·1 ml Extraction buffer. Cells were lysed by three cycles of freeze/thawing and debris removed by centrifugation. Protein concentration was determined by the method of Bradford.[45] Cell extract was added to 100 μl of Assay buffer (100 mM potassium phosphate, pH 7·8, 3 mM magnesium sulfate, 1 mM ATP). The reaction mix was placed in a LKB 1250 luminometer and the reaction initiated by injection of luciferin to a final concentration of 0·8 mM. Readings were taken 10 s following injection. To assay intact cells, ERlucA cells and parental CHO cells were harvested by mild trypsinization. Cells were pelleted through a cushion of dialysed fetal bovine serum (dFCS), washed with medium containing 10% dFCS, and repelleted. Cells were resuspended in medium containing 10% dFCS to a final concentration of 2×10^4 cells/μl. Intact cells were assayed in 100 mM potassium phosphate, pH 7·8, in the presence and absence of 1 mM ATP. Luciferin was

injected to a final concentration of 0·8 mM to initiate the reaction. Readings were taken beginning 10 seconds following injection.

Subcellular fractionation

Subcellular fractionation was performed as described previously.[46] ERlucA cells and parental CHO DUKX cells were harvested in 2 ml of Buffer S (10 mM Hepes, pH 7·4, 10 mM KCl, 1 mM $MgCl_2$) and kept on ice for 15 min. Cells were disrupted by 15 strokes in a Dounce homogenizer. The postnuclear supernatant was made 0·1 M in sucrose and layered onto a discontinuous sucrose gradient containing 2 M (1 ml), 1·3 M (3 ml), 1 M (3 ml), 0·6 M (2 ml) sucrose in 5 mM Hepes, pH 6·8. Gradients were centrifuged 2 h at 40 000 rpm in the Beckman SW41 rotor. Fractions were collected and analysed by Western immunoblot analysis.

Western protein analysis

Cell extracts were prepared in 0·5 ml Lysis buffer (0·15 M NaCl, 0·5 M Tris-HCl, pH 7·5, 0·05% SDS, 1% Nonidet P-40) from 10 cm plates of CHO cells and ERlucA cells untreated or treated for 16 h with 10 μg/ml tunicamycin. Equal volumes of cell extract corresponding to 3·3–3·7 × 10^4 cells were electrophoresed on a 10% polyacrylamide-SDS gel and transferred to nitrocellulose. The blot was reacted with rabbit antiserum directed against firefly luciferase (developed at Genetics Institute) and visualized by the chemiluminescence procedure as recommended by the supplier (Amersham).

Results

Resident ER protein levels and secretion capacity

Cultured cell lines have been derived from tissues that *in vivo* secrete high levels of protein. Among these are HepG2 cells which were derived from a human hepatoma[47] and AtT-20 cells that secrete at least 20 serum proteins and were derived from a murine anterior pituitary gland.[48] We have compared the levels of mRNA encoding the resident ER proteins GRP78, GRP94, PDI, and ERp72 in these cell lines to CHO cells by Northern analysis (Table 1). These results show that the levels of PDI and ERp72 in HepG2 cells and AtT-20 cells were elevated compared to CHO cells. Elevated levels of PDI were previously correlated *in vivo* with increased secretory activity of tissues such as liver and kidney consistent with its role in protein folding.[11] It is of particular interest that the level of ERp72 mRNA was also elevated in these cell lines. Since ERp72 contains regions homologous to the active site domains of PDI and is a mem-

Table 1. Survey of ERp and GRP mRNA level

	GRP78	GRP94	ERp72	PDI	Ref.
A. Cell line					
CHO-DUKX	1	1	1	1	66
HepG2	1	1	4	10	47
AtT-20	1	1·5	4	12	48
B. CHO cells expressing					
tPA (AJ19) (20 pg)	1	1	1	2	49
Factor VIII (10A1) (0·2 pg)	1	1	1	1	50
Factor VIII + vWF (10A1C6) (0·4 pg FVIII + 5 pg vWF)	4	6	12	6	51
B-domain △ FVIII (LA 3-5) (1·6 pg LA VIII)	5	2	4	1	62
M-CSF (5 pg)	0·7	0·6	0·3	2	63
tPA FE1X (12 pg)	1	6	9	6	64

Autoradiograms of Northern blots were quantitated using a laser densitometer. RNA loading levels were normalized using an actin-specific hybridization probe. Numbers shown are relative to the level detected in CHO cells not expressing heterologous proteins. Cellular productivity of secreted protein is shown in parentheses (pg/cell/day). References are provided for derivation of cell lines.

ber of the glucose regulated protein family,[13] ERp72 may display disulfide isomerase activity under certain conditions and its elevated levels in HepG2 and AtT20 cells may suggest a role for ERp72 in protein folding, similar to PDI. Interestingly, GRP78 and GRP94 mRNA levels were not elevated in HepG2 or AtT20 cells. This observation suggests that these cell lines are not under stress conditions that induce GRPs and that elevated levels of GRP78 and GRP94 do not correlate with the increased secretion capacity of HepG2 and AtT20 cells.

We have developed CHO cell lines that overexpress human recombinant proteins. These cell lines were obtained after subjecting parental cell lines to rigorous selection. Overexpression of some proteins in CHO cells results in significant stress to the cells and induction of GRPs (see below). We compared the amount of protein secreted from HepG2 cells and two CHO cell lines expressing high levels of heterologous glycoproteins. Cells were radiolabeled with [^{35}S]-methionine followed by a chase in complete medium. Total conditioned medium from equivalent cell numbers was resolved by SDS-polyacrylamide gel electrophoresis. CHO cells that do not express foreign genes secrete very little radiolabeled protein (Fig. 1, lane 1). Following selection and amplification by methotrexate selection, cells lines were derived that express high levels of tissue plasminogen activator[49] or coagulation factor VIII and von Willebrand factor[50] (Fig. 1, lanes 2, 3). HepG2 cells naturally secrete a variety of proteins at greater levels than observed for the selected CHO cell lines (Fig. 1, lane 4). This suggests that cells whose primary function is secretion are adapted to process and secrete proteins efficiently, and may contain altered levels of resident ER protein and/or other processing enzymes within the secretory pathway. For example, the Northern blot analysis indicated that PDI and ERp72 were elevated in HepG2 cells and AtT2O cells, whereas GRP94 and GRP78 mRNA levels were not.

The variation in the mRNA levels for different ER proteins led us to survey the levels of mRNAs encoding ER protein in different CHO cell lines that were selected to express different secreted proteins at different levels. The results indicated that overexpression of different secretory proteins at different levels did not correlate with any specific overexpression of selected ER proteins (Table 1B). In particular, high level expression of tissue plasminogen activator (tPA) did not elicit any increase in GRP78, GRP94, or ERP72 mRNA and only a slight increase in PDI mRNA. In contrast, expression of factor VIII with von Willebrand factor elicited large increases in GRP78, GRP94, ERp72 and PDI mRNA levels. It is possible that the different proteins examined require different ER proteins for optimal secretion so no correlation was observed, or that the variation detected is characteristic of normal variation between highly selected clonal cell lines.

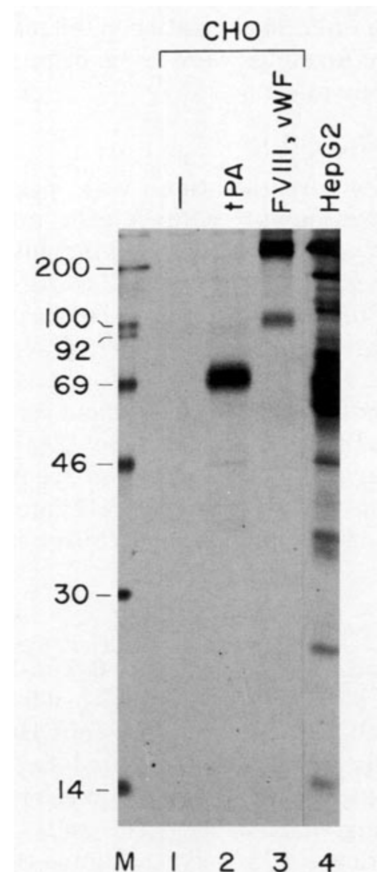

Figure 1. Secretion of proteins from HepG2 and CHO cells. Control CHO cells, CHO cells that express tPA or factor VIII with von Willebrand factor, and HepG2 cells were labeled with [^{35}S]-methionine and aliquots of conditioned medium from equal numbers of cells were analysed by SDS-PAGE and autoradiography.

Association of GRP78 with human recombinant glycoproteins in CHO cells

We have developed a model system to examine the role of GRP78 association in the secretion process. Previous studies demonstrated that inefficiently secreted proteins such as coagulation factor VIII and unglycosylated tissue plasminogen activator (tPA) were detected in stable association with GRP78 in the ER and this association correlated with retention in that compartment (Table 2).[20] Other proteins such as von Willebrand factor (vWF) and wild-type (wt) tPA were efficiently secreted from CHO cells and dis-

Table 2. GRP78 association, secretion efficiency and effect of altered GRP78 levels on secretion of heterologous proteins in CHO cells

Protein	GRP78 association	Secretion efficiency	Level of GRP78	
			Increased	Reduced
wt FVIII	Extensive Stable & transient	*	−	+
FVIII-LA	Extensive Transient	**	−	+
vWF	Slight/transient	***	−	N.D.
tPA3X	Extensive	*	−	+
M-CSF	Nondetectable	***	none	N.D.

The extent of relative association of the indicated human proteins with GRP78 in CHO cells is indicated. The relative secretion efficiency is indicated with three stars being most efficient. The relative effect of alteration of GRP78 levels on secretion is indicated (− = reduced secretion; + = increased secretion). FVIII-LA is a factor VIII molecule that is deleted for 880 amino acids within the B-domain. tPA3X is a nonglycosylated tPA molecule that has the three N-linked glycosylation sites mutated to glutamine.[37,38] N.D. = not determined.

played only slight and transient association with GRP78.[20,50] In addition, some proteins such as M-CSF (CSF-1) could not be detected in association with GRP78.[38] These studies indicated that the secretion efficiency inversely correlated with the extent of association with GRP78. Extensive stable association correlated with retention in the ER while transient association may represent an early step in the normal secretion pathway.

Effect of high level factor VIII synthesis on CHO cells

Treatment of CHO cells expressing heterologous genes with sodium butyrate increases transcription from vectors containing the adenovirus major late promoter in combination with the SV40 enhancer.[19] We examined the effect of increased wt factor VIII expression on CHO cells coexpressing factor VIII and vWF. Treatment with sodium butyrate elevated levels of factor VIII and vWF protein synthesis.[19] In addition, elevated levels of GRP78 and GRP94 were observed. While the newly synthesized vWF was efficiently secreted, factor VIII displayed a post-translational block to secretion. The induction of GRP78 and GRP94 mRNA and protein suggested that high level synthesis of factor VIII induced a stress response in CHO cells. By electron microscopy we observed dramatic dilation of the endoplasmic reticulum with juxtaposed mitochondria in cells producing high levels of factor VIII that did not occur in control cells.[19] The results demonstrated that newly synthesized factor VIII failed to be secreted and elicited a stress in the ER monitored by induction of GRPs. The presence of mitochondria adjacent to the ER suggested the requirement for ATP in that compartment.

Effect of alteration of GRP78 levels on secretion

We have studied the effect of altered GRP78 levels on the secretion of associated proteins from CHO cells. Expression of factor VIII or an unglycosylated form of tPA (tPA3×), proteins that stably associate with GRP78, induced GRP78 expression.[19,20] This indicated that protein binding to GRP78 mediates GRP78 induction.[19] An antisense RNA strategy was used to reduce GRP78 levels in cells expressing tPA3× and resulted in increased secretion of tPA3×.[37] In cells with reduced GRP78 levels, the association of tPA3× with GRP78 was decreased. tPA3× secreted from cells with reduced levels of GRP78 had a similar specific activity as that secreted from the cells prior to reduction of GRP78 levels,[37] indicating that the secreted tPA was functional and properly folded. These results show that reduction of GRP78 levels allowed tPA3× to bypass association with GRP78 resulting in increased secretion of functional protein. Similarly, expression of an immunoglobulin heavy chain with a deletion in the peptide domain that binds GRP78, did not associate with GRP78 and was secreted.[39] These results implicate that one function of GRP78 is retention of associated proteins in the ER.

The effect of GRP78 overexpression on secretion and the stress response was examined in CHO cells.[38]

Treatment of cells with tunicamycin to block N-linked glycosylation or with the calcium ionophore A23187 to alter intracellular calcium levels significantly induces transcription from the GRP genes.[10] However, when GRP78-overexpressing cells were treated with tunicamycin or A23187, reduced induction of GRP78 and GRP94 mRNAs was observed.[38] This suggested that constitutive overexpression of GRP78 may protect the cell from stress in analogy to reports on hsp70 overexpression.[51] Although the mechanism for transcriptional induction may involve a serine/threonine protein kinase localized to the lumen of the ER,[52,53] the specific mechanism is unknown. The results with GRP78 overexpression suggest that reduction in the level of free GRP78 may be the signal that initiates stress induction of GRP gene transcription.

The secretion capacity of GRP78-overexpressing cells was examined by transient transfection of expression vectors encoding a B-domain deleted form of factor VIII (FVIII-LA),[20] vWF, or M-CSF and determining secretion efficiency by [^{35}S]-methionine pulse labeling and chase analysis.[38] Secretion of FVIII-LA and vWF, proteins that transiently associate with GRP78, was reduced in cells that overexpress GRP78 compared to parental CHO cells. Co-immunoprecipitation detected FVIII-LA and vWF in association with GRP78 in extracts from the GRP78-overexpressing. In contrast, the secretion efficiency of M-CSF was unaffected by overexpression of GRP78 and M-CSF was not detected in association with GRP78. These results indicate that elevated levels of GRP78 may increase stable association and decrease secretion efficiency of selective proteins that normally only transiently associate with GRP78. However, the level of GRP78 had no effect on the secretion efficiency of a protein that was not detectably associated with GRP78.

The role of ATP in the secretion of GRP78-associated proteins

The ATP requirement for the secretion of GRP78-associated proteins from CHO cells was examined by treatment of cells with carbonylcyanide m-chlorophenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation. This treatment rapidly depletes intracellular ATP levels[54] and inhibits transport from the ER.[55] In cells that coexpress factor VIII and vWF, factor VIII secretion was blocked at concentrations of CCCP as low as 10 μM, while vWF secretion was not blocked until the CCCP concentration was increased to 200 μM.[36] The efficient secretion of vWF from cells treated with low concentrations of CCCP precluded a general defect in secretion. Examination of the intracellular association of GRP78 with factor VIII by co-immunoprecipitation experiments showed that the factor VIII-GRP78 complex failed to dissociate in the presence of CCCP. Similarly, secretion of unglycosylated tPA was blocked by low CCCP concentrations compared to wt tPA.[36] Thus, GRP78-associated proteins displayed increased sensitivity to reduction of ATP levels compared to unassociated proteins. These results indicate that proteins association with GRP78 require ATP dependent release in order to transit the ER and that some proteins, such as factor VIII, may have an unusually high ATP requirement for GRP78 release and secretion.

Firefly luciferase can detect ATP in the ER

Firefly luciferase is a peroxisomal enzyme that catalyses light production. The reaction requires luciferin, O_2, and ATP. The enzyme has been used extensively as an *in vitro* assay to measure ATP concentration in cell extracts.[56] Light production is almost constant and proportional to the limiting substrate concentration under nonsaturating conditions.[57] The cDNA encoding firefly luciferase has been cloned and expressed in mammalian cells.[58] When expressed in mammalian cells, firefly luciferase is targeted to the peroxisomes by virtue of a tripeptide (Ser-Lys-Leu) at its carboxy terminus.[59] The enzyme is active in cell extracts in the presence of ATP. Since luciferin can diffuse across cellular membranes, intact cells that express firefly luciferase also display low levels of activity when incubated in the presence of luciferin alone. In this case, the source of ATP for the reaction is derived from intracellular stores. Firefly luciferase has been targeted to the mitochondria of yeast[57] and chloroplasts in tobacco plants[60] by construction of a chimeric luciferase genes that contains the N-terminal targeting sequences from mitochondrial or chloroplast proteins. These fusion proteins were shown to be appropriately localized and displayed activity in both cell extracts and intact cells. This work suggested that luciferase could be engineered for localization to specific cellular compartments to monitor local ATP levels.

We have engineered firefly luciferase to be directed to the lumen of the ER. The signal peptide and propeptide from human tPA was fused to the amino terminus of luciferase. At the carboxy terminus the ER localization signal Lys-Asp-Glu-Leu was added to the carboxy terminus to retain the luciferase in the lumen of the ER. The construct was transfected into CHO cells with DHFR as a selectable marker. A cell line

designated ERlucA was characterized for luciferase expression. Cell extracts were prepared and assayed for luciferase activity in the presence and absence of 1 mM ATP, 3 mM $MgSO_4$, and 0·8 mM luciferin. Control CHO cells displayed no activity in the presence or absence of ATP. CHO ERlucA cells displayed 3·5 units/μg cellular protein in the presence of ATP and no activity in the absence of ATP. This indicated that the ER directed luciferase protein was functional in cell lysates.

The localization of the luciferase in ERlucA cells was studied by isolation of cytoplasmic fractions of rough ER and Golgi complex by sucrose gradient sedimentation and analysis by Western immunoblotting using a rabbit anti-luciferase antibody. This analysis detected a 69 kDa polypeptide in isolated fractions of the rough ER that was much less abundant in the Golgi fraction (Fig. 2A). To further characterize luciferase expression, total cell extracts were examined by Western immunoblot analysis. Extracts from ERlucA cells displayed a major reactive species at 69 kDa that was not present in control CHO cells (Fig. 2B). In addition, inhibition of N-linked glycosylation with tunicamycin treatment for 12 h increased the mobility on the major reactive species. This is consistent with addition of N-linked oligosaccharides onto luciferase at one or multiple of the three potential sites for N-linked glycosylation. The mobility of the luciferase was also increased by digestion with either endoglycosidase H or N-glycanase (not shown). These results demonstrate the presence of high-mannose containing oligosaccharides on the expressed luciferase, consistent with the transport of the protein into the lumen of the ER.

The activity of the ER luciferase in intact cells was tested by incubating viable cells with 0·8 mM luciferin. Background light was detected in control CHO cells whereas ERlucA cells displayed a 100-fold increase in light emission (Fig. 2C). These cells produced luciferase activity at 150 μ units/cell. In addition, inhibition of N-linked oligosaccharide addition by overnight treatment with tunicamycin increased the activity of the ER luciferase by 8-fold, whereas there was no change in the steady state level of luciferase by Western immunoblotting analysis (Fig. 2B). These results indicate that the addition of N-linked oligosaccharides reduced the specific activity of the luciferase. The results show that luciferase can be active in the ER compartment and support the hypothesis that ATP is present in that compartment. Consistent with this conclusion, it was recently shown that ATP can be transported across ER membranes in a saturable manner with a K_M of 3–5 μM.[61]

The ability to detect luciferase activity in the lumen of the ER may provide an avenue to study the importance of the ATP concentration on protein folding and secretion. However, several obstacles preclude a correlation between the amount of light emitted and the

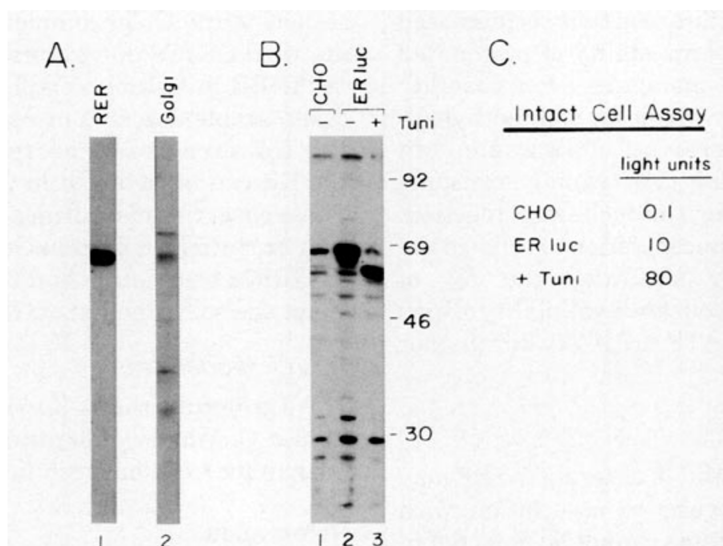

Figure 2. Expression of firefly luciferase in the lumen of the ER. Panel A. Control CHO cells or ERluc cells were analysed by cell fractionation and Western immunoblot analysis with an anti-luciferase antibody as described in Materials and methods. Panel B. Total cell extracts analysed by Western immunoblot analysis. Panel C. Luciferase activity in intact cells was monitored in the presence of 0·8 mM luciferin.

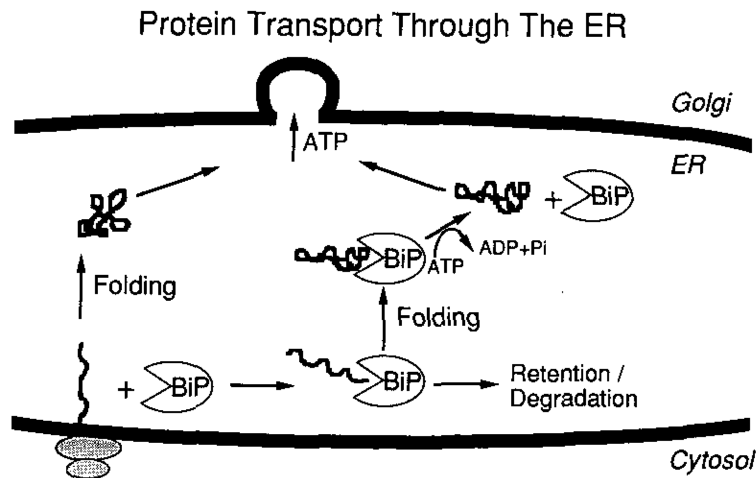

Figure 3. ATP dependent steps during transit through the endoplasmic reticulum. Protein translocation, folding and exit from the ER are shown. ATP dependent release from GRP78 (BiP) is necessary for transit of proteins that display extensive association with GRP78. It is not known if GRP78 association facilitates folding. Stable association of secretion incompetent proteins results in retention. Other proteins, both secretion competent and incompetent, are not detected in association with GRP78.

ATP concentration in the ER. First, the activity of luciferase may be significantly affected by the accessibility of substrates and other environmental conditions occurring locally. The most likely limiting factor of light emission in intact cells is the intracellular concentration of luciferin diffusing in from the medium. Intracellular luciferase activity is increased at low pH due to better permeability of protonated luciferin through the cell membrane. Permeability can be further improved by addition of dimethylsulfoxide, or nigericin in the presence of potassium.[59] In addition, actively respiring cells rapidly consume oxygen that is required for the luciferase reaction. Finally, the average ATP concentration in cells (in the millimolar range) easily saturates the K_M of luciferase. Thus, in intact cells the availability of oxygen and luciferin, and not ATP, are likely limiting for luciferase activity.

Conclusions

We have identified that a protein entering the ER may have several fates in regard to association with GRP78 (Fig. 3). Some proteins cannot be detected in association with GRP78. These proteins may fold properly, are efficiently transported to the Golgi complex and appear to not require high ATP levels for transport. A protein may also transiently associate with GRP78. The protein may fold while associated with GRP78 and upon attaining an appropriate conformation dissociate. Release from GRP78 association is an ATP dependent step and one reason for retention of selective, apparently normal proteins in the ER may be due limiting amounts of ATP that are required for protein dissociation from BiP. Upon release from GRP78, further folding occurs prior to transit to the Golgi complex. Other proteins associate with GRP78 due to misfolding and are retained in the ER in a stable complex with GRP78. In these cases, stable association results in retention in the ER. However, not all aberrant proteins retained in the ER can be detected in association with GRP78. These observations indicate that retention of all aberrant or malfolded proteins in the ER is not mediated by GRP78 association and that retention of proteins is not the sole function of GRP78.

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