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The role of endoplasmic reticulum quality control system in the biology of the major myelin glycoproteins

by

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I would like to dedicate this thesis to

my parents, Janina and Edward Jung.

ABSTRACT

Endoplasmic reticulum (ER) plays an essential role in the proper folding, maturation and quality control of newly synthesized membrane and secretory proteins. The ER contains molecular chaperones and unique enzymes that assist in protein folding and catalyze co- and post-translational modifications. The two homologous ER chaperones, calnexin and calreticulin are key components of the quality control in the secretory pathway. These chaperones are also involved in mediating interactions between newly synthesized proteins and ERp57, a thiol-disulfide oxidoreductase catalyzing disulfide bond formation and isomerization. Deletion of calreticulin or ERp57 in mice leads to an early death *in utero*, surprisingly, deficiency in calnexin is not embryonic lethal and results in a phenotype that includes alterations in morphology of myelin of peripheral and central nervous systems. Two important glycoproteins of compact peripheral myelin that are involved in its formation and maintenance are P0 and PMP22. Many of the mutations within P0 and PMP22 genes are associated with human hereditary neuropathies. In this study we created calreticulin-, ERp57- and calnexin-deficient cell lines stably expressing myelin glycoproteins and used them to investigate the role of ER

chaperones calnexin, calreticulin and ERp57 in expression, cellular trafficking, proper folding and function of myelin specific glycoproteins.

We showed novel interaction between P0 and calnexin as well as myelin oligodendrocyte glycoprotein (MOG) and calnexin. Moreover, we investigated cellular trafficking of myelin glycoproteins and discovered that P0, PMP22 and MOG localize to the plasma membrane in all cell types tested. However, the adhesive function of P0 and PMP22 was compromised in the absence of calnexin or ERp57. Limited trypsin digestion of PMP22 and P0 revealed that the cell surface targeted myelin proteins were misfolded when expressed in calnexin- or ERp57-deficient cells. We also show that expression of myelin glycoproteins in the absence of ER chaperones does not induce endoplasmic reticulum stress.

Research presented in this thesis highlights the diversity of the roles of ER chaperones calnexin, ERp57 and calreticulin in the maturation of major myelin glycoproteins.

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LIST OF ABBREVIATIONS

APMSF	4-amidinophenyl-methanesulfonyl fluoride hydrochloride monohydrate
ATF4	activating transcription factor-4
ATF6	activating transcription factor-6
ATP	adenosine triphosphate
Bap31	B cell receptor-associated protein 31
BiP	binding immunoglobulin protein
BSA	bovine serum albumin
bZip	basic leucine zipper domain
CFTR	cystic fibrosis transmembrane conductance regulator
CHOP	CCAAT/enhancer binding protein (C/EBP) homologous protein
CMT	Charcot Marie Tooth
CMV	cytomegalovirus
CNH	congenital hypomyelinating neuropathy
CNS	central nervous system
CNX	calnexin
ConA	concanavalin A
CTLs	cytotoxic T-lymphocytes
CRE	cAMP response element
CRT	calreticulin
Cx32	connexin 32
DMEM	Dulbecco's modified eagle medium
DSS	Dejerine-Sottas Syndrome

EAE	experimental autoimmune encephalomyelitis
ECL	electro chemiluminescence
EDEM	ER degradation-enhancing α-mannosidase
EDTA	ethylene diamine tetraacetic acid
EF1α	cellular polypeptide chain elongation factor 1 alpha
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
eIF2α	eukaryotic initiation factor 2α
EndoH	endoglycosidase H
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ERSE	ER response element
FACs	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GATA6	GATA-binding protein 6
GAT1	γ- aminobutyric acid transporter 1
GFP	green fluorescent protein
GlucI/II	glucosidase 1 and II
GRp78	glucose-regulated protein-78
GRp94	glucose-regulated protein 94
HCN1	hyperpolarization-activated cyclic nucleotide-gated
HMSNs	hereditary motor and sensory neuropathies
Ig	immunoglobulin

$InsP_3$	inositol 1,4,5-triphosphate
$InsP_3R$	inositol 1,4,5-triphosphate receptor
IRE1	inositol-requiring kinase 1
K_d	rate of disassociation
KDEL	lysine-aspartate-glutamate-leucine
MBP	myelin basic protein
MEFs	mouse embryonic fibroblasts
MEF2C	myocyte enhancer factor 2C
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
NCV	nerve conduction velocity
NFAT	nuclear factor of activated T-cells
NH^{3+}	amino-terminal
Ni-NTA	Ni^{2+}-nitrilotriacetic acid-agarose
NMR	nuclear magnetic resonance
NP-40	nonyl phenoxylpolyethoxylethanol
OST	oligosaccharyltransferase
P0	myelin protein zero
PBA	sodium 4-phenylbutyrate
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PERK	double-stranded RNA-activated protein kinase-like ER kinase

PNGaseF	peptide: N-glycosidase F
PLP	proteolipid protein
PMP22	peripheral myelin protein 22
PMSF	phenylmethylsulfonyl fluoride
PNS	peripheral nervous system
PrP^{SC}	misfolded prion proteins
P-STAT3	phosphorylated-STAT3
QDEL	glutamine-aspartate-glutamate-leucine
RFUs	relative fluorescence units
RIP	regulated intramembrane proteolysis
RKPRRE	arginine-lysine-proline-arginine-arginine-glutamate
RLUs	relative light units
RT	room temperature
RT-PCR	reverse transcriptase –PCR
RUs	resonance units
RyR	ryanodine receptor
S1P	site-1 protease
S2P	site-2 protease
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SER	smooth endoplasmic reticulum
SERCA	sarcoplamsic/endoplasmic reticulum Ca^{2+}-ATPase
SH2	Src homology-2
siRNA	small interfering RNA

SP	signal peptidase
SRP	signal recognition particle
STAT3	signal transducer and activator of transcription 3
STIM1	stromal interaction molecule 1
SVF	Semliki Forest Virus
TAP	peptide transporter
Tg	thapsigargin
Tr	trembler
Tr-J	trembler J
TRPC	transient receptor potential channels
UGGT	UDP-glucose:glycoprotein glucosyltransferase
UPR	unfolded protein response
UPRE	UPR element
wt	wild-type
Xbp1	X-box binding protein

Table 1 – Primers used in this study

Lentivirus Primers *att*B1(forward) *att*B2 (reverse) recombination site (bold), Kozak sequence for expression in mammalian cells (italics) ,gene specific nucleotides (underlined)			
Gene	**Forward**	**Reverse**	**Chapter**
P0-GFP	5′-GGGG**ACAAGTTTG TACAAAAAAGCAGGC** T*ATACCATG*CTCCGGGC CCCTGCCCCT-3′	5′-GGGG**ACCACTTTG TACAAGAAAGCTGGGT** TTTACTTGTACAGCTCGT CCATGCC-3′	2
PMP22-GFP	5′- GGGG**ACAAGTTT GTACAAAAAAGCAGG CT**AT*ACCATG*TCGACGG TACCGCGGGCCC -3′	5′-GGGG**ACCACTTTG TACAAGAAAGCTGGGT** TTTACTTGTACAGCTCGT CCATGCC-3′	2
GFP	5′GGGG**ACAAGTTTGTA CAAAAAAGCAGGCT**TC *ACCATG*GAAGGGAAGT GGTTACT-3′	5′-GGGG**ACCACTTTG TACAAGAAAGCTGGGT** TTTACTTGTACAGCTCGT CCATGCC-3′	2, 3
MOG-GFP	5′GGGG**ACAAGTTTGTA CAAAAAAGCAGGCT**TC *ACCATG*GAATATCAGAT ACTGAAAATGTC-3′	5′-GGGG**ACCACTTTG TACAAGAAAGCTGGGT** TTTACTTGTACAGCTCGT CCATGCC-3′	3
Mutagenesis Primers			
Mutation	**Forward**	**Reverse**	**Chapter**
P0 N^{93}del	CAACCTAGACTACAGT GACGGCACGTTCACTTG	CAAGTGAACGTGCCGTC ACTGTAGTCTAGGTTG	2
MOG N^{31}A	CTCTCCTGGGAAAGCTG CCACGGGCATGGA	TCCATGCCCGGGCAGCT TTCCCAGGAGAG	3

Table 2 – Antibodies used in this study

Name	Western Blot Dilution	Source	Chapter
rabbit anti-calnexin	1:1000	StressGen Inc.	2, 3
goat anti-calreticulin	1:300	generated in our laboratory	2, 3
rabbit anit-ERp57	1:300	generated in our laboratory	2, 3
rabbit anti-GAPDH	1:1000	Abcam	2
rabbit anti-GRP78(BiP)	(used for immunostaining at 1:50)	StressGen Inc.	2, 3
rabbit anti-GFP	1:10000	Gift from Dr. Berthiaume	2, 3
Goat anti-GFP	1:10000	Gift form Dr. Berthiaume	2, 3
rabbit anti-MOG	1:300	Santa Cruz	3
rabbit anti-goat	1:10000	Invitrogen	2, 3
goat anti-rabbit	1:1000	Invitrogen	2, 3
anti-rabbit Alexa Fluor 546	(used for immunostaining at 1:100)	Invitrogen	2

Table 3 – Cell lines used in this study

cell lines	references	Chapter
wild-type MFs and MEFs	Kraus, A., et al., J Biol Chem, 2010. **285**; Mesaeli, N., *et al*., J. Cell Biol., 1999. **144;**	2, 3
calnexin-deficient MFs	Kraus, A., et al., J Biol Chem, 2010. **285**	2, 3
ERp57-deficient MEFs	generated in this study	2, 3
calreticulin-deficient MEFs	Mesaeli, N., *et al*., J. Cell Biol., 1999. **144**	2, 3
ERp57-deficient MEFs expressing:		
P0-GFP	generated in this study	2
PMP22-GFP	generated in this study	2
MOG-GFP	generated in this study	2
P0 N^{93}del-GFP	generated in this study	2
MOG N31A-GFP	generated in this study	3
calreticulin-deficient MEFs expressing:		
P0-GFP	generated in this study	2
PMP22-GFP	generated in this study	2
MOG-GFP	generated in this study	3
P0 N^{93}del-GFP	generated in this study	2
MOG N^{31}A-GFP	generated in this study	3
calnexin-deficient MEFs expressing:		
P0-GFP	generated in this study	2
PMP22-GFP	generated in this study	2
MOG-GFP	generated in this study	2
P0 N^{93}del-GFP	generated in this study	2
MOG N^{31}A-GFP	generated in this study	3
wt MFs or MEFs		
GFP	generated in this study	2
P0-GFP	generated in this study	2
PMP22-GFP	generated in this study	2
MOG-GFP	generated in this study	3
P0 N^{93}del-GFP	generated in this study	2, 3
MOG N^{31}A-GFP	generated in this study	3

Table 4 – Plasmids used in this study

Plasmid Description	**Name**	**Source**	**Chapter**
Xbp1 splicing *Firefly* luciferase, CMV *Renilla* luciferase	pRL-XFL	Dr. R. Kaufman	2, 3
p2K7 myelin proteins plasmids:			
Plasmid Description	**Name**	**Source**	**Chapter**
GFP containing plasmid	2K7-GFP	generated in this study	2, 3
human myelin P0 fused to GFP at C-termini with bds resistance	2K7-P0-GFP	generated in this study	2
N-glycosylation mutant of P0 fused to GFP at C-termini with bds resistance	2K7 P0(N^{93}del)GFP	generated in this study	2
mouse PMP22 fused to GFP at C-termini with bds resistance	2K7 PMP22-GFP	generated in this study	2
Mouse MOG fused to GFP at C-termini with bds resistance	2K7 MOG-GFP	generated in this study	3
N-glycosylation mutant of MOG P0 fused to GFP at C-termini with bds resistance	2K7 MOG(N^{31}A)-GFP	generated in this study	3

Chapter One

Introduction

Introduction

The Endoplasmic Reticulum: *a Multifunctional Organelle*. Compartmentalization is an essential feature of multicellular organisms. It allows for efficient communication, diversity of environments and specialization within the cell. The intracellular membrane system in eukaryotic cell includes plasma membrane, Golgi complex, endosome, lysosome and endoplasmic reticulum (ER) [1]. ER, whose lumen contains about 10% of total volume of the cell [2, 3], is an essential sub-cellular compartment in eukaryotic cells. It forms a network of membranes that extends through the cytoplasm of the cell and it contiguous with the nuclear envelope (Figure 1-1) [4]. The environment of the ER lumen has a high concentration of Ca^{2+}, is oxidizing relative to the cytosol and contains a set of specialized chaperones and enzymes [3]. The ER is involved in diverse functions within the cell, including translocation of proteins into the ER lumen, folding and post-translational modification of proteins in the ER lumen, synthesis of phospholipids and steroids on the cytosolic side of the ER membrane and storage of Ca^{2+} ions in the lumen and their controlled release into the cytosol [5, 6].

There are two types of ER found in eukaryotic cells: smooth and rough ER [2]. They differ in physical appearance as well as in function. The differences in appearance between the two types of ER are related to the presence of membrane-bound ribosomes on the rough ER [2]. Moreover, the two types of ER also differ in membrane protein composition. Rough ER is enriched in proteins required for protein folding and "packaging" [7]. The smooth ER acts mainly as a synthesis site

of phospholipids and steroids on the cytosolic side of the ER membrane and it forms an important metabolic compartment [3]. In muscle cells, smooth ER is called sarcoplasmic reticulum and is mainly involved in Ca^{2+} handling required for muscle contraction [3]. On the other hand, rough ER contains associated ribosomes and is involved in protein synthesis [2].

Beside processing and "packaging" of proteins, the ER also plays a central role in various processes such as carbohydrate metabolism, steroid metabolism and protein processing [8]. For example, it is closely involved in regulating apoptosis, various transcriptional cascades and stress signaling pathways [8]. To perform its many functions efficiently, the ER is closely connected with other cellular organelles like the plasma membrane, the Golgi, vacuoles and mitochondria [3].

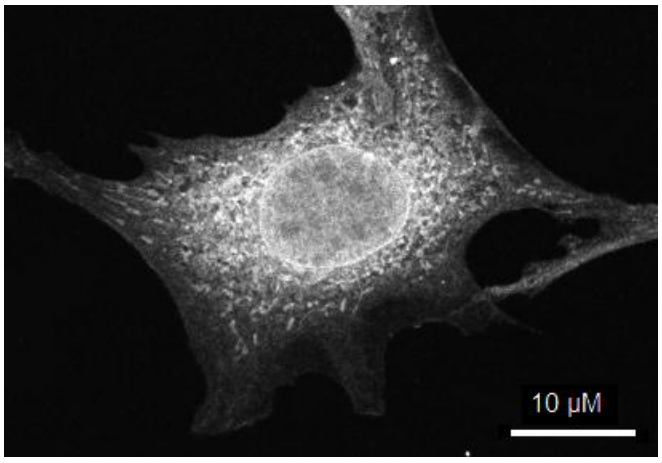

Figure 1-1. The endoplasmic reticulum membrane network in a mouse fibroblast cell. Confocal microscopy of ER visualized by staining of calnexin, an ER membrane protein, with anti-calnexin antibodies. A version of this figure has been previously published in Jung, J *et al*., *Calnexin. An Endoplasmic Reticulum Integral Membrane Chaperone*. Calcium Binding Proteins, 2006 1(2).

Protein folding and maturation may be the most important functions of the ER as approximately one-third of all the proteins in the cell are targeted for the secretory pathway where the ER is a first compartment [9]. The ER contains a set of molecular chaperones and folding enzymes that assist in protein folding. To ensure that only properly folded proteins leave the ER and are sent to their final destination within the cell, all the proteins folded in the ER are subject to ER quality control [10]. The ER quality control system closely monitors protein folding by using specific factors that assist newly synthesized proteins to achieve their final conformation [11]. The quality control system comprises of molecular chaperones and folding enzymes that increase the efficiency of the folding process in the very crowded ER environment. Molecular chaperones of the ER promote efficient protein folding by binding to hydrophobic patches on unfolded proteins [11]. Calnexin and calreticulin, two ER-resident lectin chaperones, have a central role in protein folding in the ER [12]. These two proteins ensure that only correctly folded proteins reach their final destination. The majority of proteins that enters the ER associates with calnexin and/or calreticulin in the calnexin–calreticulin cycle [12]. The role of chaperones is to increase the efficiency of the folding process. However, if folding is delayed and the final native protein conformation cannot be achieved, the protein enters a degradation pathway called ER-associated degradation (ERAD) [13]. Many human diseases, including neurodegenerative disorders, are associated with dysregulation of protein folding [14, 15]. This makes the calnexin–calreticulin chaperone system an important target for therapeutic approaches [16]. Additionally, if misfolded proteins escape the quality control system and are not degraded via ERAD, they

can accumulate in the lumen of the ER [17]. This accumulation leads to induction of the unfolded protein response (UPR), which, by several mechanisms, reduces ER-protein overload [18].

1.0 Endoplasmic Reticulum and Calcium Homeostasis. Ca^{2+} is a very potent molecule that regulates many cellular processes such as exocytosis, contraction, metabolism, transcription, protein synthesis, fertilization and proliferation [19]. Cell development and survival depend on Ca^{2+} mediated signaling and homeostasis [20]. Initiation of Ca^{2+} dependent processes or sustaining Ca^{2+} homeostasis in the cell include both entry of external Ca^{2+} and its release from the intracellular stores [21]. There are many different channels that control Ca^{2+} entry from the extracellular space in response to numerous stimuli including receptor-operated channels, second-messenger-operated channels and store-operated channels [22]. The ER has a total Ca^{2+} concentration of 1-3 mM [23] and it is a main source of intracellular Ca^{2+} ions critical for cellular signaling [24]. However, the concentration of free ER Ca^{2+} varies from 1 to 400 μmol/l [24]. These drastic changes in free ER Ca^{2+} concentration are related to release and uptake of Ca^{2+} upon signaling. Ca^{2+} fluctuations in the lumen of ER not only allow cellular signaling but also affect many functions of the ER [24]. In order to perform its function as a signaling molecule, Ca^{2+} release and uptake must be quick and efficient [24]. Release of Ca^{2+} from the intracellular stores is mediated by Ca^{2+} itself or by various messengers like $InsP_3$ or cyclic ADP [24]. Ca^{2+} released is facilitated by Ins(1,4,5)P_3R (activated by $InsP_3$, Ca^{2+} or phosphorylation/dephosphorylation) [24] and ryanodine receptors (RYR) [24]. Whereas, refilling the ER Ca^{2+} stores is mediated by the sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) [25].

Recently stromal interaction molecule 1 (STIM1) has been shown to be involved in the communication of intracellular Ca^{2+} stores to the plasma membrane [26, 27]. STIM1 acts as a sensor to detect ER lumen Ca^{2+} concentrations but also as a messenger that translocates and accumulates at the plasma membrane to activate store-operated channels upon depletion [27]. Upon store depletion, STIM1 together with Ca^{2+} channel (Orai1) accumulates in punctuate clusters at the plasma membrane to activate and increase Ca^{2+} influx into the cell [27].

1.1. Calcium Binding Proteins of the Endoplasmic Reticulum. The ER has a high capacity for Ca^{2+} because of ER resident proteins that buffer Ca^{2+} ions [28]. Ca^{2+} binding residents of the ER often function ER chaperones or folding enzymes during protein folding in the ER lumen [28]. In the lumen of the ER, there are several Ca^{2+} buffering chaperones. One of the major Ca^{2+} binding/buffering proteins in the ER is calreticulin [29].

Calreticulin is a well-known Ca^{2+} buffering protein of the ER [30]. Many of calreticulin's cellular functions are related to its Ca^{2+} binding properties [30]. Calreticulin is a soluble ER protein with three distinct structural domains: a globular N-domain, an extended proline-rich arm (P-domain) and an acidic C-domain (Figure 1-3) (see section 2.1a). Its C-domain binds Ca^{2+} with low affinity (K_d=2 mM) and high capacity (25 mol of Ca^{2+} per mol of protein). The C-domain of calreticulin alone is responsible for retaining over 50% of ER Ca^{2+} [30]. The P-domain is also able to bind Ca^{2+} however, unlike the C-tail, it binds Ca^{2+} with high affinity (K_d=1 μM) and low capacity (1 mol of Ca^{2+} per mol of protein) [31]. The binding properties of calreticulin's C-domain are related to its very acidic amino acid sequence consisting of aspartic and glutamic acid residues

interspersed by highly basic lysine and arginine residues [32]. Interestingly, the basic residues are related to calreticulin's Ca^{2+}-binding properties [32].

Direct evidence for the importance of calreticulin for Ca^{2+}-signalling is shown by changes in calreticulin expression that have a direct effect on Ca^{2+}-signalling in the cell [33]. Studies with animal models deficient in calreticulin revealed an essential role for calreticulin in Ca^{2+}-signalling [34]. Calreticulin-deficient mice are embryonic lethal and further studies showed this is due to impaired cardiac development. These studies show that it is the function of calreticulin as a Ca^{2+}-buffering protein that is critical for embryo development rather than its role as a molecular chaperone [35]. In agreement, fibroblasts deficient in calreticulin showed significantly decreased ER Ca^{2+} capacity while the concentration of free ER Ca^{2+} remains unaltered [33]. However, the chaperoning feature of calreticulin has also been shown to be important through studies with calreticulin-deficient cells. A decrease in the activity of the bradykinin receptor (a substrate of calreticulin) in calreticulin-deficient cells was observed. This observation suggests misfolding and subsequent degradation of the bradykinin receptor in calreticulin-deficient cells [33, 35]. Experiments with the bradykinin receptor confirmed the dual activity of calreticulin. Misfolding of the bradykinin receptor resulted in a decrease in Ins_3PR-ER-Ca^{2+} release and, thus in the impairment of nuclear translocation of transcription factors essential during cardiac morphogenesis like nuclear factor of activated T-cells (NF-AT) and myocyte enhancer factor 2C (MEF2C) [35, 36]. Interestingly, lethality

caused by calreticulin deficiency was rescued by overexpression of the activated serine/threonine phosphatase calcineurin [37].

Calreticulin function as an ER chaperone involved in folding of newly synthesised proteins, also depends on the presence of Ca^{2+} [12, 38]. These data suggest that Ca^{2+} influences calreticulin's conformation and subsequently its function as a molecular chaperone [12, 38]. Moreover, drugs that disrupt ER Ca^{2+} homeostasis, like thapsigargin, a Ca^{2+} ATPase inhibitor cause accumulation of misfolded proteins in the lumen of ER and activation of UPR [39].

Calnexin is a structural and functional homolog of calreticulin [40]. Although it binds Ca^{2+}, it does not appear to play an important role in Ca^{2+}-buffering in the lumen of ER [34]. However, the highly acidic C-terminal domain of calnexin binds Ca^{2+} with moderate affinity and may affect its function.

Immunoglobulin binding/glucose-regulated regulated protein 78 (BiP/GRP78) and glucose-regulated binding protein 94 (GRP94) are additional proteins in the lumen of ER that possess Ca^{2+} binding properties. GRP94 as well as BiP bind Ca^{2+} with a low capacity (1-2 mol of Ca^{2+} per mol of protein). BiP, like calreticulin is also an ER molecular chaperone [41]. The functions of GRP94 and BiP can be regulated by Ca^{2+} [28]. The ATPase activity of BiP is regulated upon depletion of ER Ca^{2+} and binding of Ca^{2+} by GRP94 results in significant conformational changes of the protein structure affecting functional activity of the proteins [42]. These data show that Ca^{2+} fluctuations in the ER lumen influence activity of ER chaperones [43].

Although the ER has many functions in the cell, its role as the main Ca^{2+}-storage compartment is critical [28]. It is demonstrated by early embryonic lethality of calreticulin-deficient mice (due to disruption in Ca^{2+}-homeostasis) [35]. Furthermore, this suggests that the role of the ER as a main Ca^{2+}-storage organelle has the most dramatic impact on development. This not only indicates a crucial role for Ca^{2+} but also an essential role of the ER as a Ca^{2+} store.

2.0 Protein Folding in the Endoplasmic Reticulum. Rough ER, with its associated ribosomes, is actively involved in protein synthesis [44]. In eukaryotic cells about one-third of all newly synthesized proteins (secretory or plasma membrane) are synthesized on ER membrane-bound ribosomes and undergo maturation and posttranslational modifications in the lumen ER [9].

The ER is the first compartment of secretory pathway and, as such, plays a critical role in the synthesis, folding and maturation of membrane and secretory proteins [2]. This includes roles in posttranslational modification of newly synthesized proteins and quality control during synthesis and maturation of proteins produced in the ER [45].

Protein folding in the ER is one of the fundamental processes in the living cell [46]. To perform these functions it contains a wide range of molecular chaperones, folding enzymes and other proteins. The process of protein folding involves a series of steps during which an unfolded polypeptide chain achieves its final structure. Only proteins that have achieved their native conformation and meet strict quality control criteria can then be sent to their final destination [44]. The environment of the ER

is similar to the extracellular space; it has a high Ca^{2+} concentration, is highly oxidizing and is crowded with a large number of chaperones and foldases [47-49]. The lumen of ER is not only crowded with its resident chaperones and foldases but also with "clients", which are often misfolded substrates. Client proteins found in the lumen of ER have different conformations depending on the stage of maturation. This results in a large number of partially folded polypeptide chains that have not achieved their final, lowest-energy structures [46]. The minimization of the number of hydrophobic amino acid residues at the surface of the protein and obtaining the lowest-energy state are the driving forces of protein folding [17]. Molecular chaperones present in the ER lumen bind to these incompletely folded proteins and prevent inappropriate interactions with other molecules in the ER lumen, which has a high protein concentration (~100 g/l or 2 mM) [50]. In mammalian cells, proteins are targeted to the ER by a hydrophobic signal sequence composed of approximately the 20 first amino acids [51]. Upon translation, the mRNA sequence is recognized by the signal recognition particle (SRP), which targets the translating ribosome to the ER membrane by binding to the SRP-receptor and making sure that folding will only begin once the polypeptide has reached the ER [51]. In eukaryotic cells, polypeptide chains are translocated into the ER *via* a conserved, translocon complex formed by the heterotrimeric Sec61 channel [52]. Although the same translocation machinery is used by secretory and membrane proteins, only integral membrane proteins are integrated into the ER membrane and must do so in their proper orientations [52].

As a nascent protein enters the ER lumen through the translocon, it is recognized by a set of proteins associated with the channel. The hydrophobic signal sequence is cleaved off by a signal peptidase (SP) and oligosaccharyltransferase (OST) catalyzes the addition of a preassembled oligosaccharide core (N-acetylglucosamine$_2$-mannose$_9$-glucose$_3$ or $Glc_3Man_9GlcNAc_2$) at selected asparagine residues on the nascent polypeptide. This post-translational modification taking place in the lumen of ER is called N-glycosylation (1-2) [53, 54]. Multiple N-glycans are attached to most of the proteins that enter the secretory pathway, and the glycosylation is one of the most important post-translational modifications. The addition of glycan changes the general properties of the protein, and is important for protein transport, interactions with other proteins and degradation. At the same time, the glycan provides binding sites for lectin chaperones that residue in the ER [55]. Glycans are covalently attached as a side chain on asparagines in the consensus sequence N-X-S/T [56]. Coordination of posttranslational modification is a challenging task for the protein folding machinery of the ER. The initial glycan is composed of three glucose, nine mannose and two N-acetyl glucosamine residues ($G_3M_9GlcNAc_2$) [11]. This structure is transferred onto the polypeptide chain by OST from dolichol, a lipid donor that resides in the ER membrane [57]. Immediately after glycan transfer, it is trimmed by the sequential actions of glucosidase I and glucosidase II to generate monoglucosylated side chains ($G_1M_9GlcNAc_2$) (Figure 1-2 A) [58]. This allows for initial interactions with calnexin and calreticulin, which have a high affinity for monoglucosylated glycans (Figure 1-2) [11]. The chaperone–glycan interaction is later disrupted by removal of the third

glucose by glucosidase II to generate a nonglucosylated intermediate product. However, if the protein displays a non-native structure, it will be recognized by UGGT (uridine diphosphate glucose:glycoprotein glycosyltransferase), a folding sensor, which will add back the glucose, allowing another pass through the calnexin–calreticulin cycle [11]. Upon substrate binding, calnexin and calreticulin associate with ERp57, a member of the protein disulfide isomerase (PDI) family, and a thiol oxidoreductase in the ER that catalyzes the formation and isomerization of disulfide bonds between two cysteines in the correct proximity [59]. Disulfide bond formation is a very common covalent modification among newly synthesized proteins in the lumen of ER. The chaperone–ERp57 interaction brings ERp57 and its protein substrates in close proximity, and results in increased catalysis of disulfide-bond formation in newly synthesized glycoproteins [60].

2.1. The Calnexin-Calreticulin Cycle. The calnexin-calreticulin cycle involves a sophisticated and complex machinery that assists in folding of newly synthesized glycoproteins and is able to distinguish terminally misfolded proteins without prematurely interrupting folding process (Figure 1-2 B) [12, 61-63]. The ER quality control system is comprised of the ER lectin chaperones calnexin and calreticulin and of the ERp57 oxidoreductase that interacts with calnexin and calreticulin to catalyze disulfide bond formation on newly synthesized glycoproteins [64]. Other components of the quality control system, like UGGT, are involved in sensing unfolded proteins and sending them for ERAD [65].

Having two lectin chaperones with different topologies broadens the scope of protein substrates that they can assist. It is important to

recognize that calnexin and calreticulin, in addition to being lectin-like chaperones, function as bona fide molecular chaperones, capable of interacting with the polypeptide segments of their glycoprotein substrates [66, 67]. As calnexin and calreticulin bind monoglucosylated glycans, the cycle is closely regulated by the action of glucosidase I, which removes the outermost glucose first [11]. Other enzymes in the calnexin–calreticulin cycle such as glucosidase I, glucosidase II and UGGT have key roles in the quality control of glycoproteins in the ER. Glucosidase II is a soluble homodimer that consists of two subunits (α and β) [68]. This enzyme removes the second-outermost glucose on the N-linked glycan and generates monoglucosylated substrates that can enter the calnexin-calreticulin cycle [11]. Glucosidase II also removes a final glucose, creating a nonglucosylated substrate after it is released from the cycle. UGGT controls re-glucosylation of substrates, as it can transfer a single glucose residue from UDP-Glc (uridine diphosphate glucose) to the terminal mannose of the substrate side chains of glycoproteins that contain non-native structures [69]. UGGT combines chaperone and glycosyltransferase functions, and exhibits high affinity towards misfolded or incompletely folded glycoproteins by recognizing the core structure of the glycan [70]. In the calnexin–calreticulin cycle, UGGT acts as a folding sensor that re-glucosylates substrates and enables rebinding to calnexin and/or calreticulin. This cycle continues until a native conformation is achieved or ERAD is initiated [13]. Although the actions of UGGT are closely dependent on calnexin-calreticulin substrate binding, these proteins do not form complexes [71].

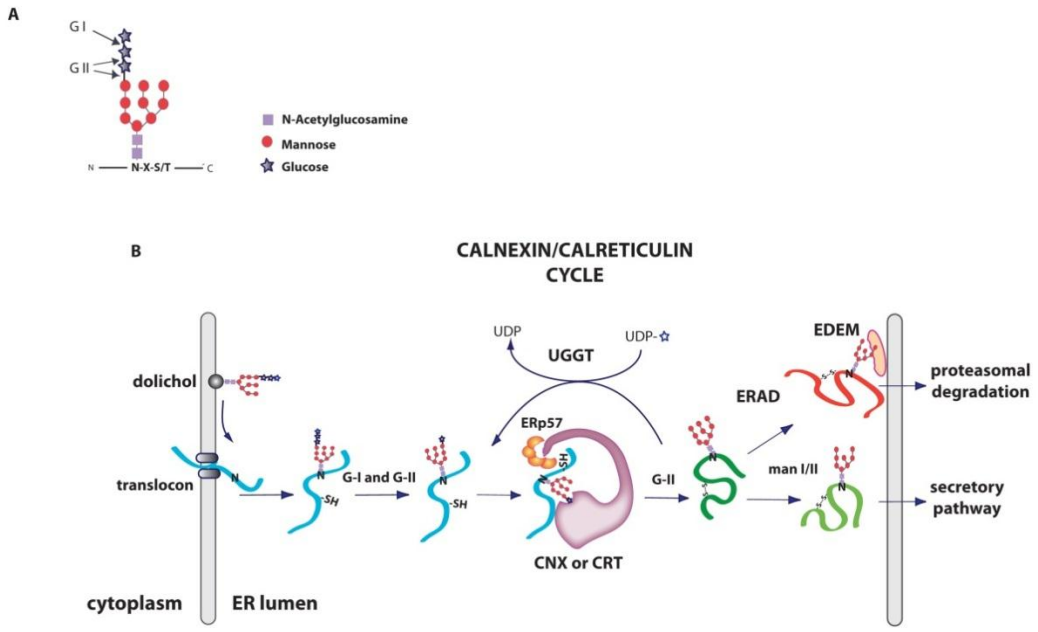

Figure 1-2 A step by step figure of the calnexin-calreticulin cycle and the quality control system in the endoplasmic reticulum. A. Schematic representation of N-linked glycan and sites of cleavage for glucosidase I and II. B. Proteins entering the ER are often modified with glycan added to an Asn (N) residue in the consensus N-X-S/T. First, N-glycan is transferred from the membrane bound donor dolichol to substrate protein. Next, glucosidase I and II remove two terminal glucoses, resulting in formation of monoglucosylated substrate that is recognized by the lectin chaperones calnexin or calreticulin. These interactions facilitate folding of newly synthesised glycoproteins. ERp57 associates with the P–domain of the lectin chaperones (calnexin or calreticulin) and catalyzes formation and isomerization of disulfide bonds. After release from the calnexin-calreticulin cycle, glucosidase II removes the final glucose. If the substrate protein achieved its final conformation, a mannosidase present in the ER removes one of the mannose residues and the correctly folded protein leaves the ER lumen and proceeds to its final destination. However, some proteins require multiple rounds of association with the chaperones; such substrates are re-glucosylated by UGGT (uridine diphosphate glucose:glycoprotein glycosyltransferase). This folding sensor recognizes the non-native state of the protein and by adding glucose facilitates it entering calnexin-calreticulin folding cycle. Terminally misfolded proteins are targeted for ER-associated degradation that includes binding by factors facilitating ERAD, like EDEMs (ER degradation enhancing α-mannosidase-like lectins).

Both calnexin and calreticulin have a high affinity for glycoproteins [72]. Extensive mapping studies helped to identify oligosaccharide binding sites within the globular domains of calnexin and calreticulin [73]. Mutations of residues within the lectin-binding sites of either calnexin or calreticulin result in a decrease in lectin function [74-76]. However, multiple studies show that calnexin and calreticulin interact with folding glycoproteins not only through the glycan, but also through polypeptide binding. In this dual-binding model, chaperones bind to the hydrophobic segments of their substrates [66, 67]. Evidence for polypeptide-based interactions has shown that removal of glycan did not abolish the interaction between the unfolded protein substrate and the chaperone [66, 67, 77-79]. Calnexin and calreticulin have overlapping but distinct substrate specificities [80, 81]. As the glycan-binding sites in calnexin and calreticulin are virtually identical [82], the basis for substrate specificity is likely related to topology and polypeptide-binding sites [83].

Folding, assembly and peptide-loading of major histocompatibility complex (MHC) class I molecules in the ER by calnexin and calreticulin have been extensively studied and provide an excellent model for describing the mechanisms of action of the two lectins. The MHC class I peptide-loading complex consists of TAP (transporter associated with antigen processing), the chaperone-like molecules tapasin, calnexin and calreticulin, which work together to promote complex formation [84, 85], and ERp57, which assists in disulfide-bond formation. The first complex formed by TAP and tapasin is recognized by calnexin and ERp57 in a glycan-independent manner. Next, calnexin is released from the intermediate complex [84] and the MHC class I molecule associates with

calreticulin , which results in the MHC class I peptide-loading complex. Studies involving knockouts of genes encoding different members of the MHC class I peptide-loading complex revealed that in B cell specific knockouts of ERp57 mice, assembly of the MHC class I molecules is disrupted (Table 1-1) [86]; in calreticulin-deficient cells, accelerated export of MHC class I molecules from the ER is observed; and in calnexin-deficient cells, formation of the peptide-loading complex is unaltered [87]. Importantly, calnexin or calreticulin that lack the chaperone motif within the globular domain can still bind the MHC class I peptide-loading complex, suggesting peptide–based interactions between chaperones and the complex. Lectin-independent interactions between calnexin and its substrates are further supported by studies with calnexin mutants that are unable to bind glycan [75]. These lectin-binding-deficient calnexin mutants are still able to prevent aggregation of non-glycosylated proteins [75].

2.1a Structures of Calnexin and Calreticulin. Calnexin (predicted molecular weight of 67-kDa) and calreticulin (46-kDa) share 40% amino acid sequence similarity and 30% amino acid sequence identity [40]. They are both composed of three distinct structural and functional domains: a globular N-terminal domain, an extended P-arm and an acidic, charged C-terminal domain (Figure 1-3). The key difference between the two proteins is that calnexin is a type I transmembrane ER protein with a single transmembrane helix, whereas calreticulin is a resident, soluble ER luminal protein. Both proteins are ubiquitously expressed in all cells that have ER membrane. The calnexin amino acid sequence is highly conserved between species (93–98% identity). Calreticulin is a Ca^{2+}-

binding chaperone that has a role in many processes, including Ca^{2+} storage and signalling and regulation of gene expression [30]. Calnexin and calreticulin both contain an N-terminal signal sequence that targets them to the ER [12, 30].

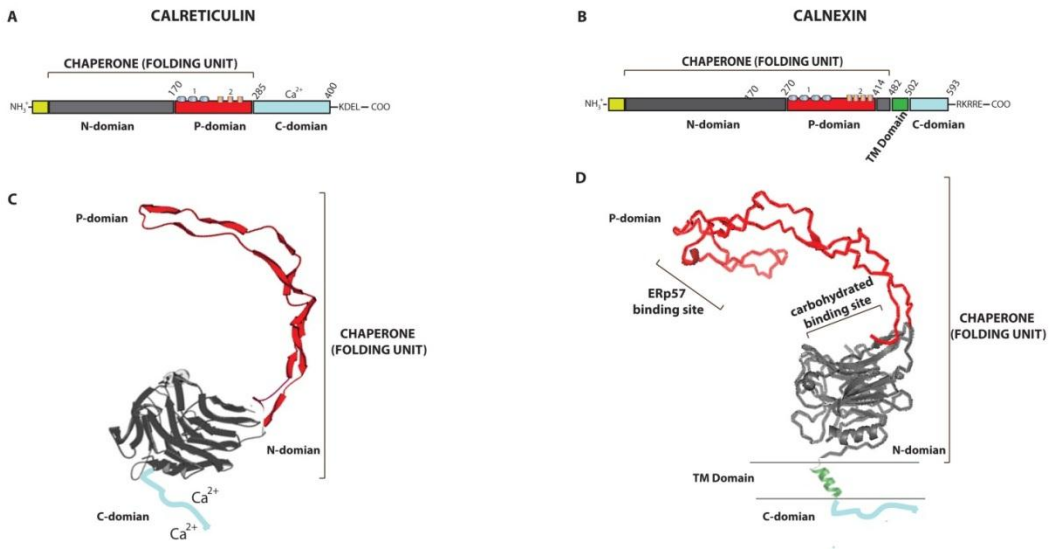

Figure 1-3 Linear models and structures of two homologous chaperones: calreticulin and calnexin. **A** and **B**. Linear, schematic representation of calreticulin's domains. Numbers represent the first amino acid residue of a distinct domain. The ER signal sequence is in yellow; the N and P-domains form the chaperone unit essential for substrate folding. Numbers 1 and 2 indicate repeats (**A,** repeat 1: PXXIXDPDAXXPEPWDE and repeat 2: GXWXPPXIXNPXYX; **B**, repeat 1:IXDPXA/DXKPEDWDX and repeat 2: GXWXPPXIXNPXYX) within P-domain. The C-domain contains Ca^{2+} binding sites. The KDEL sequence is an ER retrieval signal at the C-terminus of the protein. **C**. Three dimensional model of calreticulin based on NMR studies of the P-domain [88] and crystallographic results for calnexin (PDB code 1JHN) [89]. The N-globular domain is in grey and the extended P-arm is in red. The N and P domains form the chaperone unit critical for folding of newly synthesized proteins. The C-domain (in blue) contains many negatively charged amino acids responsible for the high Ca^{2+}-binding capacity of calreticulin. **D**. Three dimensional model of calnexin created based on crystallography results for globular the N-domain (PDB code 1JHN) [89]. The N-domain is in gray and the extended P-domain in red; these domains form chaperone unit. The tip of the P-domain is a site of interaction between calnexin and the oxidoreductase ERp57. Green represents the transmembrane helix (TM) and blue, the cytosolic tail (C-domain).

The luminal domain of calnexin has been resolved by X-ray crystallography [89]. It consists of two distinct regions: a compact globular domain formed by β-sheets (amino acid residues 1–270 and 414–482), and a 145-residue region that forms an extended proline-rich arm called the P-arm (or P-domain) [89]. Based on this information, the secondary structure of the N-domain of calreticulin is also predicted to be a globular domain comprising eight anti-parallel β-strands [34] Recently, an X-ray structure of the human calreticulin globular domain has been solved. Detailed analysis revealed that the N-terminal extension binds to the end of lectin-binding site, providing a larger substrate binding region [90]. These results reveal the interesting peptide-binding properties of calreticulin and provided new, important insights into the chaperone activity of calreticulin. The N-terminal domains of both chaperones contain binding sites for carbohydrate, polypeptide [73, 74], Ca^{2+}, Zn^{2+} and ATP [73, 91]. Within the N-terminal domain of calreticulin, Y^{109} and D^{135} have been identified as essential for the lectin–glycan interactions between calreticulin and its substrates [74]. Recently, Kozlov and colleagues crystallized calreticulin's lectin domain in complex with the pentasaccharide (Glc_1Man_4), providing important insight into the architecture of the lectin-binding site. Importantly, they show that the monoglucosylated-glycan-binding sites in calnexin and calreticulin are nearly identical, and they bind glycans in a very similar manner [82]. The residues responsible for glycan binding are well-conserved in both chaperones. This structure also shows that Ca^{2+} influences glycan binding by stabilizing calreticulin's lectin domain. Moreover, disulfide bonds between C^{88} and C^{120}, and (I didn't know T formed disulfide bonds!) T^{244} and T^{302}, within the globular domain, are essential for the chaperone

function of calreticulin [92]. For calnexin, Y^{156}, K^{167}, Y^{186}, M^{189}, E^{217} and E^{426} have been identified as essential for oligosaccharide binding [75].

The P-domains in both calnexin and calreticulin contain pairs of amino acid repeats (repeat 1: IXDPXA/DXKPEDWDX, and repeat 2: GXWPPXIXNPXYX). Calreticulin's P-domain has three sets of repeats 1 and 2, whereas calnexin's P-domain has four sets of repeats 1 and 2. This results in calnexin's P-domain being longer than that of calreticulin [88]. These sets of repeats are essential for the formation and maintenance of the specific structures of the P-domains that are formed by antiparallel β-sheets [88]. The P-domain contains secondary binding sites for oligosaccharide and polypeptide [93]. The tip of the P-domain associates with ERp57, and brings ERp57 in close proximity to the substrates. The interaction of calreticulin and ERp57 is abolished when E^{239}, D^{241}, E^{243} and T^{244} at the tip of the P-domain are mutated [92].

The C-terminal domain of calnexin is formed by amino acid residues 509–605, and it is a short extension after the transmembrane domain. The C-terminal domain is an acidic, negatively charged cytosolic domain that can be phosphorylated by several kinases, and binds Ca^{2+} with moderate affinity. It also takes part in protein–protein interactions [94]. The C-terminal domain of calreticulin is also negatively charged but, unlike calnexin's, has a low affinity and high capacity for Ca^{2+}, binding over 50% of the Ca^{2+} present in the ER lumen. Both calnexin and calreticulin contain ER retention signals within their C-terminal domains. For calnexin, the ER retention signal is RKPRRE, whereas calreticulin is a classical ER-resident protein that is retained in the lumen of ER by the KDEL-receptor-driven ER retrieval system [83].

2.2b Structure of ERp57. Many transmembrane and secretory proteins that proceed through the secretory pathway contain a disulfide bond that stabilizes their final structure and is crucial in protein maturation. The ER lumen is a more oxidizing environment than the cytoplasm making it an ideal location for disulfide-bond formation. Formation of disulfide bonds is crucial for protein maturation. Lack of disulfide bond formation often results in terminally misfolded proteins that are retained in the ER [59]. Proteins entering the ER contain reduced cysteines and leave ER with oxidized cysteines that have formed disulfide bond(s). For proteins containing multiple disulfide bonds the isomerization (or do you mean exchange) of disulfide bonds is also required [95].

Disulfide bond formation and isomerization is catalyzed by a group of ER resident proteins that belongs to the PDI family [96]. The PDI group of proteins consists of over 20 different oxidoreductases that reside in the ER lumen [97]. ERp57 is an extensively studied member of the PDI family; it interacts with lectin chaperones of ER and assists in folding of newly synthesized glycoproteins [98]. The interactions between ERp57 and the lectin chaperones occur primarily through the P-domains of either calnexin or calreticulin (Figure 1-4) [99, 100].

The structure of ERp57, which has been resolved by nuclear magnetic resonance (NMR) spectroscopy, shows that it has a "U" shape similar to the structure of the other members of the PDI family. ERp57 has four distinct domains: a b b' a' (4). Overall, ERp57 and PDI share 33% amino acid sequence identity [101]. However, unlike PDI, the C-domain of ERp57 is not acidic but basic and this mediates the substrate specificity of ERp57 [102].

The a and a′ domains are responsible for reductase and isomerase activities of ERp57 [103]. These domains share 50% amino acid identity with PDI [103]. The b and b′ domains are crucial for binding to the P-domains of the lectin chaperones calnexin or calreticulin [101]. These interactions have been confirmed by mutational studies; when positively charged residues in ERp57 are mutated ($K^{274}A$ and $R^{282}A$), binding of ERp57 to calnexin is decreased [101].

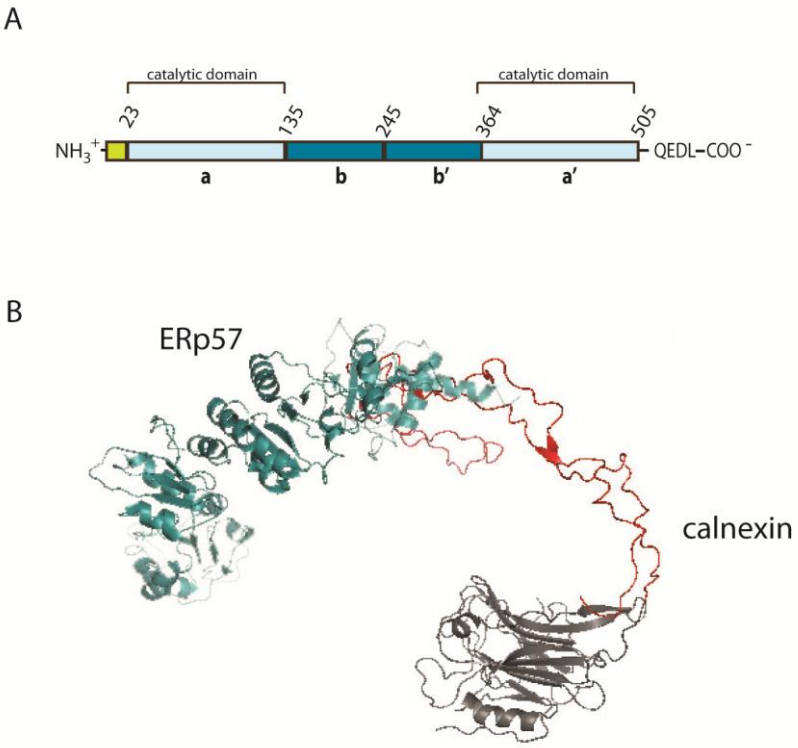

Figure 1-4 A model of ERp57 and its interaction with calnexin. **A**. Linear representation of ERp57 domains; numbers correspond to the beginning of distinct structural domains. ERp57 consists of an N-terminal signal sequence shown in yellow, two catalytic domains *a* and *a′* (light blue), substrate binding domains b and b′ (dark blue) and a C-terminal ER retention signal (QEDL). **B**. Interaction between the P-domain of calnexin and ERp57. The three-dimensional structure of ERp57 is based on NMR studies of the *a* and *a′* domains of protein disulfide isomerase (PDI) and the *b* and *b′* domains of ERp57 [101].

2.2 Additional Functions of Endoplasmic Reticulum Chaperones. All the ER chaperones are involved in a great number of other cellular processes [16]. Despite their structural similarities and roles as molecular chaperones in the ER, both calnexin and calreticulin are involved in other important cellular events inside and outside the ER lumen. Calnexin is involved in cell–cell adhesion through interactions with integrins [104]. Calnexin is involved in the assembly of β_1-integrins; the largest subgroup of the β-chain family is known to bind many adhesion proteins, such as collagen, laminin, fibronectin, vitronectin and many others [104].

Calnexin has also been shown to be involved in the process of apoptosis, as calnexin-deficient cells are more resistant to stress-induced apoptosis; calnexin also interacts with apoptotic proteins, such as Bap31 and caspase 12 [105, 106].

Calnexin is a molecular chaperone for a number of substrates. Therefore, it has been implicated in many diseases and pathological conditions (Table 1-1). Calnexin has been found to be involved in the ER retention of the ΔF508 protein, a mutant form of the CFTR (cystic fibrosis transmembrane conductance regulator) chloride channel [80]. The ER retention results in the CFTR channel not being localized to the plasma membrane and this dysfunction is known to contribute to the disease [107, 108].

Many functions of calreticulin are related to its ability to bind Ca^{2+} with high capacity at its C-terminal domain. In the ER lumen, calreticulin is involved in Ca^{2+} buffering and storage, and in Ca^{2+} signalling. Over-expression of calreticulin results in increased amounts of ER Ca^{2+}, whereas

calreticulin deficiency leads to a reduced Ca^{2+}-storage capacity in the ER [109-111]. Although calreticulin has been widely studied for its role in protein folding and for its role as the major Ca^{2+}-buffering chaperone of the ER, it plays a number of other roles within the cell [30]. Calreticulin is the most potent buffer of ER Ca^{2+} and is responsible for binding over 50% of ER luminal Ca^{2+} [33]. This ability to essentially regulate ER luminal Ca^{2+} concentrations inevitably affects other biological functions within the cell [30, 112-119].

Interestingly, calreticulin has also been found outside the ER, where it is involved in other biological functions [115, 120]. It has been proposed that calreticulin translocates to the cell surface with ERp57 [120]. This exposure of calreticulin to the cell surface has been shown to be critical for immunogenic cell death [120].

Calreticulin is involved in a variety of biological pathways and its absence can lead to a range of pathological conditions (Table 1-1) [30]. Calreticulin has also been shown to be important in cardiac disease through its modulation of intracellular Ca^{2+} stores [30]. Interestingly, calreticulin has been found helpful in wound healing, as it is expressed in fibroblasts of injured dermis and has been shown to promote healing [117]. Experiments showed that when calreticulin is topically applied to the wounds of diabetic mice or cortisone-impaired pigs, their wounds heal faster than wild-type controls [30]. The numerous functions of calreticulin within the cell make it an interesting target for treatment of various of diseases.

ERp57 has been implicated in a number of different functions within the cell [121]. The most widely studied functions of ERp57 are its role in quality control with calnexin and calreticulin in the folding of

newly synthesized proteins and its involvement in the assembly of the MHC class I molecules [12, 121, 122]. ERp57 associates with the lectin-like ER chaperones calreticulin and calnexin to catalyze the isomerization of disulfide bonds of newly synthesized glycoproteins with unstructured disulfide-rich domains [12]. ERp57 is recruited into the calnexin-calreticulin cycle, where the *b* and *b′* domains of ERp57 interact with the extended P-domains of both calnexin and calreticulin [12]. ERp57 is an important part of quality control system; it has been shown that its isomerase activity increases significantly when ERp57 forms complexes with either calnexin or calreticulin [60, 123].

Beside its ability to catalyze disulfide bond formation and isomerisation, ERp57 is involved in many other cellular functions. ERp57 has been extensively studied from the perspective of the folding of MHC class I molecules. ERp57 in complex with calnexin binds glycosylated heavy chain of MHC I and catalyzes disulfide bond formation allowing the heavy chain to assemble with the β_2-microglobulin (β_2-m). At the late stage, heterodimer complexes of heavy chain and β_2-m acquire peptides associated with calreticulin, ERp57, TAP, tapasin (a glycoprotein critical for peptide loading of MHC class I) forming the peptide loading complex [122]. Interestingly, mutagenesis studies have shown that the catalytic activity of ERp57 is not required for MHC class I assembly, as catalytically inactive ERp57 is able to promote peptide loading in a similar manner to wild-type ERp57 [124]. Recently, ERp57 has also been localized outside the ER, in the nucleus, on the cell surface and in the cytoplasm, suggesting that it may have other functions other than the ones indicated above [125]. ERp57 has been shown to be involved in gene regulation [121, 126]. ERp57 also known as GRP58 [121, 127-133] has been reported to affect STAT3

signaling [126]. STAT3 is involved in many physiological processes including oncogenesis, tumour growth, early embryogenesis, lymphocyte growth, wound healing and postnatal survival [134]. Although it is not clear how STAT3 activity is negatively regulated by ER resident ERp57/GRP58, it has been suggested that the effect of ERp57 on STAT3 may be due to formation of inhibitory complexes between ERp57 and the STAT3 protein, either in the cytoplasm or the nucleus [125, 127, 135-137]. Our data showed that ERp57-deficient cells have significantly increased STAT3 activity [126]. Moreover, we showed that the ER, and not the cytosolic form of ERp57 is responsible for inhibition of STAT3 activity [126]. Furthermore, we were able to identify that the ERp-57-dependent modulation of STAT3 is enhanced by ER luminal interactions between ERp57 and calreticulin [126]. In summary, our results showed that, *in vivo*, ERp57 and STAT3 may not interact and that the observed inhibition of STAT3 activity is due to ERp57-dependent signaling from the ER lumen [126].

ERp57 has been linked to many human diseases, such as cancer, prion disorders and Alzheimer's disease (Table 1-1) [128, 131, 138-141]. Increased expression of ERp57 has been seen in transformed cells [129]. It is possible that the impact of ERp57 on the modulation of STAT3 activity could also contribute to its oncogenic properties [126, 127]. At early stages of prion disease, ERp57 may have a neuroprotective role [128]. In patients with Alzheimer's disease, ERp57 together with calreticulin have been found in the cerebrospinal fluid, where they might be involved in prevention of the aggregation of β-amyloids, a toxic component of plaques found in Alzheimer's patients. Although detailed studies are still critical,

the numerous functions of ERp57 make it an interesting candidate for treatment and diagnosis of diseases.

Interestingly, in the absence of calnexin, calreticulin or ERp57, eukaryotic cells in culture survive with no severe problems [126, 142]. Gene-knockout technology has been used to create mice deficient in ER chaperones. Deletion of such chaperones as BiP (GRP78) [143], GRP94, ERp57 [126, 144], calreticulin and UGGT [145] result in embryonic lethality (Table 1-1). These observations show the importance of ER chaperones for early development, as well as the specificity of each chaperone, as the absence of one chaperone cannot be compensated for through the presence of others. Surprisingly, calnexin deficiency results in neurological disorders [146]. Mice lacking calnexin are born alive but exhibit severe neurological disorders, as well as a decrease in size and weight compared with wild-type mice [146].

3.0 The Endoplasmic Reticulum Homeostasis. In the living cell, protein homeostasis is achieved by coordinated and closely controlled actions of protein synthesis, trafficking machinery and protein degradation. In eukaryotic cells, protein homeostasis, also referred to as proteostasis, is a series of processes that controls protein synthesis and protein degradation, leading to balance and integrity of the cell [147]. Balance between protein synthesis and degradation is critical for a living cell survival. Risks related to compromised balance in the cell, include misfolded proteins that contribute to many conformational diseases.

The difficult process of protein folding takes place in the complex and crowded ER environment. There are many factors that can compromise proper protein folding, such as errors occurring during

transcription or translation, genetic mutation, cellular stress and many others. Moreover, accumulation of misfolded proteins is extremely toxic to the cell. To prevent this, the mammalian cell developed mechanisms and processes to minimize the risk of accumulation of misfolded proteins. When quality control fails, these mechanisms ensure that only properly folded proteins leave the ER. Those proteins that did not meet quality control criteria are subject to degradation.

3.1 Endoplasmic Reticulum Associated Degradation (ERAD). After a nascent protein is released from calnexin–calreticulin cycle, the next step depends on its folded state. If the protein has achieved its final, native conformation with no exposed regions (usually hydrophobic patches), the final glucose is trimmed through de-mannosylation by ER mannosidases I and II (Figure 1-2). Consequently, the protein exits the ER and continues through secretory pathway. However, if the protein is recognized as terminally misfolded, it is targeted for ERAD to prevent accumulation of incorrectly folded peptides and terminally misfolded proteins [148, 149]. Sequential steps of ERAD include: recognition of terminally misfolded proteins, translocation of misfolded proteins to the cytosol and degradation through the ubiquitin–proteasome system [13]. For selection of misfolded proteins, ERAD relies on factors that recognize native misfolded regions, including hydrophobic patches, unpaired cysteines and extensively de-mannosylated N-glycans. Terminally misfolded "clients" of the calnexin–calreticulin cycle can be recognized by EDEM (ER degradation-enhancing 1,2-mannosidase-like protein), a factor that delivers calnexin substrates to the ERAD pathway [150, 151] (Figure 1-2). EDEM has been shown to interact with calnexin through its

transmembrane domain [150]. Interestingly, it has been shown that over-expression of EDEM leads to accelerated release of unfolded glycoproteins from the scalnexin–calreticulin cycle [151]. The mechanisms of retro-translocation of ERAD substrates to the cytosol are not very well understood. Derlin-2 and derlin-3 translocation channels and/or Sec61 might be involved in retro-translocation [152-156]. These translocation channels have been found to associate with EDEMs [65]. This suggests that EDEMs may be involved in delivering ERAD substrates to translocation channels [150]. ERAD substrates are translocated into the cytoplasm and as they exit the retrotranslocon, they are polyubiquitinated in the cytoplasm by E3 ubiquitin ligases. Polyubiqitination for integral membrane proteins can occur at the same time as retrotranslocation, whereas for soluble proteins it takes place once they are in the cytosol [13]. The misfolded protein tagged with a polyubiquitin chain is recognized by a proteasome subunit [157]. The proteasome consists of two subunits: 19S and 26S. Ubiquitin receptors are localized to the 19S subunit [158]. High affinity ubiquitin-binding molecules drive substrates to the 26S subunit, where the protein proteolysis takes place [157].

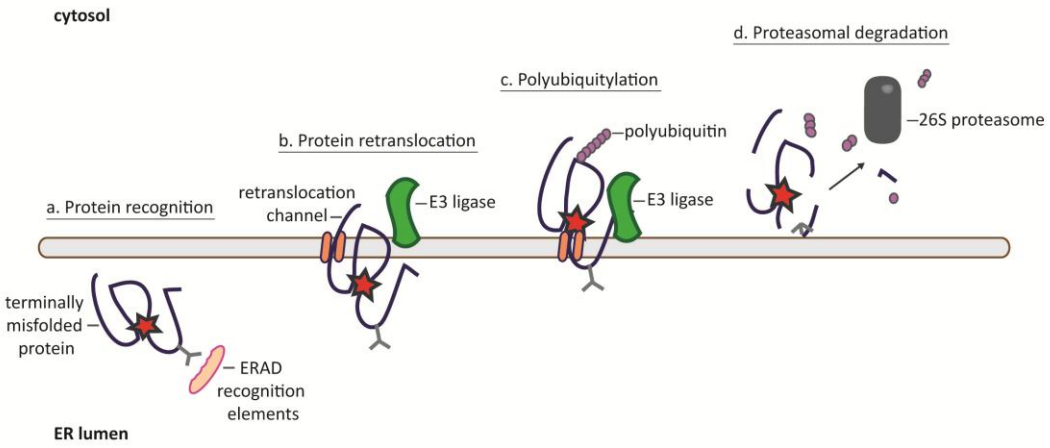

Figure 1-5 Schematic representation of sequential steps of endoplasmic reticulum-associated degradation (ERAD). a. Protein recognition. Terminally misfolded proteins are recognized by ERAD-recognition elements like EDEM. **b.** Protein retranslocation. Proteins recognized as ERAD substrates are translocated back into cytosol, retrotranslocation is facilitated by different channels and E3 ligases. **c**. Polyubiquitination. As ERAD substrates enter the cytosol, they are polyubiquitinated by E3 ubiquitin ligases. **d**. Proteasomal degradation. Polyubiquitinated substrates are recognized by receptors of the 26S proteasome and targeted for degradation.

Spontaneous errors during transcription or translation, genetic mutation and environmental factors may contribute to compromised protein folding efficiency. Therefore, ERAD ensures a balance between protein synthesis, folding and degradation, which is essential for the homeostasis of every cell. Imbalance between these three processes leads to accumulation of misfolded proteins in the cytoplasm [65].

Often, physiological conditions require an enhanced activity of ERAD. However, uncontrolled ERAD activity might interfere with folding processes ongoing in the ER [65]. Regulation of ERAD and maintaining of its basal steady state is achieved by the rapid removal of selected ERAD regulators from the lumen of ER and their degradation via proteasome and lysosomal proteases [65].

3.2 Unfolded Protein Response (UPR). A number of stress conditions can contribute to accumulation of misfolded proteins in the lumen of ER; such a condition is referred to as ER stress [5]. For example, when newly-synthesised proteins in the ER fail to achieve their correct conformations, escape the quality control machinery, and are not recognized as ERAD substrates, they accumulate in the ER lumen and result in activation of ER stress [10, 159, 160]. ER stress is a term that describes a condition where ER homeostasis is lost due to the protein folding capacity of the ER being overwhelmed [161]. To deal with ER stress, the eukaryotic cells have evolved an adaptive complex response, the unfolded protein response (UPR) [161]. Subsequently, UPR is activated to deal with the increased load of misfolded proteins. The UPR is a collection of cellular pathways that function to maintain homeostasis in the ER and the whole cell. Mammalian UPR is represented by three ER-resident transmembrane

proteins: inositol-1-requiring enzyme (IRE1), PKR-like endoplasmic reticulum kinase/pancreatic eukaryotic initiation factor-2α (PERK/PEK) and activating transcription factor 6 (ATF6) (Figure 1-6) [162]. Together, these three proteins serve as proximal sensors of ER stress [49, 163-165]. In summary, UPR decreases the load of accumulated misfolded proteins by activating the transcription of genes encoding ERAD proteins and genes involved in increasing the ER folding capacity, as well as inhibiting general protein translation [160, 166-170].

Activation of UPR sensors includes IRE1, a transmembrane proteins kinase. Its oligomerization into dimers or higher order structures is a first step of the UPR pathway [171, 172]. IRE1 is a type I membrane protein that contains three distinct domains: an N-terminal luminal domain, a carboxy-terminal kinase domain and an endoribonuclease (RNase) domain [173, 174]. Oligomerization of IRE1 results in trans-autophosphorylation of its kinase domain, which in turn activates the RNase domain. Two models have been proposed to explain IRE1 activation. In the first model, the ER resident chaperone BiP is bound to IRE1 under steady state conditions and inhibits IRE1 oligomerization. However, when misfolded proteins accumulate in the ER lumen, BiP dissociates from IRE1, resulting in IRE1 homodimer *trans*-autophosphorylation and activation of RNase activity [175]. In the second model, misfolded proteins bind directly to the luminal domain of IRE1, resulting in its oligomerization [176]. Activation of IRE1 results in the rapid degradation of ER-localized mRNA and reduces protein overload in the ER. The active RNase domain of IRE1 is responsible for non-conventional splicing of *xbp1*u into *xbp1s,* which encodes XBP1 (X-box

binding protein 1), the bZiP transcription factor [161]. The XBP1s translocate to the nucleus and activate the transcription of genes encoding ER molecular chaperones like BiP, GRP94, calreticulin and proteins involved in the ERAD pathway [173].

PERK is also a type I transmembrane ER protein containing an N-terminal luminal domain and cytosolic kinase domain, and it is thought to be activated by similar mechanisms as those involved in the activation of IRE1 [177]. When activated, the kinase domain of PERK phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2α) [175, 178]. Activation of PERK causes increased transcription of genes involved in cell survival under cellular stress [179].

The third branch of the UPR consists of ATF6, a type II ER transmembrane protein (Figure. 1-6) [180, 181]. Under basal conditions, ATF6 is associated with BiP; however, when unfolded proteins accumulate in the ER, ATF6 dissociates from BiP. Dissociated ATF6 is reduced and monomeric and translocates to the Golgi apparatus, where it is subject to regulated intramembrane proteolysis (RIP) sequentially by Site-1 protease (S1P) and Site-2 protease (S2P) [180, 182]. This sequential cleavage releases nuclear-ATF6 (N-ATF6) transcription factor, which can then translocate to the nucleus and induce transcription of genes encoding ER stress proteins, including BiP, calreticulin and GRP94 [183].

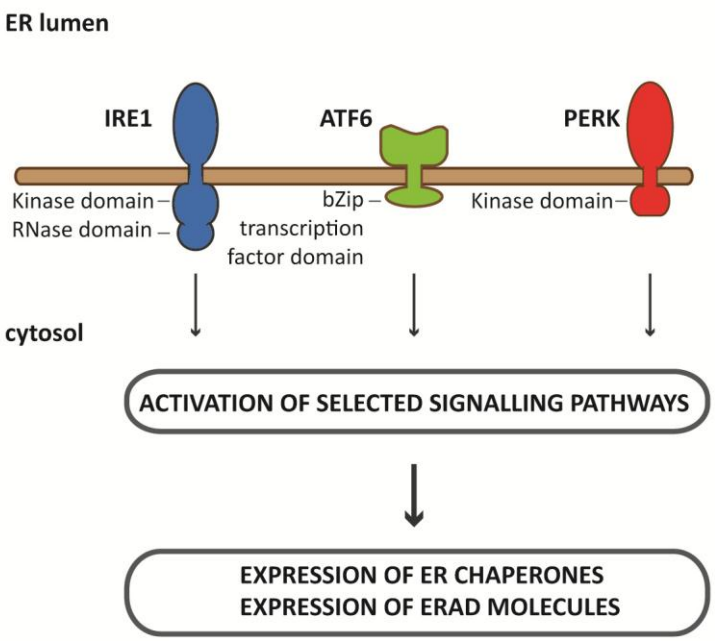

Figure 1-6 Three branches of the unfolded protein response (UPR). Mammalian UPR consists of three main branches distinguished by three ER membrane proteins: IRE1, ATF6 and PERK, that serve as a UPR sensors under ER stress. IRE1 consists of luminal and kinase domains that activate the RNase domain; ATF6 has a luminal domain, and a cytosolic domain that contains a bZip transcription factor that is being released upon activation. PERK has a similar structure to IRE1 but lacks the RNase domain. After the sensors are activated, they initiate several different responses that result in activation of a number of signalling pathways that may result in the increased expression of ER chaperones and proteins involved in ERAD.

Depending on the severity of the ER stress, UPR can simultaneously activate survival and apoptotic signals. One of the factors that influences the decision of the cell to activate survival or cell death is time. Under conditions of sustained ER stress and when cell homeostasis cannot be reached, there is an increase in the likelihood of activating apoptotic signals *via* UPR [184].

3.3 The Interplay Between Endoplasmic Reticulum Chaperones. Proteins that undergo folding in the ER can use more than one chaperone system simultaneously; this also occurs when access to one chaperone is limited. The mammalian ER chaperone BiP is an ortholog of Hsp70, and has important roles in the maturation of nascent proteins and targeting of terminally misfolded proteins for degradation [185]. Unlike calnexin and calreticulin, which recognize both N-linked glycan and unfolded polypeptides, BiP binds to exposed short hydrophobic regions within polypeptides that are commonly present in incomplete proteins. Therefore, most of the BiP substrates are non-glycosylated proteins [186] or glycoproteins that have N-glycans close to the end of their linear sequences [187]. Exposure of N-glycans favours interactions with calnexin or calreticulin; however, in the absence of glycan or upon blocking of de-glucosylation, calnexin and/or calreticulin substrates may associate with BiP [185]. Interestingly, unlike calnexin and calreticulin, BiP binds its substrates in an ATP-dependent manner. BiP also has an important role in the UPR [188], specifically through direct interaction between misfolded proteins and membrane sensors or titration of BiP [43]**.** Activation of the UPR leads to activation of various signalling pathways, including the induction of transcription factors that are required for ERAD [18], chaperones and other factors.

GRP94 is another important ER chaperone. It is the most abundant protein in the ER and plays an important role in ER protein quality control. GRP94 is a very selective chaperone, with specificity for many secretory proteins. GRP94 levels increase during the induction of the UPR to reduce the chance of misfolded proteins leaving the ER. Interestingly, the deletion of BiP induces the expression of GRP94, PDI, calreticulin and ERp57, whereas the silencing of calreticulin results in increased expression of GRP94 [35]. These compensatory effects are still not well understood, although they seem to be very specific and well-controlled processes. For example, *grp94*-null cells do not show increased expression of any other chaperone [189].

4.0 Deletion of Endoplasmic Reticulum Protein Gene. To gain more insight into the physiological roles of endoplasmic reticulum molecular chaperones, knockout mice deficient in calnexin, calreticulin, or ERp57 have been generated [190].

4.1 Calreticulin-deficient Mouse Models. Calreticulin deficiency in mice is embryonic lethal [191]. Embryonic lethality of the calreticulin-deficient mouse is due to impaired cardiac development, including a significant decrease in ventricular wall thickness and deep intrabecular ventricular walls [191]. Calreticulin-deficient embryos die at day 14.5 of gestation as a result of insufficient development of the ventricular wall [191]. Impaired cardiogenesis in calreticulin-deficient embryos is a result of disruption of the Ca^{2+}-signalling pathway [37].

Calreticulin-deficient mouse cardiomyocytes and ES cell-derived cardiomyocytes have disorganized myofibrils, indicating that calreticulin plays an important role during myofibrillogenesis [192, 193]. Further

studies with embryonic fibroblasts show inhibition of agonist-mediated Ca^{2+} release from the ER in the absence of calreticulin [33, 191]. Store-operated Ca^{2+} influx was significantly affected as a result of the reduced capacity for Ca^{2+} of ER stores in the absence of calreticulin, affecting proper function of the store-operated Ca^{2+} influx [33]. Calreticulin-deficient cells have inhibited nuclear import of the transcription factor, nuclear NF-AT [37, 191] and MEF2C [194]. These data suggest that calreticulin controls the availability of Ca^{2+} ions required for the activation of the NF-AT/GATA-4/MEF2C/calcineurin transcriptional pathway. Lethality of calreticulin-deficient embryos can be rescued by the expression of activated calcineurin in the heart [37]. This confirms the hypothesis that calreticulin-deficient cells have inhibited calcineurin activity and, consequently, impaired cardiac-specific transcriptional processes [191].

4.2 ERp57-deficient Mouse Model. Ubiquitous deletion of ERp57 in mice results in embryonic lethality [126, 144]. This demonstrates the importance of ERp57 during mouse development. High activation of the ERp57 gene can be detected at the very early stage of the blastocyst and abundantly in the inner cell mass [126]. ERp57 is involved in assembly of the heavy chain of MHC class I molecules [84]. Tissue-specific deletion of ERp57 in B cells shows that although ERp57 is not crucial for the development of function of B cells, it is essential for assembly of the peptide-loading complex and contributes to MHC I antigen presentation [144]. The role of ERp57 in the oxidative folding of glycoproteins that are substrates in the calnexin-calreticulin cycle has been well established [195-197]. However, studies using small interfering RNA (siRNA) technology to create different cell lines deficient in ERp57 have shown that ERp57 is not essential for

disulfide bond formation during folding of glycoproteins [86, 128, 198, 199]. However, loss of ERp57 affects the post-translational phase of oxidative folding. This results in a significant loss of folding efficiency, suggesting that ERp57 has a role in reshuffling of the native set of disulfide bonds, as well as demonstrating that there is some level functional redundancy amongst ER oxidoreductases [86].

4.3 Calnexin-deficient Mouse Models. Deletion of the calnexin gene in different model organisms has been an extremely useful tool in understanding calnexin's diverse functions. *Dictyostelium discoidium* is an organism that expresses both of the lectin chaperones calreticulin and calnexin [200]. A dramatic decrease in the rate of phagocytosis was observed in the calreticulin/calnexin double knockout; however, only a mild decrease in each single knockout. These results suggest that calreticulin and calnexin may have redundant functions in *Dictyostelium* [200]. *Saccharomyces cerevisiae* is another interesting model of organism used to study calnexin's function. Disruption of the calnexin gene (CNE1) results in over-secretion of glycoproteins, supporting the role of calnexin/CNE1 in quality control of the secretory pathway [201]. However, in *Saccharomyces pombe,* calnexin is essential for its survival [202, 203]. The most apparent difference between calnexin found in these two strains of yeast is the presence of TM and C-tail domains in *Saccharomyces pombe* [204, 205]. It has been proposed that the cytosolic tail present only in calnexin homolog in *Saccharomyces pombe* is required for apoptotic signalling. Disruption of the calnexin gene in *Drosophila melanogaster* affects the expression and function of rhodopsin, resulting in age-related retinal degradation and defects in Ca^{2+} buffering. Gene knockout of

calnexin is viable in *Caenorhabditis elegans*, suggesting that another chaperone system may compensate for its loss [206, 207]. A homolog of the calnexin gene has also been identified in the plant, *Arabidopsis thaliana,* where it might play a role as a molecular chaperone [208].

In contrast to calreticulin or ERp57-deficient mice, calnexin-knockout mice are viable [146, 190]. Surviving calnexin-deficient mice are reported to be 30-50% smaller than their wild-type littermates, have severe motor disorders characterized by ataxia, tremor of the forelimb and abnormal and unstable gait [146, 190]. Recent work on the calnexin-deficient mouse reports significant changes in the histology of myelin in the central (CNS) and peripheral nervous systems (PNS). Electron micrographs of the spinal cord and sciatic nerve indicate severe dysmyelination in both CNS and PNS [146]. Furthermore, calnexin-deficient mice have a severely reduced nerve conduction velocity due to dysmyelination, appearing as wavy, disorganized and decompacted myelin in the PNS [146]. To gain insights into which domain of calnexin is responsible for this phenotype, calnexin-deficient mice expressing a truncated form of calnexin (deleted region includes carbohydrate binding site within chaperone motif) have been created [190]. Mice expressing the truncated form of calnexin were phenotypically identical to the calnexin-deficient mouse, suggesting that the chaperone function of calnexin is responsible for the observed phenotype [190]. Although extensive characterization of calnexin-deficient mice showed defects in the CNS and PNS, no other major abnormalities were observed in tissues throughout the mouse [146]. The phenotype of peripheral neuropathy in the calnexin-deficient mouse is a result of alterations in the morphology of the myelin.

The phenotype of neuronal disorder in the calnexin-deficient mice is very strong evidence for the critical role of the ER quality control system in the formation of compact, functional myelin. The relationship between calnexin and myelination originates in its chaperone function for the newly synthesized glycoproteins.

Myelin is the multilamellar sheath that surrounds large axons in the PNS and CNS and facilitates rapid conduction of action potentials [209]. Two important glycoproteins of compact peripheral myelin involved in its formation, maintenance and degeneration are myelin protein zero (P0) and peripheral myelin protein 22 (PMP22) [210]. Their adhesive function is essential for formation of functional myelin. Many of the mutations within the P0 and PMP22 genes are associated with human hereditary neuropathies [211]. Importantly, it has been reported that calnexin is a molecular chaperone for PMP22 [212]. A protein that is specific to CNS myelin is myelin oligodendrocyte glycoprotein (MOG); it has an important role in myelin formation [210].

5.0 Perspectives

Defects in the protein folding process are the source of a large number of human diseases. Impairment of protein folding may influence disease causation in different ways; for example: insufficient levels of functional protein, lack of protein in its correct localization, loss of function of the protein, gain of toxic function in which toxic aggregates form (a common feature of many neurodegenerative disorders) and impaired proteasomal degradation and activation of the UPR, which may result in cell death. But there is another aspect of activation of ER quality control; retaining

misfolded proteins in the ER can increase the level of toxic aggregates in the cell, as well as lead to induction of the UPR [213]. In neurodegenerative diseases, therapeutic approaches often involve disruption of aggregate formation or disruption of the existing aggregates. These approaches have very limited success and undesired side effects. Detailed studies of ER quality control provide important insights of medical significance and new opportunities for therapeutic approaches, including limiting ER-stress-induced cell death [214] or stimulation of proteasome activity [215].

The ER chaperones are targets for small-molecule compounds. Therapeutic approaches focus on enhancing the cell's quality control machinery and restoration of cell homeostasis. Another benefit of enhancing the function of ER chaperones might be a slowdown in the aging process, as aging is closely related to the accumulation of misfolded proteins and it has been shown that expression of some ER chaperones decreases with age [216].

Table 1-1

Summary of function and disease relevance of selected ER chaperones and folding enzymes.

Protein	Main function	Mouse model	Diseases	Reference
Calnexin	Chaperone; Glycoprotein folding	Dysmyelination; Motor disorders	Alzheimer's disease; CMT; cystic fibrosis	[107, 146, 217]
Calreticulin	Ca^{2+} buffering; Chaperone; Glycoprotein folding	Embryonic lethality at E14.5 due to impaired cardiac development	Cardiac hypertrophy; Autoimmune diseases; Cancer	[35, 218-220]
ERp57	Thio-oxidoreductase; Formation and isomerization of disulfide bonds; MHC I assembly	Embryonic lethality ; B cell-conditional *ERp57*$^{-/-}$ knockout results in defective antigen presentation	Cancer; prion diseases; Alzheimer's disease	[128, 140, 144]
BiP/GRP78	Chaperone; Ca^{2+} binding; ER stress sensor; UPR regulator	Embryonic lethality at E 3.5; defective pre-implantation of embryo	Cancer; Alzheimer's disease; prion disease; Parkinson's disease	[217, 221]
UGGT	Glucosyltransferase; Recognition of misfolded glycoproteins; reglucosylation	Embryonic lethality at E13	ND	[145]

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7.0 Research Objective

The objective of my PhD Thesis was to investigate the role of the endoplasmic reticulum (ER) molecular chaperones calnexin, calreticulin and ERp57 in the biology of central and peripheral myelin glycoproteins, with an emphasis on the expression, cellular localization, folding and function of these proteins in the absence of selected ER chaperones. Considering the surprising phenotype of the calnexin-deficient mouse showing peripheral neuropathy resulting in the severely altered morphology of myelin, it is important to fully understand the critical and novel roles of ER molecular chaperones in the formation of myelin sheaths. The contribution of ER chaperones to the maturation, folding and function of major myelin glycoproteins needs to be elucidated in order to better understand the molecular mechanisms underlying the pathology of hereditary neuropathies. ER molecular chaperones could be potential therapeutic targets in treating human neuropathies.

8.0 Research Hypothesis

The hypothesis of this study is that the essential endoplasmic reticulum molecular chaperones calnexin, calreticulin and ERp57 play an important role in the biology of glycoproteins of the peripheral and central nervous systems. To address this hypothesis, I focused on the role of calnexin, calreticulin and ERp57 in the intracellular trafficking, folding and function of the major peripheral myelin glycoproteins PMP22 and P0, as well as the important central nervous system glycoprotein MOG. We designed a set of experiments to answer three main questions: (i) Does the absence of calnexin, calreticulin or ERp57 affect intracellular trafficking of PMP22, P0 or MOG? (ii) Does the absence of calnexin, calreticulin or ERp57 result in impaired folding of PMP22 or P0? (iii) Are the functions of P0 or PMP22 compromised in the absence of calnexin, calreticulin or ERp57?

Chapter Two

Specialization of Endoplasmic Reticulum Chaperones for the Folding and Function of Myelin Glycoproteins P0 and PMP22

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Author contributions:
H.C. designed and performed experiments (Unfolded protein responses measured using the luciferase reporter gene assay, Figure 2-13).

Introduction

Myelin is the multilamellar sheath that surrounds large axons in both peripheral (PNS) and the central nervous systems to facilitate rapid conduction of action potentials and integrity of the axons [1, 2]. In the PNS, the myelin sheaths are generated by an extension of the Schwann cells that wrap repeatedly around individual axons (Figure 2-1). By increasing the resistance, myelin reduces current flow across the axon, facilitating signal conduction at nodes of Ranvier. Compact myelin consists mainly of the Schwann cell's plasma membrane, which mostly contains lipids; however, it also contains a set of myelin-specific proteins that play a key role in the formation of the functional myelin sheath. The major proteins of peripheral myelin sheaths are the glycoproteins P0 and PMP22 (Figure 2-1) [3].

Myelin proteins and neurological disorders - Mutations in genes encoding Schwann cell-specific proteins cause hereditary peripheral neuropathies characterized by myelin abnormalities [4]. In order to understand the complex effects of diseases linked to genetic mutations, it is necessary to elucidate the structure, stability and folding of Schwann cell-specific proteins. Mutation in myelin specific proteins can cause peripheral nerve disease through a variety of general and/or Schwann cell-specific cellular mechanisms, such as abnormal biosynthesis, processing and abnormal targeting of protein to the myelin and function in the myelin. The molecular mechanisms by which different mutations, including missense, nonsense, deletion or substitution, in P0 and PMP22 genes, result in peripheral neuropathy are unclear; however, altered processing and retention of the mutated proteins along the ER and secretory pathway are

suspected to play important roles [5]. Hereditary peripheral neuropathies are common human genetic conditions. There are several different hereditary peripheral neuropathies such as Charcot-Marie-Tooth disease (CMT), Dejerine-Sottas Syndrome (DSS) and congenital hypomyelinating neuropathy (CNH). All of the conditions are related to mutations in myelin proteins. These peripheral neuropathies are defined as alterations in myelination, reduced nerve conduction velocity and muscle weakness [6]. Different mutations cause phenotypes with varying degrees of disease severity as a result of nonfunctional protein being produced, altered protein-protein interactions or imbalance between protein synthesis/folding, and degradation. All of these ultimately lead to an unstable myelin structure [7-9]. An imbalance between synthesis, correct folding and degradation often leads to accumulation of misfolded proteins. The formation of aggregates is associated with various neurodegenerative conditions of PNS. For example, PMP22 is known to accumulate in aggresomes when carrying a K16P mutation in the first transmembrane domain [8]. Cytosolic accumulation of PMP22 has been found in nerve biopsies of CMT type 1A patients. Mutations in the P0 gene cause a CMT type1B neuropathy, a very complex neurological disorder. A very severe phenotype is associated with the S^{63} deletion mutant in the extracellular domain of P0. This mutant, which causes demyelination in Schwann cells, is misfolded and retained in the ER, and activates the unfolded protein response (UPR) [10].

Myelin Protein P0 – P0 is a type I membrane glycoprotein involved in the formation and compaction of PNS myelin (Figure 2-1, 2-2A) [3]. P0 is a major protein of PNS myelin; this 30-kDa glycoprotein accounts for about

50% of the total protein in compact PNS myelin [11]. The protein is specific to Schwann cells in the PNS [12]

Structure and Function of P0 - P0 consists of three domains: an N-globular domain, a single transmembrane domain and a highly basic 69-residue long C-terminal domain [13-15]. The N-domain is 124 amino acids in length and has a similar structure to immunoglobulins [16]. Hemophilic interactions of P0's extracellular domain are thought to be involved in formation of myelin sheaths. Hemophilic properties of the extracellular domain of P0 have been demonstrated in several ways [9, 17, 18]. The structure of the P0 extracellular domain has been determined by X-ray crystallography [15]. These data confirmed oligomerization properties of the P0 extracellular domain. It has also confirmed the immunoglobulin-like structure of the N-domain, with a hydrophobic core and a single intersheet disulfide bond. P0 has a single disulfide bond formed by C^{21} and C^{98} within its extracellular domain [19]. Mutagenesis studies have shown that the disulfide bond is important for P0's adhesive properties, since it is essential for the correct conformation of the extracellular domain and plays a role in the intracellular transport of P0 [15, 19, 20]. The extracellular domain of P0 contains a single N-glycosylation site (at the position N^{93}) within an extracellular immunoglobulin-like domain [21]. Glycosylation has been shown to be important for the homophilic interactions of P0 [22, 23]. P0 stabilizes compact myelin by a homophilic interaction as well as a heterophilic interaction with PMP22 [24]. Although it is the extracellular domain that is involved in the formation and maintenance of the myelin sheath, the cytosolic domain has also been reported to play a role in these processes *via* interacting with different

molecules in the cytoplasm [17, 25]. Mutations within the cytosolic tail result in disruption of P0 adhesive properties *in vitro* [17]. The cytosolic region of P0 contains several phosphorylation sites; S^{197}, S^{199}, S^{204}, S^{214} and Y^{191}. Point mutations at either S^{199} or S^{204} abolish the adhesive properties of P0, indicating that phosphorylation of the cytosolic tail is important for the function of the protein [26, 27].

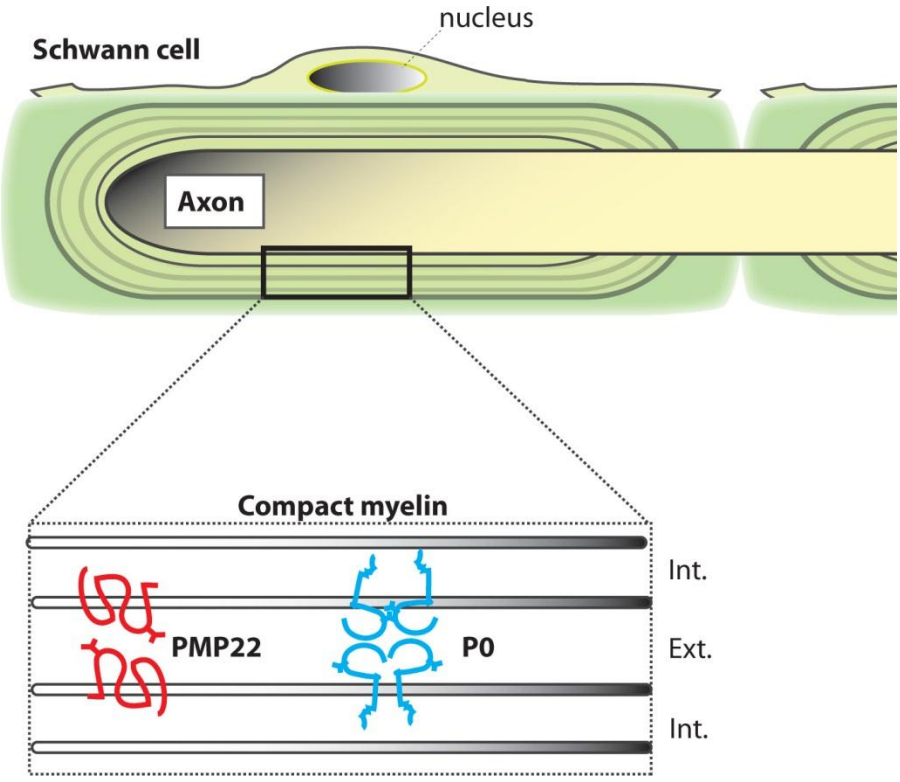


Figure 2-1 Schematic overview of myelinated axon in the peripheral nervous system. The cartoon shows that compacted myelin forms from plasma membrane extensions of a Schwann cell that tightly wrap around the axon. The enlarged area shows two main myelin glycoproteins involved in formation of compact myelin sheath. Ext.- extracellular, Int.- intracellular; P0- myelin protein zero, PMP22 – peripheral myelin protein 22.

Mutations in the Myelin Protein Zero gene – More than 100 mutations within the P0 gene are associated with human neuropathies; most of them are localized in the extracellular domain [28]. Many of the mutations in P0 lead to CMT1B and DSS syndromes. Mutation in P0 can disrupt the myelination process during development, resulting in severe early onset neuropathies or disrupt myelin structure, leading to late onset, adulthood neuropathies [29]. Patients with early onset of a disease present a very severe peripheral demyelinating neuropathy occurring at very early age with very slow NCV [29]. One of the well characterized mutants of P0 causing early onset of disease symptoms is S^{34}del (Figure 2-2B). The mutant protein is retained in the ER, activates ER stress and contributes to the apoptosis of myelinating Schwann cells. Late (adult) onset neuropathies are characterized mostly by axonal loss. An example of the mutation resulting in the late onset neuropathy is $I^{62}F$ within the cytosolic tail that correctly localizes to plasma membrane but causes disruption of P0 adhesive properties *in vitro* [17]. $I^{62}F$ is related to CMT1B in patients.. Several results suggest that many of the P0 mutants causing early onset of disease are retained in the ER, causing ER stress and activating the UPR, whereas mutants related to late onset syndromes are trafficked to the plasma membrane, but cause a loss of cellular adhesion properties [9, 30, 31].

The site and nature of amino acid changes in P0 are related to the adhesive properties of the protein, and therefore, to clinical phenotypes. However, the relationship between mutation, cellular localization and function and the molecular mechanisms underlying human diseases remains unknown. Other underlying mechanisms, such as interactions

with ER chaperones that assist in folding of myelin proteins might be related and affect the pathological mechanisms of the disease.

Mouse Models - P0 is believed to be essential for myelin formation and maintenance. Severe hypomyelination in mice with a disrupted P0 gene supports these beliefs [32]. Mice deficient in P0 exhibit abnormal motor coordination and tremors that are the result of impaired myelin compaction [32]. Moreover, expression of the correct amount of P0 protein has been shown to be essential for normal myelin formation and its maintenance. Mice heterozygous for a P0-null mutation appear normal, but with age develop progressive demyelination and chronic inflammatory demyelination polyneuropathy [33]. On the other hand, mice over-expressing P0 display remarkably delayed nerve development due to deregulation in the expression of other myelin genes and altered trafficking to the plasma membrane [34, 35]. More than 100 mutations in the human P0 gene are associated with hereditary neuropathies. They cause the demyelinating peripheral neuropathy Charcot-Marie-Tooth disease 1B (CMT1B), the more severe DSS and congenital hypomyelination, all associated with muscle weakness, atrophy and sensory loss. The pathomechanism of these neuropathies remains unknown.

P0 is thought to play a role in myelin compaction by promoting adhesion between myelin wraps; however, as an adhesion molecule, it also has a regulatory function; it is involved in regulation of expression of other myelin related genes. Schwann cells deficient in P0 show down-regulation of PMP22 specific to P0 deficiency [36].

Mice deficient in both P0 and connexin 32 (Cx32), an important component of gap junction channels between membrane layers of myelin sheaths in the PNS, have a phenotype identical to mice lacking P0 alone [37]. This observation shows that although Cx32 is essential for myelin formation in case of disrupted myelin sheaths (in the absence of P0), its function is dispensable [38]. It shows that the phenotype of P0 mice does not depend on Cx32.

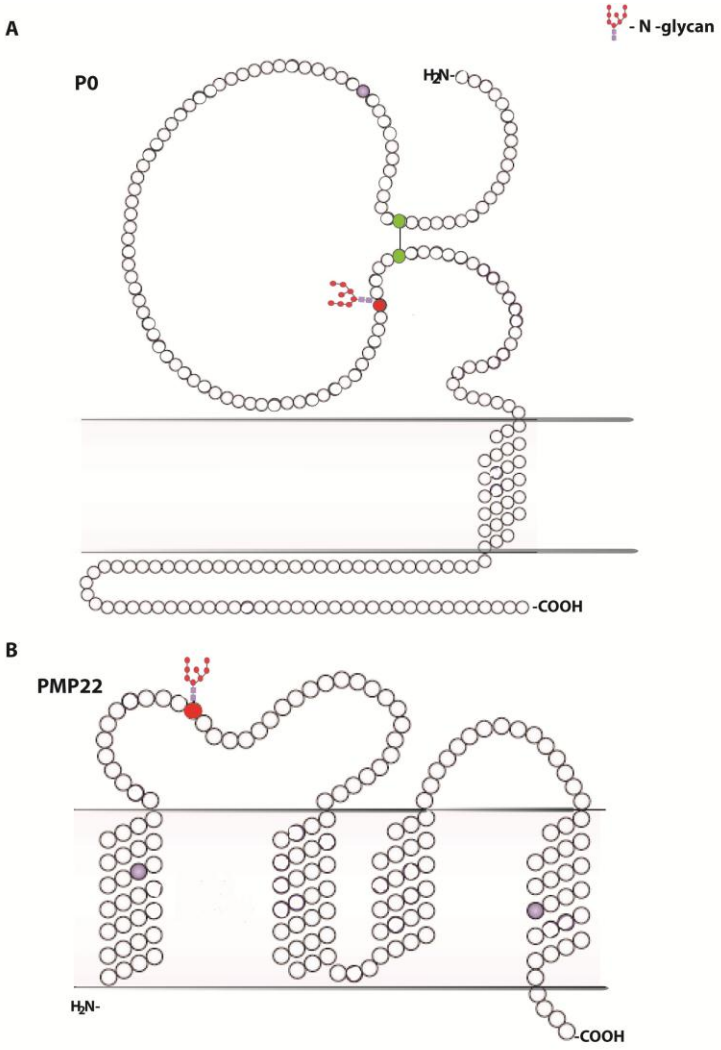


Figure 2-2 Schematic representation of structures of P0 and PMP22. **A**. Schematic representation of P0, a type I membrane protein, consisting of an N-globular domain, a single transmembrane domain and a cytosolic C-domain. The N-domain has an Ig-like structure. It contains a single N-glycosylation site at the position N^{93} (red circle). C^{21} and C^{98} (green circles) form a single disulfide bond within N-domain. The purple circle represents the S^{24}del mutation related to human hereditary neuropathy. **B**. Schematic representation of the PMP22 protein with four predicted transmembrane segments and C- and N-termini in the cytosol. A single N-glycosylation site is located at N^{41} (red circle). Sites of mutations linked to human neuropathies are shown as purple circles.

Peripheral Myelin Protein 22 – PMP22 is a 22-kDa myelin membrane glycoprotein with four putative transmembrane segments (Figure 2-2B) [39]. It is expressed by Schwann cells and represents 2-5% of the total protein content of peripheral myelin membranes [40]. Interestingly, in healthy Schwann cells, 80% of newly synthesized PMP22 protein is rapidly degraded through the proteasomal pathway, because of inefficient protein folding [41-43].

Structure and Function - PMP22 is a hydrophobic membrane glycoprotein with four predicted transmembrane segments and cytosolic N- and C-termini (Figure 2-2A) [39]. However, the structure of PMP22 remains unsolved and there is a confusion concerning the PMP22 transmembrane domains. Studies with epitope tagging led to the conclusion that PMP22 has only two transmembrane segments [44]. Unlike P0, PMP22 accounts for less than 5% of total PNS myelin protein [45]. The pattern of expression of PMP22 is synchronous with myelin formation of healthy myelin sheaths. Just like P0, PMP22 is exclusively localized in compact myelin [46]. PMP22 has been shown to transiently associate with calnexin in a glycan-dependent manner [47].

The main function of PMP22 involves promoting and maintaining the myelin sheath [3, 48]. Although adhesion between myelin layers is primarily mediated by P0, PMP22 assists in P0's adhesive function through interaction with P0, as well as through homophilic interactions [3, 49]. Like P0, the correct amount of PMP22 is crucial for normal formation and maintenance of myelin. Both human studies and animal models reveal that alteration in PMP22 expression has consequences during development of peripheral myelin [50].

Mutations in PMP22 gene – Mutations in the PMP22 gene are associated with several neurodegenerative diseases of PNS, including CMT1A, DSS and hereditary neuropathy with liability to pressure palsies (HNPP). There are many mutations in the PMP22 gene linked to human peripheral nervous system pathologies; however, two mutations occurring in the first and fourth transmembrane segments result in severe phenotypes. $Leu^{16}Pro$ (the same mutation is found in mice and is called Trembler-J), causing CMT1A and $Leu^{150}D$ (in mice Trembler) related to DSS, are very well characterized [43, 51]. Both of the mutants are recognized as folding-defective by the ER quality control machinery and sent for proteasomal degradation [43]. However, under disease condition, degradation of PMP22 mutants is accompanied by its ER retention and toxic accumulation in the ER lumen and the cytosol. It has been shown that calnexin is responsible for ER retention of the Tr-J mutant through prolonged interaction with mutated PMP22, consequently contributing to the pathology caused by this mutant [47]

Mouse Models - PMP22-deficient mice are born live and develop normally until the second week of life when they start to have difficulties with walking. One year postnatal PMP22-deficient mice show severe hypomyelination and axonal atrophy [48, 52]. Overexpression of PMP22 also results in a CMT-like neuropathy, characterized by unstable gait, reduced NCV and loss of muscle strength, resulting from severe hypomyelination [53]. Thus, over-expression of PMP22 in mice serves as a great model to study CMT1A, the most prevalent form of human neuropathy, often related to duplication of the PMP22 gene.

Objectives - The functions of P0 and PMP22 are to promote and maintain the compact myelin sheath [3]. Alteration in P0 or PMP22 expression or specific mutations in these myelin proteins are associated with hereditary demyelinating peripheral neuropathies in mice and human [48], characterized (among other symptoms) by unstable gait and reduced nerve conduction velocity [53]. Mutations in P0 and PMP22 cause phenotypes with varying degrees of disease severity depending on whether the mutation results in the production of nonfunctional myelin protein, altered protein-protein interactions or an imbalance between protein synthesis/folding, degradation and/or retention in the ER [10]. All of these molecular events ultimately lead to an unstable myelin structure; yet, little is known about the role of the ER quality control machinery in the biology of myelin proteins.

Calreticulin, calnexin and the oxidoreductase ERp57 are ER chaperones and a folding enzyme, respectively, responsible for the quality control of glycoproteins [54]. ERp57, a folding enzyme, catalyzes the formation and rearrangement of disulfide-bonds within the calnexin and calreticulin substrate proteins [55]. Calreticulin and ERp57 deficiencies are both embryonic lethal [56, 57]. In contrast, calnexin-deficient mice are viable and develop peripheral neuropathy due to dysmyelination [58]. Calnexin-deficient mice have reduced nerve conduction velocity and impaired myelin formation [58]. Electron microscopy analysis of calnexin-deficient sciatic nerve showed wavy and decompacted myelin indicating a defective formation of myelin in the absence of calnexin [58]. These findings indicate that calnexin may play an important role in the

development, function and pathology of myelin. Yet, the molecular events responsible for dysmyelination in the absence of calnexin are not known.

In this study, I show that different components of the ER quality control system (calnexin, ERp57 and calreticulin) have distinct effects on myelin protein folding and function (adhesiveness). The function and folding of PMP22 and P0 were not affected in the absence of calreticulin. However, they were severely impaired in cells deficient in calnexin (for PMP22 and P0) or ERp57 (for P0). These findings indicate that ERp57 and calnexin, but not calreticulin, play important roles in the biology of myelin proteins and consequently, ERp57 and calnexin may contribute to the diversity of myelin diseases. We believe that the calnexin-deficient phenotype of neuronal disorder is the result of impaired myelination.

Materials and Methods

Vector DNA - The full-length cDNAs encoding human myelin protein zero (P0) and mouse PMP22 were cloned into pEGFP-N1 vector to create vectors encoding P0 or PMP22 fused to GFP (green fluorescent protein) at their carboxyl termini and designated P0-GFP or PMP22-GFP, respectively. The following DNA primers containing an *attB* recombination site (Gateway Cloning, Invitrogen) were used: for PMP22-GFP forward primer 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT ACG CCA CCA TGC TCC TAC TCT TGT TGG GGA-3′ for P0-GFP forward primer: 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TAC CAT GC TCC GGG CCC CTG CCC CT-3′ and the same reverse primer for both PMP22-GFP, P0-GFP: 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TTA CTT GTA CAG CTC GTC CAT GCC-3′. First the PCR products were cloned into the pDONR vector (Invitrogen) using LR Clonase Enzyme (Invitrogen). Next recombination reaction was carried out using BP Clonase Enzyme Mix (Invitrogen) with the gene of interest, the ubiquitously expressed promoter, EF1α (cellular polypeptide chain elongation factor 1 alpha) and a destination vector $2K7_{bsd}$ containing a blasticidin resistance gene that allows the easy selection of transfected cells [59]. Site specific mutagenesis was carried out to generate the expression vector encoding non-glycosylated P0 [P0(N^{93}del)-GFP]. For site specific deletion of the N^{93} glycosylation site on P0 the following DNA primers were use: forward primer 5'-CAA CCT AGA CTA CAG TGA CGG CAC GTT CAC TTG-3' and reverse primer 5'-CAA GTG AAC GTG CCG TCA CTG TAG TCT AGG TTG-3′. Expression vector encoding P0-GFP was used as a template. Site directed mutagenesis was carried out using QuickChange Site-Directed Mutagenesis Kit

(Stratagene). Virus isolation and transduction were carried out as described previously [59].

Cell Culture, Cell Lines and Immunofluorescence - Wild-type (K41) and calreticulin-deficient (K42) mouse embryonic fibroblasts were used in this study [60]. ERp57-deficient mouse embryonic fibroblasts were isolated from *ERp57*$^{-/-}$ embryos and immortalized through transfection with SV40 [57, 60]. Wild-type and calnexin-deficient mouse embryonic fibroblasts were isolated from newborn mice and immortalized through transfection with SV40 [61]. Lentiviral constructs encoding P0-GFP or PMP22-GFP were used to create wild-type, *cnx*$^{-/-}$, *crt*$^{-/-}$ and *ERp57*$^{-/-}$ cell lines stably expressing recombinant PMP22-GFP or P0-GFP. Cells were cultured in the presence of 7 μg/ml blasticidine for 4 days. Expression of P0-GFP and PMP22-GFP proteins was monitored by Western blot analysis using anti-GFP antibody at 1:10,000 dilution or by immunofluorescence, co-staining with ER marker anti-BiP/GRP78 antibodies using confocal microscopy. For immunofluorescence analysis, cells expressing P0-GFP or PMP22-GFP were grown for 24 hours after seeding in culture media and fixed in 4% paraformaldehyde for 15 minutes [61]. Images were collected by spinning-disk microscopy (WaveFx from Quorum Technologies, Guelph, Canada) set up on an Olympus IX-81 inverted stand (Olympus, Markham, Canada). Images were acquired through a 60X objective (N.A. 1.42) with an Electron Multiplying Charge Coupled Device (EMCCD) camera (Hamamatsu, Japan). Fluorescein isothiocyanate (FITC) (488 nm) and Texas Red (520 nm) wavelengths were used. Quantifications of plasma membrane localized P0-GFP or PMP22-GFP was carried out using ImageJ (http://rsbweb.nih.gov/ij/index.html). Three images were analyzed for

each cell line and the fluorescence at plasma membrane was reported as arbitrary units ± standard deviation (SD).

Western Blot Analysis, Metabolic Labeling and Immunoprecipitation - Sciatic nerves from mice were isolated and crushed in liquid nitrogen with a RIPA lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.5% Nonidet P-40, 1% deoxycholate, 0.1% sodium dodecyl sulfate and protease inhibitors [62]. Crushed tissue was incubated on ice for 45 minutes followed by sonication for 20 minutes in RIPA lysis buffer. For Western blot analysis, cells expressing P0-GFP or PMP22-GFP were lysed on ice in RIPA buffer [61]. The following antibodies were used: goat anti-GFP 1:10,000, rabbit anti-GFP 1:10,000, rabbit anti-P0 1:300, rabbit anti-calnexin (Stressgene), rabbit anti-calreticulin 1:300, rabbit anti-ERp57 1:1000, and rabbit anti-GAPDH 1:1000.

Metabolic labeling was performed using HEK293T cells transfected with expression vectors encoding P0-GFP or P0(N^{93}del)-GFP (glycosylation site mutant). Cells were first incubated for 30 min in cysteine/methionine-free medium (Invitrogen) containing 10% of dialyzed FBS (Invitrogen). Cells were than incubated for 15 minutes in the cysteine/methionine-free medium containing 125 μCi of [^{35}S]methionine (EasyTag EXPRESS^{35}S Protein Labeling Mix, PerkinElmer). Next, cells were incubated in fresh complete medium (DMEM, 10% FCS) supplemented with 5 mM cysteine, methionine (Sigma) for periods up to 2h. The cells were then washed once with cold PBS containing 20 mM of *N*-ethylmaleimide (Sigma), followed by immunoprecipitation. Labeled samples were than separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Gels were treated for 1h with EN^{3}HANCE (PerkinElmer) according to the manufacturer's instructions.

Dried gels were exposed to Fuji RX film and developed on a film processor.

For immunoprecipitation, cells were grown to 80-90% confluency followed by the addition of 500 μl per 10 cm dish of lysis buffer containing 50 mM Hepes, pH 7.4, 200 mM NaCl, 2% Chaps and protease inhibitors [63]. Cells were also incubated with 1 mM castanospermine for 16 hours followed by application of the lysis buffer. Samples were incubated on ice for 30 minutes and then spun at 11,600 x *g* for 15 minutes to remove insoluble material. The supernatant was pre-cleared with the addition of a 1/15th portion of 10% protein A/G Sepharose bead suspension in an HBS buffer containing 50 mM HEPES, pH 7.4 and 200 mM NaCl for 30 minutes at 4 ^{o}C. Beads were spun down for 10 seconds and 2 μl of specific antibody were added. The samples were incubated overnight at 4 ^{o}C with rotation followed by addition of 100 μl of 10% protein A/G Sepharose in HBS buffer and incubated for an additional 4 hours. Beads were spun down, washed 3 times with HBS containing 1% Chaps, one time with HBS, followed by addition of 30 μl of Laemmli sample buffer [64]. Proteins were separated by SDS-PAGE (10% acrylamide).

Endoglycosidase Digestions, Cell Surface Biotinylation and Trypsin Digestion - Cellular proteins were extracted with RIPA buffer and 20 μg of total protein were incubated with EndoH or PNGaseF at 37^{o}C for 3 hours according to the manufacturer's protocols. Digested and undigested samples were separated by SDS-PAGE (10% acrylamide) followed by Western blot analysis with rabbit anti-GFP antibodies at 1:10,000.

For cell surface biotinylation, cells stably expressing P0-GFP PMP22-GFP (in 10 cm dishes) were first washed with phosphate-buffered

saline (PBS) and then with 5 ml of cold borate buffer [65]. Biotinylation was in a buffer containing 10 mM boric acid, pH 9.0, 154 mM NaCl, 7.2 mM KCl, and 1.8 mM $CaCl_2$ followed by incubation with sulfosuccinimido-NHS-biotin (0.5 mg/ml of borate buffer) at 4 ^{o}C for 30 minutes. The biotinylation reaction was stopped by addition of 5 ml of quenching buffer containing 192 mM glycine, 25 mM Tris, pH 8.3. Cells were carefully washed 3 times with cold quenching buffer followed by lysis for 20 minutes on ice in 500 μl of lysis buffer containing 10 mM Tris-HCl, pH 7.5, 1% NP40, 5 mM EDTA, 150 mM NaCl, 0.5% deoxycholate and protease inhibitors: 200 μM PMSF (phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride), 100 μM benzamidine, and SL inhibitors (0.05 μg/mL aprotinin, 0.025 μg/mL phosphoramidone, 0.05 μg/mL TLCK, 0.1 μg/mL TPCK, 0.05 μg/mL APMSF, 0.05 μg/mL E-64, 0.025 μg/mL leupeptin, and 0.01 μg/mL pepstatin [62]. Samples were centrifuged at 13,400 x *g* for 20 min. The supernatant was used from the protein assay (250 μl) and for incubation with streptavidin agarose (150 μl). Samples were incubated with streptavidin agarose overnight at 4 ^{o}C. Resin containing biotinylated plasma membrane proteins was spun down at 7500 x *g* for 2 minutes followed by 5 washes with lysis buffer without protease inhibitors. The percent of biotinylated proteins was determined using densitometry and normalized to equivalent levels of GAPDH: %biotinylated at cell surface = (total protein – unbound) * 100/ total protein). The percentage of biotinylated, plasma membrane localized P0 or PMP22, was expressed as a percentage of total P0 and PMP22 content as determined by Western blot analysis. Twenty μg of biotinylated proteins were digested with trypsin at a 1:10 protein/trypsin ratio (w/w) at 37^{o}C for 5 or 30 min. Digestion was stopped by the addition of the Laemmli sample

buffer [64]. Samples were boiled for 5 minutes and followed by SDS-PAGE (10% acrylamide) and Western blot analysis using rabbit anti-GFP antibodies at 1:10,000 dilution.

Cell Adhesion Assay - Cell layers of wild-type, *cnx*$^{-/-}$, *ERp57*$^{-/-}$ or *crt*$^{-/-}$ cells expressing P0-GFP or PMP22-GFP were plated on a 96-well tissue culture plate at 5x10^3 cells/well overnight followed by incubation with 5x10^3 cells tested for adhesiveness as indicated under the legend to the Figure 2-4 in DMEM supplemented with 1 mM EDTA for 2 hours at 37 ^{o}C. Nonadherent cells were removed by several washes with phosphate-buffered saline. The number of adherent cells to the monolayers of cells was detected using the CellTiter 96 Aqueous Cell Proliferation Assay (Promega). To determine the number of adhering cells, the monolayers of each cell line were measured and subtracted as background. Each experiment was repeated four times. Adhesion of wild-type, *cnx*$^{-/-}$, *ERp57*$^{-/-}$ or *crt*$^{-/-}$ cells not transfected with P0 or PMP22 (after the background was subtracted), was defined as 100%. Statistic were performed using two-sample unpaired *t* tests to compare the means of adhesiveness between cells that do not express P0 or PMP22 and cells stably expressing these proteins.

Miscellaneous Procedures - Unfolded protein responses were measured using the luciferase reporter gene assay [61]. In brief, wild-type, *cnx*$^{-/-}$, *ERp57*$^{-/-}$, and wild-type, *cnx*$^{-/-}$, *ERp57*$^{-/-}$ stably expressing PMP22-GFP or P0-GFP were transfected with pRL-XFL vector encoding *Renilla* luciferase and firefly luciferase reporter genes as described previously [61, 66]. Briefly, 1 μg of DNA was transfected using Effectene (Qiagen). Cells were treated with 1 μM thapsigargin for 16 hours, lysed, diluted and assayed

with the Dual-Luciferase Assay Kit (Promega) using a luminometer (Berthold-Lumat LB 9501). Relative light units (RLUs) were normalized to an internal control. Average values ± standard deviation of three independent experiments (n = 3) are reported.

Protein concentration was measured using the Bio-Rad protein assay [61].

Results

To assess the role of ER quality control in the folding and function of the most abundant peripheral myelin proteins PMP22 and P0 required for the formation of functional myelin sheaths, we created stable cell lines deficient in ER chaperones (*cnx*$^{-/-}$, *crt*$^{-/-}$ or *ERp57*$^{-/-}$ cells) expressing PMP22-GFP and P0-GFP fusion proteins. The C-terminal GFP fusion to P0 is known not to affect P0 function [67]. These cells were tested for expression, intracellular trafficking and function (adhesiveness) of the peripheral myelin proteins PMP22 and P0 in the absence of the ER quality control components.

Cell Surface Targeting of Myelin Proteins in the Absence of the ER Quality Control Chaperones-Western blot analysis showed that wild-type, *cnx*$^{-/-}$, *crt*$^{-/-}$ and *ERp57*$^{-/-}$ cells expressed PMP22-GFP and P0-GFP (Figure 2-3), indicating that expression of these myelin proteins was not affected by the absence of the chaperones. Next, we used confocal microscopy to investigate the intracellular localization of PMP22-GFP and P0-GFP in cells deficient in ER quality control chaperones. Figure 2-4 shows that both myelin proteins were localized primarily to the plasma membrane in the absence of calnexin, calreticulin or ERp57. The intracellular fluorescence

seen in all cell lines (Figure 2-4A) likely corresponds to ER localization of newly synthesized PMP22-GFP and P0-GFP. The amounts of plasma membrane-localized P0-GFP and PMP22-GFP were quantified using ImageJ (Figure 2-4B). Wild-type, calnexin-deficient and ERp57-deficient cells contained the same amounts of cell surface myelin glycoproteins (Figure 2-4B). This was slightly reduced in the absence of calreticulin. We concluded that the absence of calnexin, calreticulin and ERp57 did not affect cell surface targeting of P0 and PMP22.

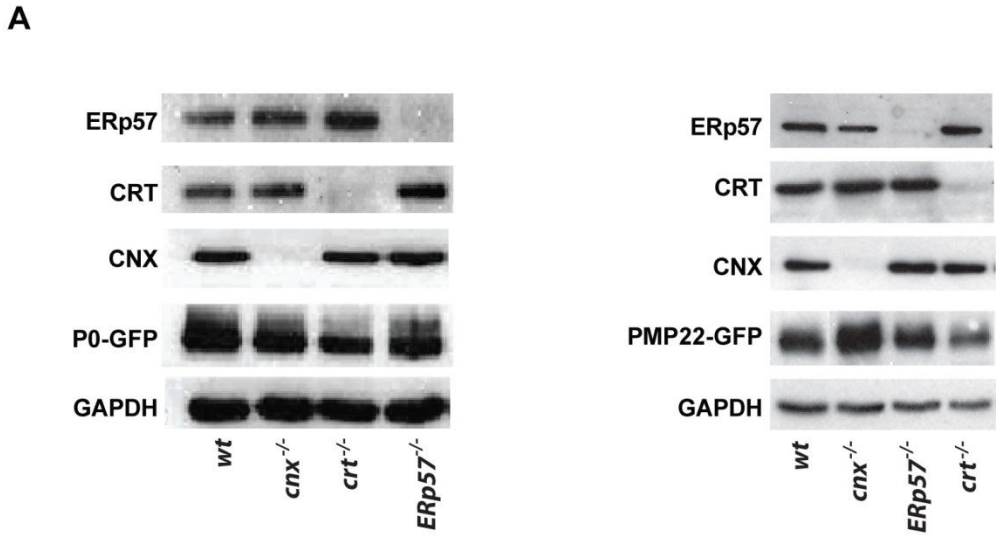


Figure 2-3 Expression of P0-GFP and PMP22-GFP in wild-type and chaperone-deficient cells. Wild-type (*wt*), calreticulin-deficient (*crt*$^{-/-}$), ERp57-deficient (*ERp57*$^{-/-}$) and calnexin-deficient (*cnx*$^{-/-}$) cells were transfected with expression vectors encoding P0-GFP (green fluorescent protein) and PMP22-GFP as described under "Materials and Methods". Western blot analysis was carried out with antibodies against ERp57, calreticulin (*CRT*), calnexin (*CNX*), green fluorescent protein (*GFP*) and glyceraldehyde phosphate dehydrogenase (GAPDH).

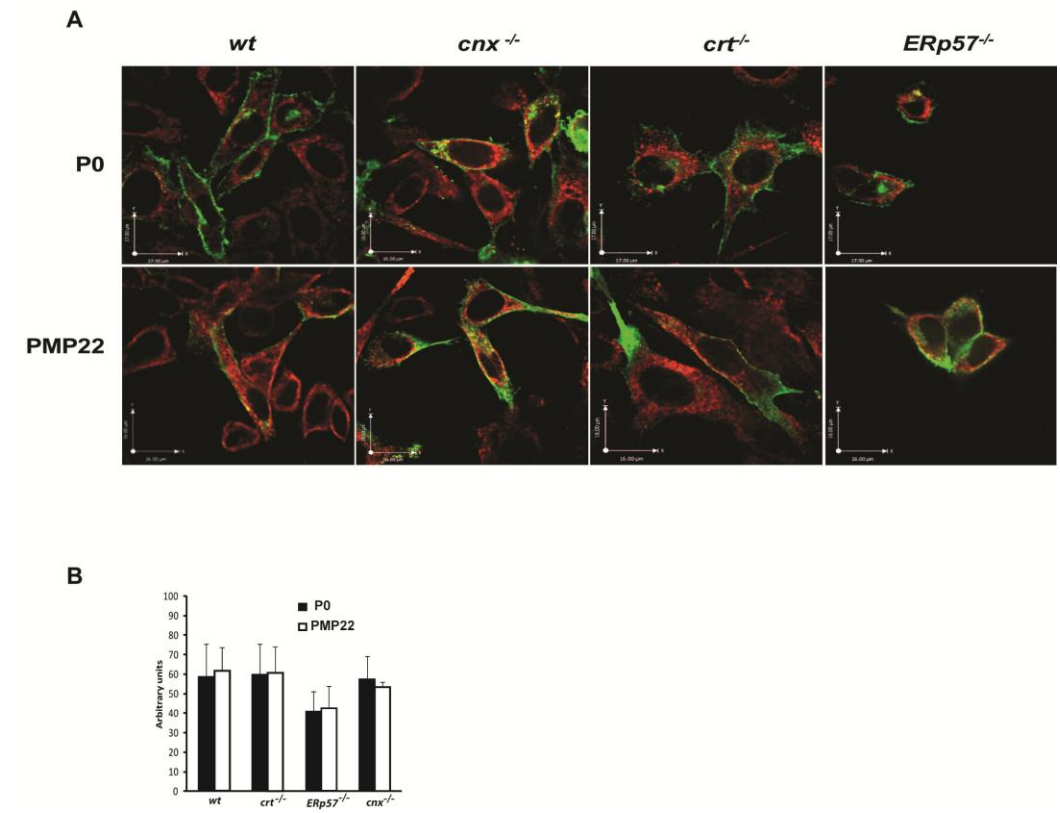


Figure 2-4 Expression and cell surface targeting of P0-GFP and PMP22-GFP in wild-type and chaperone-deficient cells. Confocal images of cell surface expression (green) of P0-GFP and PMP22-GFP in wild-type (*wt*), calnexin-deficient (*cnx*$^{-/-}$), calreticulin-deficient (*crt*$^{-/-}$) and ERp57-deficient (*ERp57*$^{-/-}$) cells. Anti-BiP/GRP78 (red) staining was used as an ER marker. Scale bar=17 μm. **B**. The graph represents qualitative analysis of cell surface localization of P0-GFP or PMP22-GFP in wild-type or chaperone deficient cells. The fluorescence at the plasma membrane is reported as arbitrary units (n=3; mean±SD).

Cell surface localization of PMP22 and P0 was further investigated using a membrane-impermeant biotin reagent that labels surface proteins. Wild-type and chaperone-deficient cell lines expressing PMP22-GFP or P0-GFP were labeled with sulfo-NHS-biotin, followed by isolation of the biotinylated proteins using streptavidin-affinity chromatography [65]. Total cellular proteins (Figure 2-5A lane *t*), streptavidin unbound (Figure 2-5A lane *ub*) and streptavidin bound (Figure 2-5A lane *b*) proteins were separated by sodium SDS-PAGE followed by Western blot analysis with anti-GFP antibodies to identify PMP22-GFP and P0-GFP. Western blot analysis of glyceraldehyde phosphate dehydrogenase (GAPDH), a cytoplasmic protein, was used as an internal control (Figure 2-5A) to indicate that the cell surface biotinylation protocol identified cell surface proteins only. Quantitative analysis of biotinylated fractions and confocal images indicated that the levels of cell surface, biotinylated P0-GFP and PMP22-GFP in wild-type and chaperone-deficient cells were relatively similar (Figure 2-5B).

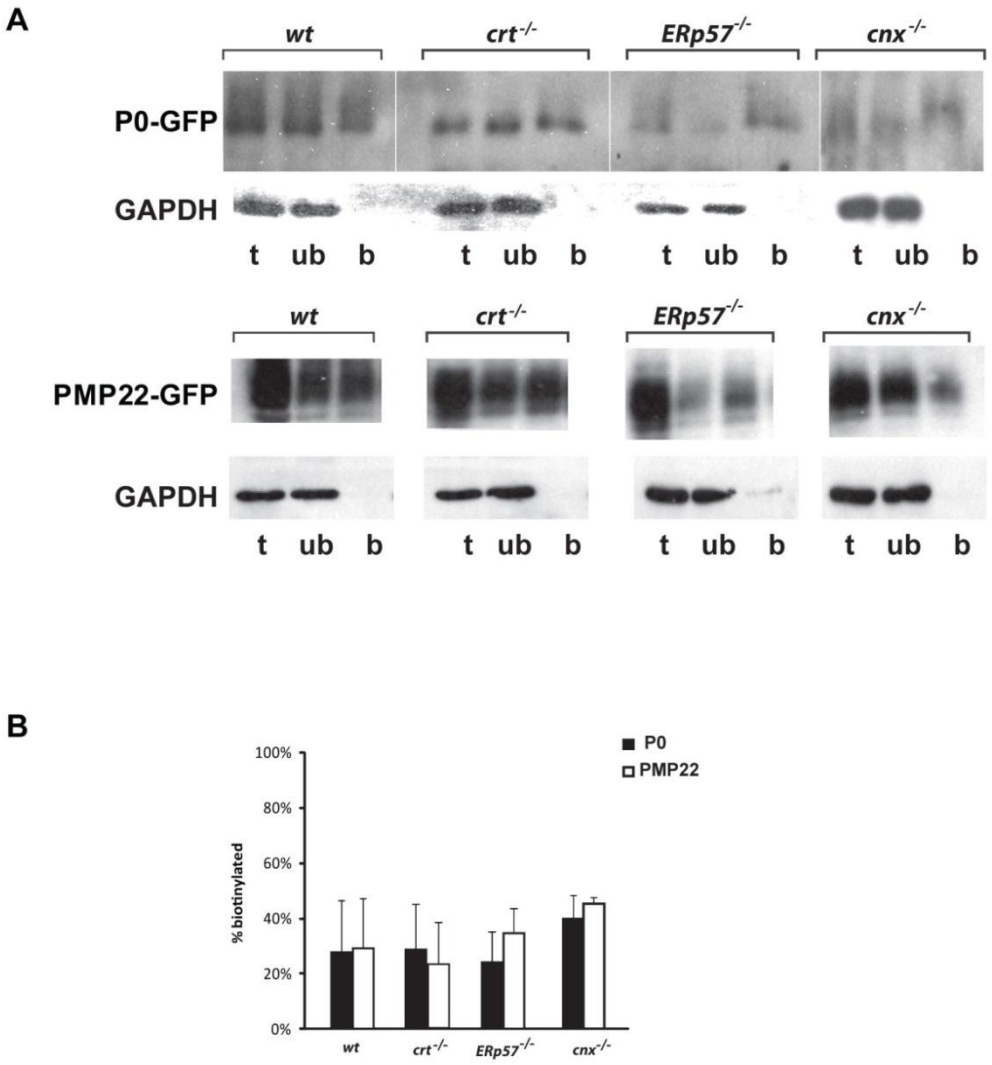


Figure 2-5 Cell surface targeting of P0-GFP and PMP22-GFP in wild-type and chaperone-deficient cells. Cell surface biotinylation of P0-GFP, PMP22-GFP expressed in wild-type (*wt*), calreticulin (*crt*$^{-/-}$), calnexin- (*cnx*$^{-/-}$) and ERp57-deficient (*ERp57*$^{-/-}$) cell lines was carried out as described under "Materials and Methods". Western blots were probed with anti-GFP or anti-GAPDH antibodies. A. *Lanes t*, total cellular extracts; *lanes ub*, material not bound to the streptavidin affinity beads; *lanes b*, streptavidin bound, biotinylated fractions. The graph shows quantitative analysis of cell surface biotinylated P0-GFP and PMP22-GFP (n=3; mean ± SD). The percentages of biotinylated, plasma membrane-localized P0 or PMP22 were expressed as a percentage of total P0-GFP and PMP22-GFP content as determined by Western blot analysis. B. Quantitative analysis of biotinylated (plasma membrane localized) P0-GFP and PMP22-GFP (n=3; mean±SD).

Both P0 and PMP22 have a single N-linked glycosylation site at N^{41} and N^{93}, respectively [3]. Calnexin, calreticulin and ERp57 play a key role in the folding of glycosylated proteins [54, 68]. Therefore, we asked whether the absence of ER quality control chaperones affects the glycosylation state of myelin proteins. The state of maturity of a specific glycoprotein can be deduced from its sensitivity to hydrolysis by specific glycosidases (endoglycosidase H [EndoH] or peptide:N-glycosidase F [PNGaseF]); digestion with EndoH removes high mannose, ER-attached, N-linked carbohydrates, whereas digestion with PNGaseF removes both high mannose and complex carbohydrates. A fully matured glycoprotein will be modified with complex oligosaccharides making it resistant to cleavage by EndoH but not PNGaseF [69]. PMP22-GFP and P0-GFP expressed in wild-type and chaperone-deficient cells were strongly resistant to EndoH digestion, indicating that the majority of PMP22-GFP and P0-GFP synthesized in wild-type and chaperone deficient cells contained complex carbohydrate typical of cell surface glycoproteins (Figure 2-6). P0 and PMP22 expressed in the absence of calreticulin exhibited some EndoH sensitivity, which corresponded to the ER-associated fraction of protein associated with the membrane during maturation process (Figure 2-6). Residual EndoH-sensitive protein was observed as a small fraction of the PMP22-GFP and P0-GFP, and likely corresponds to newly synthesized, ER-localized PMP22-GFP and P0-GFP (Figure 2-6). PNGaseF sensitivity of PMP22-GFP and P0-GFP expressed in wild-type and chaperone-deficient cells further confirmed that the myelin fusion proteins contained complex carbohydrate found on cell surface-localized but not on ER-localized glycoproteins (Figure 2-4).

Combined, confocal analysis, biotinylation and glycosidase sensitivity studies demonstrate that the absence of calnexin, calreticulin or ERp57 did not interfere with the cell surface trafficking of PMP22-GFP and P0-GFP, indicating that ER quality control components (calnexin, calreticulin and ERp57) do not affect expression and cell surface targeting of the peripheral myelin proteins PMP22 and P0.

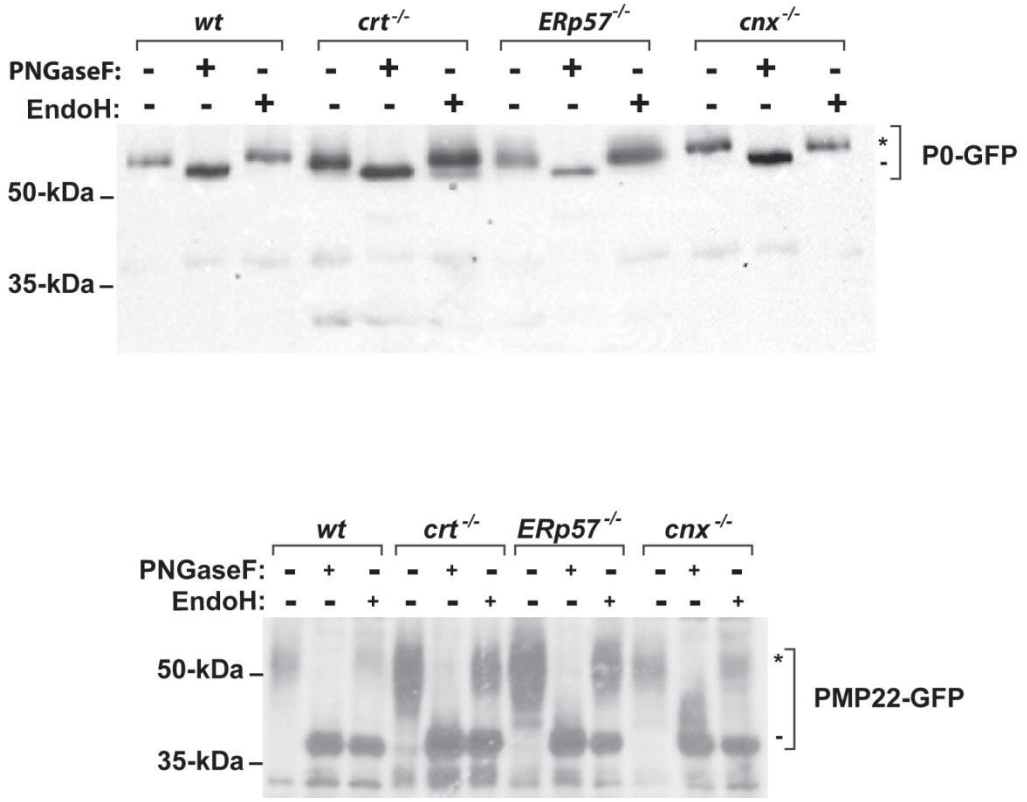


Figure 2-6 Glycosylation of PMP22 and P0. Endoglycosidase digestion of P0-GFP **(A)** and PMP22-GFP **(B)** expressed in chaperone-deficient cell lines (calnexin-deficient (*cnx*$^{-/-}$), calreticulin-deficient (*crt*$^{-/-}$) and ERp57-deficient (*ERp57*$^{-/-}$). Immunoblots of cell lysates treated with PNGaseF or EndoH. (*, glycosylated, mature forms of proteins; -, de-glycosylated proteins). One representative Western blot of 8 independent experiments is shown.

Deficiencies in Calnexin and ERp57, but not Calreticulin Affect Adhesiveness (Function) of Myelin Proteins - Interactions between P0 and PMP22 are essential for the formation of compact myelin [24]. P0-GFP and PMP22-GFP were expressed in wild-type and chaperone-deficient fibroblasts to test if cell surface-targeted P0-GFP and PMP22-GFP are functional in the absence of ER quality control chaperones. To examine this, we carried out a cell adhesiveness assay on wild-type and chaperone-deficient cells expressing myelin proteins [7]. Changes in adhesion of cells expressing myelin proteins report the functionality of PMP22 and P0 [9, 70]. As expected, wild-type fibroblasts expressing PMP22-GFP and P0-GFP showed increased adhesiveness compared to the control wild-type fibroblasts (Figure 2-7A, B, C), indicating that both myelin proteins interact and are functional on the cell surface of wild-type cells. Similar to wild-type cells, calreticulin-deficient cells expressing myelin proteins exhibited increased adhesiveness (Figure 2-7A), indicating that both myelin proteins were functional on the cell surface in the absence of calreticulin. In sharp contrast, calnexin-deficient fibroblasts expressing PMP22-GFP and P0-GFP showed no significant adhesiveness (Figure 2-7B), indicating that cell surface PMP22-GFP and P0-GFP were not fully functional in the absence of calnexin. Figure 2-7C shows that the absence of ERp57 did not have significant effect on adhesiveness of cells expressing PMP22-GFP while P0-GFP adhesiveness was significantly inhibited in the absence of ERp57 (Figure 2-7), indicating that this ER oxidoreductase was essential for P0 but not PMP22 cell surface function.

Taken together, these results indicate that the absence of calnexin compromises functionality of P0 and PMP22, whereas the absence of

ERp57 affected functionality of P0 but not PMP22. Defective PMP22 and P0 processing contributes to impaired myelination and constitutes a molecular explanation for the reduced nerve conduction velocity and disturbed neurological phenotype observed in calnexin-deficient mice [58].

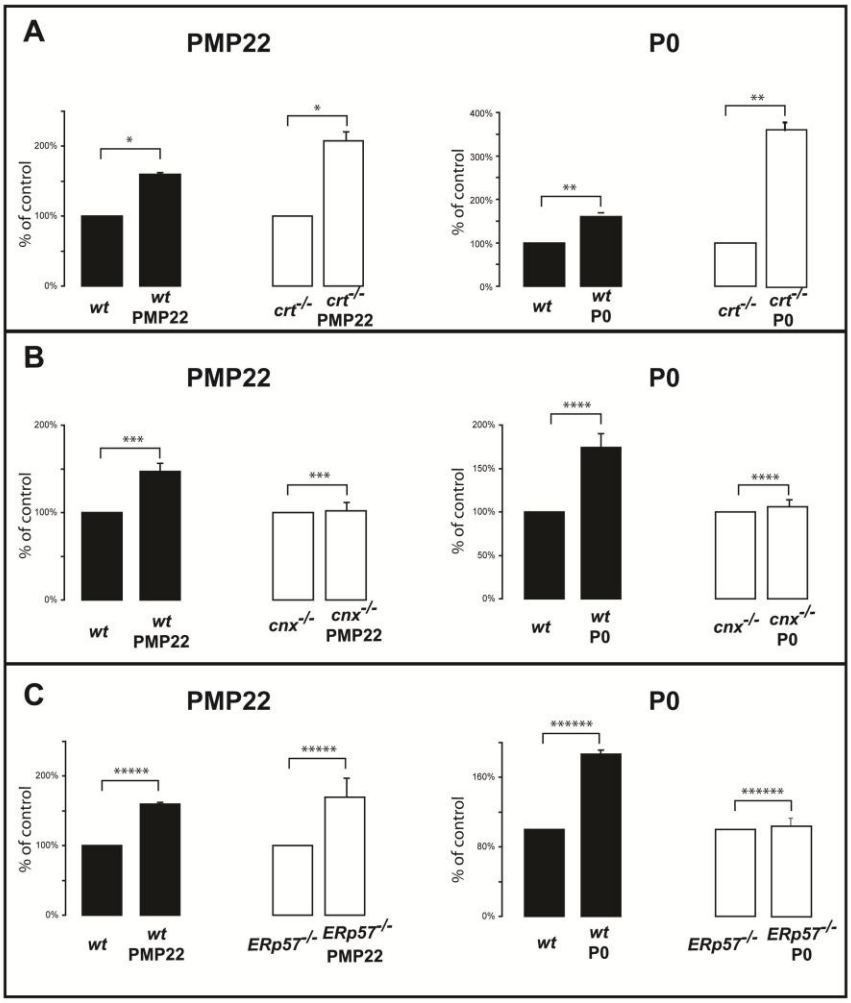


Figure 2-7 Functional analysis of myelin proteins in chaperone-deficient cells. Analysis of the adhesive properties of P0-GFP and PMP22-GFP expressed in wild-type (*wt*) and chaperone-deficient [calnexin-deficient (*cnx*$^{-/-}$), calreticulin-deficient (*crt*$^{-/-}$) and ERp57-deficient (*ERp57*$^{-/-}$)] cells was carried out as described under "Materials and Methods". Cells were cultured overnight in 96-well plates followed by the addition of wild-type (*wt*), calnexin-deficient (*cnx*$^{-/-}$), calreticulin-deficient (*crt*$^{-/-}$) or ERp57-deficient (*ERp57*$^{-/-}$) cells expressing myelin proteins. **(A)** adhesiveness of PMP22-GFP and P0-GFP in calreticulin-deficient cells; **(B)** adhesion activity of PMP22-GFP and P0-GFP in calnexin-deficient cells; **(C)** adhesiveness of PMP22-GFP and P0-GFP in ERp57-deficient cells. Error bars are the means of three separate experiments (*p value=0.61; **p value=0.9; ***p value=0.036; ****p value=0.014; *****p value=0.75; ******p value=0.17; unpaired *t* test). A 100% value corresponds to the following average arbitrary absorbance values: for *wt* cells, 0.82; for *crt*$^{-/-}$ cells, 0.56; for *cnx*$^{-/-}$ cells, 0.57 and for *ERp57*$^{-/-}$ cells, 1.03.

Folding of Myelin Proteins in the Absence of Quality Control ER Chaperones Calreticulin and Calnexin – Next, we tested whether impaired protein folding of myelin proteins in the absence of chaperone was responsible for the reduction of their adhesiveness in the absence of ERp57 or calnexin. We used limited proteolysis to investigate if folding of P0 and PMP22 was affected when myelin proteins were expressed in cells deficient in ER chaperones [71]. P0-GFP or PMP22-GFP expressed in wild-type and chaperone-deficient cells were biotinylated, isolated by streptavidin affinity chromatography followed by a limited proteolysis with trypsin, SDS-PAGE and Western blot analysis with anti-GFP antibodies. Figure 2-8 shows that P0-GFP and PMP22-GFP expressed in wild-type and calreticulin-deficient cells had similar susceptibilities to proteolysis by trypsin, indicating that P0 and PMP22 expressed in the absence of calreticulin were folded correctly and indistinguishable from that expressed in the wild-type cells.

Figure 2-8 shows that trypsin sensitivities of PMP22 and P0 were significantly increased when the proteins were expressed in calnexin-deficient cells, indicating they may be misfolded. These observations are in agreement with functional analysis of myelin proteins in the absence of ER quality chaperones where PMP22 and P0 exhibited no adhesive activity when expressed in the absence of calnexin (Figure 2-7). Taken together, these results indicate that the functionality and folding of myelin proteins, but not their cell surface targeting were affected in the absence of calnexin but not in the absence of calreticulin.

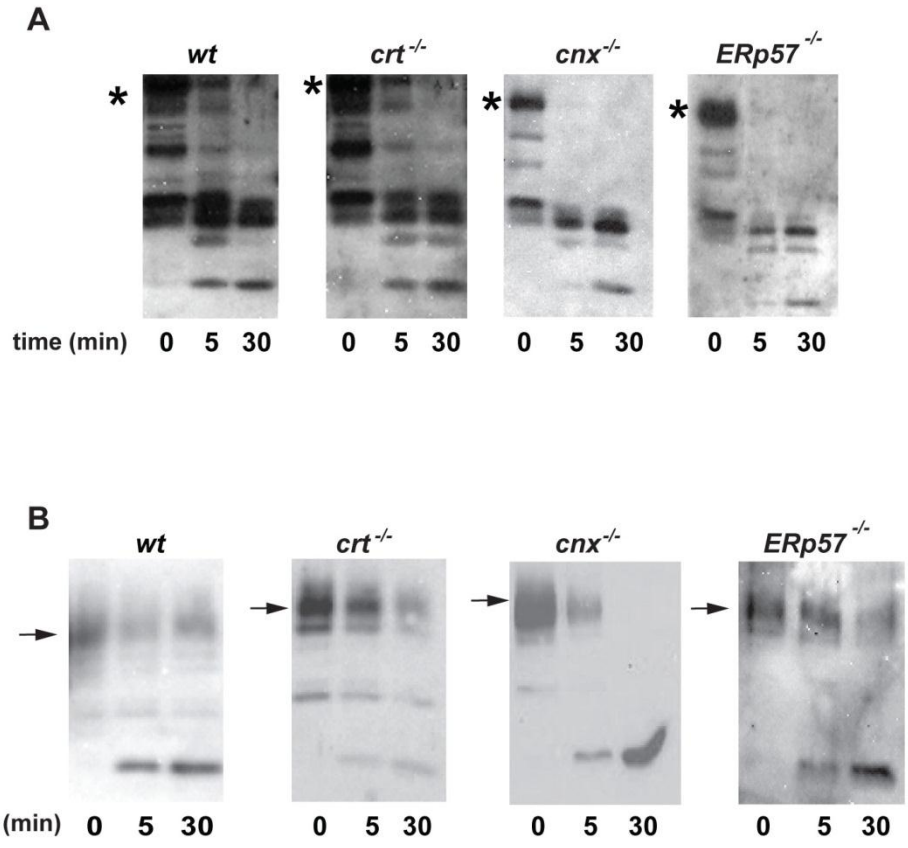


Figure 2-8 Limited proteolysis of the cell surface proteins P0 and PMP22 in the absence of ER chaperones. Trypsin digestion of biotinylated P0-GFP and PMP22-GFP was carried out as described under "Materials and Methods". Wild-type (*wt*), calreticulin-deficient (*crt*$^{-/-}$), ERp57-deficient (*ERp57*$^{-/-}$) and calnexin-deficient (*cnx*$^{-/-}$) cells expressing **(A)** P0-GFP (green fluorescent protein) or **(B)** PMP22-GFP were cell surface biotinylated followed by isolation of the biotinylated proteins. The purified cell surface proteins were incubated with trypsin at a 1:10 dilution (trypsin/protein; w/w) at 37 ^{o}C. Aliquots were taken at the time points indicated (0, 1, 5 and 30 min), and the proteins were separated by SDS-PAGE and probed with anti-GFP antibodies. Asterisks and arrows indicate the locations of PMP22 and P0, respectively.

ERp57 Deficiency Affects the Folding of Myelin Protein P0 but not PMP22 - Figure 2-6 shows that trypsin sensitivity of cell surface PMP22 was also not affected by the absence of ERp57, indicating that PMP22 folding was not affected in the absence of ERp57. In sharp contrast, myelin protein P0 expressed in ERp57-deficient cells (Figure 2-8B) was highly sensitive to proteolysis as compared to myelin proteins expressed in wild-type cells, indicating a significant alteration of P0's structure when expressed in the absence of ERp57. These findings explain why ERp57 deficiency did not affect the function (adhesiveness) of PMP22 (Figure 2-7, *left panel*) but had a significant effect on adhesiveness of P0 in ERp57-deficient cells.

P0 has a single disulfide bond in the extracellular domain formed by C^{21} and C^{98} and differential effects of ERp57 on PMP22 and P0 folding and functionally might be due to the presence of a disulfide bond. Mutations of C^{98} lead to severe early onset neuropathy, whereas mutation of C^{21} abolishes the aggregation (function) of P0 [19, 20]. Importantly, ERp57 affected the function (Figure 2-7) and folding (Figure 2-8) of P0 (containing a disulfide bond) but not PMP22 (which does not contain any disulfide bonds). Therefore, we used SDS-PAGE under reducing and non-reducing conditions to test whether disulfide bond formation in P0 was affected in the absence of the oxidoreductase ERp57. As expected, SDS-PAGE mobility was affected under non-reducing conditions (Figure 2-9), indicating that P0-GFP expressed in wild-type cells formed a disulfide bond. Surprisingly, the SDS-PAGE mobility of P0-GFP expressed in ERp57-deficient cells was also sensitive to a reduced and non-reduced environment, indicating that P0-GFP formed a disulfide bond in the absence of ERp57 (Figure 2-9). This indicates that despite observed effects

of ERp57 deficiency on function and folding of P0, the catalysis of the disulfide bond formation during maturation of P0 was not affected in the absence of ERp57.

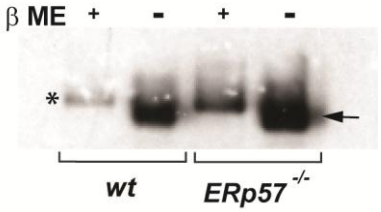


Figure 2-9 Western blot analysis of P0-GFP expressed in wild-type (*wt*) or ERp57-deficient (*ERp57*$^{-/-}$) cells under reducing (β-mercaptoethanol) or non-reducing (no β-mercaptoethanol) conditions as described under "Materials and Methods". The locations of non-reduced and reduced P0-GFP are indicated by the arrow and the asterisk, respectively. β-ME, β-mercaptoethanol.

Calnexin Transiently Interacts with P0 in a Glycan Independent Manner - It has been reported earlier that calnexin forms complexes with PMP22 [19, 20], supporting our findings that the function of PMP22 is affected in the absence of calnexin. Since the function and folding of the myelin proteins P0 was also affected in the absence of calnexin or ERp57, we asked whether these chaperones form complexes with the newly synthesized myelin protein P0. Since calnexin-PMP22 forms complexes have been reported previously [19, 20], here we focused on P0. First, we performed pulse chase experiments to test for a transient interaction between calnexin and P0-GFP (Figure 2-10). Figure 2-10 shows that P0-GFP formed transient complexes with calnexin, indicating that calnexin functioned as a P0 molecular chaperone.

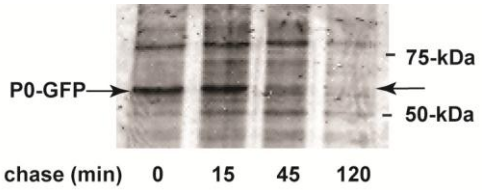


Figure 2-10 Interaction between calnexin and myelin protein P0. HEK293T cells were tranfected with expression vectors encoding P0-GFP metabolically labeled with [^{35}S]-methionine containing medium followed by chase and immunoprecipitation with anti-calnexin antibody at the times indicated in the Figure. The arrows indicate the location of P0-GFP.

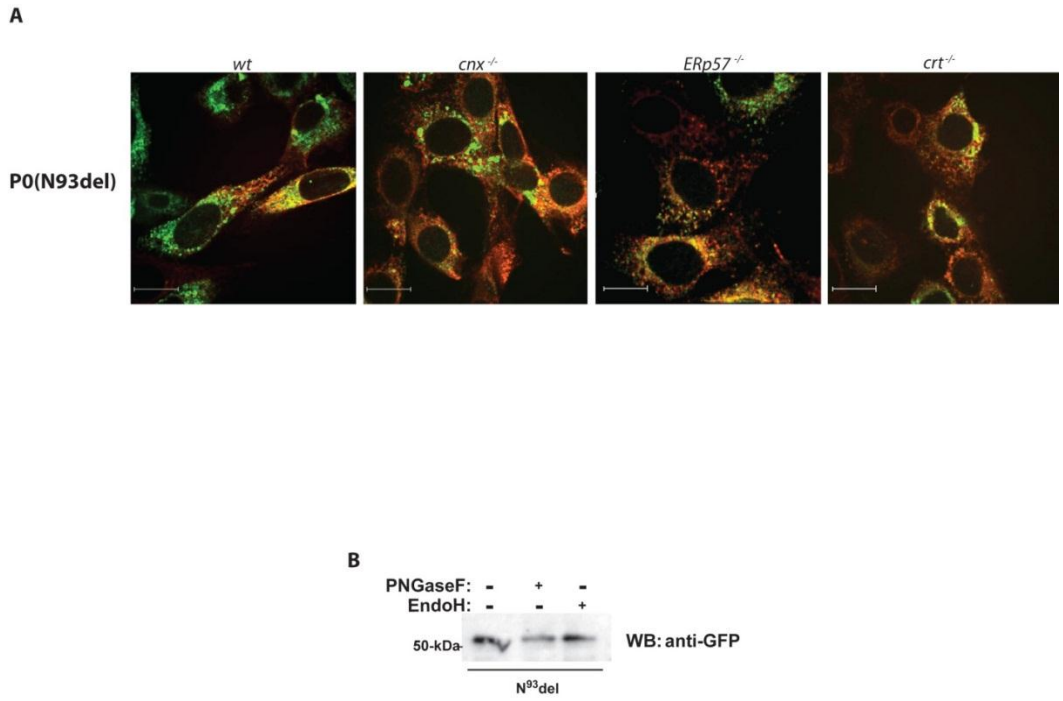


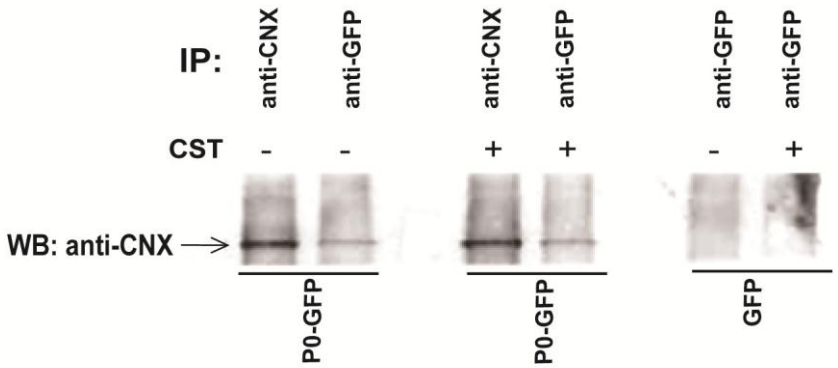
Figure 2-11 Analysis of the P0(N^{93}del) mutant. Confocal images of the cellular localization of P0(N^{93}del)-GFP (green) in *wt*, calnexin-deficient (*cnx*$^{-/-}$), ERp57-deficient (*ERp57*$^{-/-}$) and calreticulin-deficient (*crt*$^{-/-}$) cells. Concanavalin A (red) staining was used as an ER marker. The P0(N^{93}del) mutant shows high colocalization with an ER marker in all cell types, suggesting ER retention of the mutant protein. Scale bar=17 μm. **B**. Endoglycosidase digestion of P0(N^{93}del)-GFP expressed in *wt* cells**.** Immunoblot of cell lysates, untreated or treated with PNGaseF or EndoH shows no difference in migration between untreated and treated samples confirming the lack of glycan.

To determine if calnexin binding to P0 was glycan-dependent and if the glycan mediates interaction between calnexin and P0, we generated a P0 mutant lacking the N-glycosylation site (N^{93}del). First, we looked at the localization of P0(N^{93}del)-GFP in *wt* and chaperone-deficient cells (Figure 11A). Figure 2-11A shows intracellular localization of P0 N^{93}del; interestingly, deletion of the N-glycosylation site resulted in ER retention of this mutant. To confirm that P0(N^{93}del)-GFP was not glycosylated, we carried out endoglycosidase digestion analysis of the recombinant protein (Figure 11B). Figure 2-11B shows that P0(N^{93}del)-GFP was not sensitive to either EndoH or PNGaseF digestion, indicating that it was not glycosylated. To further determine if calnexin binding to P0 was glycan-dependent, we expressed P0(N^{93}del)-GFP followed by immunoprecipitation with anti-GFP antibodies and by Western blot analysis with anti-calnexin antibodies (Figure 2-12B). Figure 2-12B shows that even in the absence of glycosylation, P0 formed immunoprecipitable complexes with calnexin, indicating that interaction between calnexin and P0 was glycan-independent.

Moreover, we used castanospermine (glucosidase inhibitor) to confirm the glycan-independent interaction between calnexin and P0. Castanospermine should prevent the formation of complexes between calnexin and P0 if the interaction is glycan-dependent. Figure 2-12A shows that P0-GFP formed complexes with calnexin even in cells treated with castanospermine, indicating that calnexin interacted with this myelin glycoprotein in a glycan-independent way.

Attempts to immunoprecipitate PMP22-GFP or P0-GFP complexed with ERp57 failed, suggesting that ERp57 may not interact directly with PMP22

and P0 or that PMP22- and P0-ERp57 interactions may not be direct or as stable as those observed between these myelin proteins and calnexin. We also failed to detect any immunoprecipitable complexes of calreticulin with PMP22 or P0. This supports our cell biological and biochemical observations suggesting that calreticulin does not play a role in the biology of these myelin glycoproteins.



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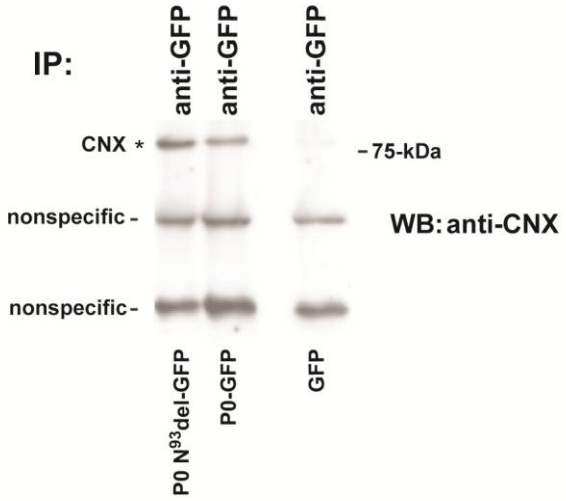

B

Figure 2-12 Interaction between calnexin and myelin protein P0. Cell lysate was collected from cells stably expressing P0-GFP followed by addition of the appropriate antibodies as indicated in the Figure and as described under "Materials and Methods". **A.** Cells were incubated with 1 mM castanospermine (*CST*) as indicated by "**+**". Proteins were separated by SDS-PAGE followed by Western blot analysis with anti-calnexin (*CNX*) antibodies. A representative Western blot of 3 independent experiments is shown. **B.** Cell lysate was collected from cells stably expressing P0(N^{93}del)-GFP, P0-GFP or GFP followed by addition of anti-GFP antibodies. Proteins were separated by SDS-PAGE followed by Western blot analysis with anti-calnexin antibodies. A representative Western blot of 3 independent experiments is shown. The asterisk indicates the location of calnexin (*CNX*).

Expression of Myelin Proteins In the Absence of ER Chaperones Does Not Activate the Unfolded Protein Response (UPR) - In the ER lumen, proteins are subjected to a quality control. However, terminally misfolded proteins may escape the quality control machinery. To avoid accumulation of misfolded protein, maintain balance between protein synthesis and degradation, cells have developed mechanisms that lead to the activation of various pathways and responses that promote the degradation of misfolded proteins. Recently, the UPR has been closely linked to peripheral neuropathies. It has been shown that the mutant of P0 (S34del) that is retained in the lumen of ER can trigger the UPR, resulting in changes in the levels of UPR markers, such as BiP and CHOP (CAATT enhancer-binding protein homologous protein), which is a transcription factor active upon UPR activation [10]. Interestingly, ablation of CHOP was able to rescue of the phenotype [10]. The UPR is an important protective mechanism that helps to reduce ER stress caused by accumulation of misfolded proteins. However, activation of the UPR can result in regained homeostasis in the cell or activation of apoptotic pathways [72]. Apoptosis will lead to Schwann cell death and consequently to peripheral neuropathy. As activation of the UPR has been proposed to contribute to the pathology of peripheral neuropathies, we tested if expression of P0-GFP or PMP22-GFP in cells deficient in ER chaperones will result in induction of ER stress and consequently the UPR. To test for UPR activation, we examined the unconventional splicing of Xbp1 mRNA downstream of activation of the IRE1 pathway (see Chapter 1 section 3.2). We used a luciferase reporter system developed by Kaufman's group [66]. Wild-type, $crt^{-/-}$, $ERp57^{-/-}$ or $cnx^{-/-}$ stably expressing P0 or PMP22 cells were transfected with pRL-IXFL vector encoding *Renilla*

luciferase (internal control) and the *Firefly* luciferase reporter gene, which is activated only when the 26-nucleotide intron from Xbp1 is removed upon activation of the UPR [66]. Figure 2-13 shows that expression of P0-GFP or PMP22-GFP in cells deficient in ER chaperones, did not result in activation of the UPR as measured by Xbp1 splicing. These data confirm the previous result that neither P0 nor PMP22 accumulate in the ER lumen in the absence of ER chaperones (Figures 2-2, 2-3, 2-4). As a positive control for activation of the UPR, cells were treated with the ER stress inducers - thapsigargin (Tg), an inhibitor of SERCA, an ER localized Ca^{2+}-ATPase.

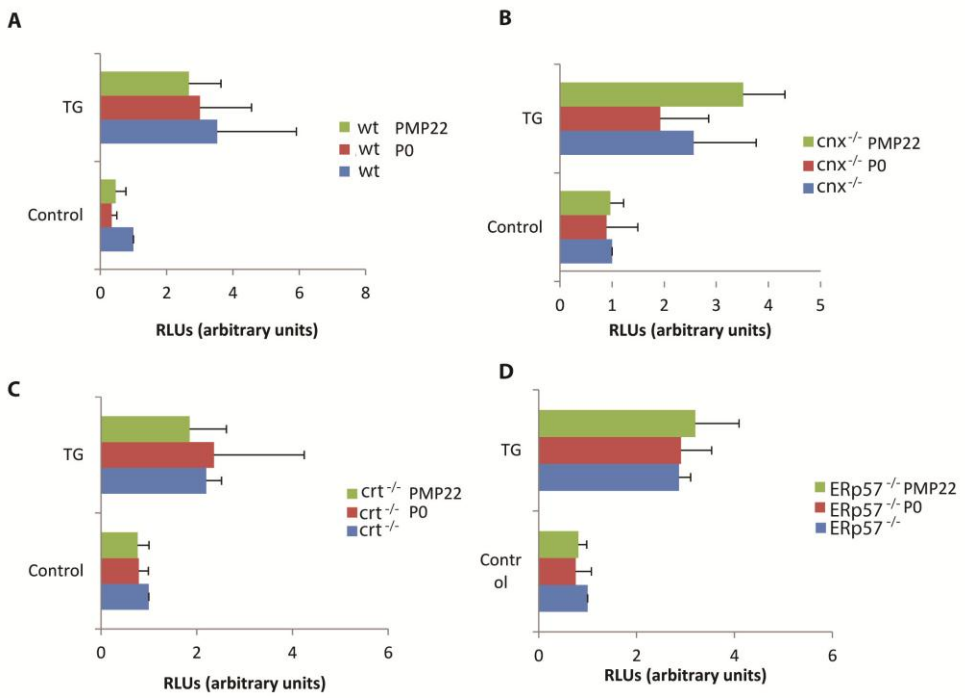

Figure 2-13 Xbp1 mRNA splicing in cells deficient in ER chaperones stably expressing myelin glycoproteins P0 or PMP22. *Renilla* luciferase and *Firefly* luciferase activities were measured as described under "Materials and Methods" and the relative ratio of *Firefly* luciferase to *Renilla* luciferase activity in each cell lysate was calculated. **A**. wild-type cells expressing PMP22-GFP (green bars), P0-GFP- (red bars) or control cell (blue bars) were transfected with reporter plasmid for Xbp1 splicing as described under "Materials and Methods". Cell lysates were assayed for luciferase activity. **B.** calnexin-deficient cells *(cnx-/-)* expressing PMP22 (green bars), P0- (red bars) or control cell (blue bars) were transfected with reporter plasmid for Xbp1 splicing as described under "Materials and Methods". Cell lysates were assayed for luciferase activity. **C**. calreticulin-deficient cells *(crt-/-)* expressing PMP22-GFP (green bars), P0-GFP- (red bars) or control cell (blue bars) were transfected with reporter plasmid for Xbp1 splicing as described under "Materials and Methods". Cell lysates were assayed for luciferase activity. **D**. ERp57-deficient cells *(ERp57-/-)* expressing PMP22-GFP (green bars), P0-GFP (red bars) or control cell (blue bars) were transfected with reporter plasmid for Xbp1 splicing as described under "Materials and Methods". Cell lysates were assayed for luciferase activity. Cells were treated with thapsigargin (*Tg*) followed by the luciferase assay. Columns and bars represent the means ± S.D. of nine measurements from three independent transfection experiments. *RLU*, relative light units.

Discussion

P0 and PMP22 are the most abundant glycoproteins in PNS myelin and they are critical for compacting and maintenance of myelin. Mutations in the P0 and PMP22 genes result in peripheral neuropathies (characterized by alterations in myelination, reduced nerve conduction velocities and muscle weakness). Recently, we showed that calnexin-deficiency leads to impaired myelin development and function. Yet, little is known about the role of ER quality control chaperones and folding enzymes in the biology of these myelin glycoproteins. Here, we used chaperone-deficient cells and showed that components of the ER quality control are essential for function and folding of peripheral myelin glycoproteins. Unexpectedly, we discovered that, cell surface targeting of the myelin proteins P0 and PMP22 is not affected in the absence of ER chaperones in fibroblasts stably expressing P0 or PMP22. Folding and function (adhesiveness) of PMP22 and P0 are significantly affected in the absence of calnexin, but not in the absence calreticulin (Figure 2-14). In the absence of the oxidoreductase ERp57, folding and function of P0, a disulfide bond containing protein, is severely affected whereas ERp57 deficiency does not affect PMP22 folding nor function (Figure 2-14). Our studies indicate that there is a functional specialization within the quality control components with respect to myelin protein folding and function. This supports the hypothesis that there is substrate specific specialization of the components of the ER quality control, including ERp57 [73].

The ER quality control consists of calnexin and calreticulin, which are molecular lectin-like chaperones, and ERp57, which is an oxidoreductase folding enzyme with specificity for calnexin/calreticulin substrates [74]. In

this study, we showed for the first time, that calnexin forms complexes with P0. Furthermore, these interactions are glycan-independent. Considering that ERp57 deficiency had significant effects on folding and function of myelin protein P0, it was surprising that we could not detect ERp57-P0 complexes by immunoprecipitation. This might be due to relatively weak and/or transient interactions between ERp57 and P0. Alternatively, ERp57 may be a part of a larger complex, which may include other chaperones and folding enzymes. Calnexin-deficient mice are viable but have impaired myelination [58]. In contrast, calreticulin-deficient mice are embryonic lethal due to a defect in cardiac development [56]. Despite the structural and functional similarities between calnexin and calreticulin, the loss of calreticulin did not have any effect on myelin proteins. The different topological environments of calnexin and calreticulin are likely critical in determining their distinct substrate specificities [73] with calnexin "preferring" integral membrane proteins (such as myelin proteins) over substrates in the mobile ER luminal environment. Calnexin's ability to chaperone the myelin proteins PMP22 and P0 provides an explanation for the dysmyelination observed in the absence of calnexin, and it provides a molecular basis for the calnexin-dependent peripheral neuropathy [58].

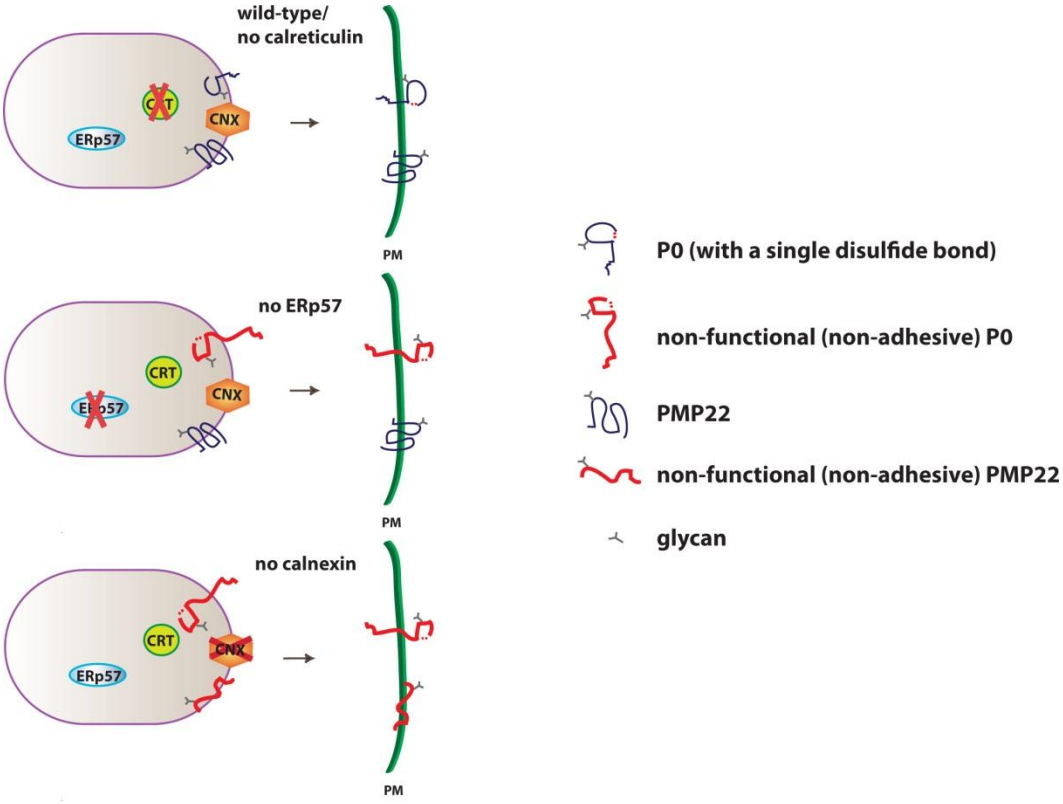

Figure 2-14 A model of PMP22 and P0 maturation and transport to the plasma membrane in the absence of ER quality control components. In wild-type (*wt*) or calreticulin-deficient (*crt*$^{-/-}$) cells, the myelin proteins P0 and PMP22 are targeted from the endoplasmic reticulum (ER) to the plasma membrane (PM), where they are functional and properly folded. In the absence of oxidoreductase ERp57 (*ERp57*$^{-/-}$), P0 and PMP22 are properly localized to the plasma membrane, but only PMP22 is functional and correctly folded, whereas the P0 protein containing a C^{21}-C^{98} disulfide bridge is non-functional and misfolded. In the absence of calnexin (*cnx*$^{-/-}$), both myelin proteins localize to the plasma membrane but they have significantly decreased function (adhesiveness) and are misfolded. CRT, calreticulin; CNX, calnexin; PM, plasma membrane.

An important observation of this work is that both peripheral myelin proteins PMP22 and P0 are efficiently targeted to cell surface in the absence of the ER quality control chaperones calnexin, calreticulin and ERp57. Furthermore, PMP22 and P0 expressed in calnexin-deficient or ERp57 deficient cells do not form aggregates nor do they accumulate in the ER. However, the functional assay and analysis of folding of these myelin glycoproteins indicated that cell surface PMP22 (calnexin-deficient cells) and P0 (calnexin- and ERp57-deficient cells) are misfolded and not adhesive. Several human peripheral neuropathies are associated with specific mutations or partial deletion of the genes encoding myelin protein P0 or PMP22 [10]. Three of the most common neuropathies are Charcot-Marie-Tooth type 1B, Dejerine-Sottas syndrome and congenital hypomyelinating neuropathy. Many myelin protein mutations produce phenotypes with varying degrees of disease severity, as a result of altered protein function and/or cell surface targeting [7-10, 34, 35, 47]. Despite these reports, the molecular mechanisms by which different mutations in P0 and PMP22 genes result in diverse peripheral neuropathies remain unclear. Accumulation of misfolded protein in the ER typically leads to activation of unfolded protein responses (ER stress) [10]. Here, we have not detected significant activation of ER stress in either calnexin-deficient or ERp57-deficient cells expressing P0-GFP or PMP22-GFP. Our results indicate that the absence of the chaperone calnexin and folding enzyme ERp57 leads to synthesis and cell surface trafficking of non-functional myelin proteins PMP22 and P0, and this may contribute to the pathology of peripheral neuropathies.

Although both myelin proteins play critical roles in the formation and maintenance of myelin sheaths, only P0's extracellular domain contains a disulfide bond [20]. The disulfide bond in the extracellular domain of P0 plays a role in its adhesive properties [20], protein stability and, it might be important for cell surface targeting of P0 [19]. ERp57 is critical for biosynthesis of functional P0, but surprisingly, ERp57 is not essential for its disulfide bond formation, suggesting that other oxidoreductase(s) in the ER lumen might be involved in disulfide bond formation on P0. Instead ERp57 may function as a molecular chaperone for myelin glycoprotein P0. This is in line with other ERp57 substrates that undergo disulfide bond formation in the absence of ERp57 with the assistance of other ER resident oxidoreductases [75, 76]. It is also possible that the timing of the oxidation is changed in the absence of ERp57 and, therefore, this may contribute to the loss of protease resistance and adhesiveness.

In summary, our work indicates that components of ER quality control (calnexin and ERp57) may play an important role in the pathology of peripheral neuropathies and contribute to the diversity of these neurological disorders. It is critical, therefore, to understand the differential contribution of the ER protein quality control machinery to the biology of myelin proteins because this could provide important information for managing specific protein folding disorders, including myelin diseases.

A novel and promising therapeutic approach for peripheral neuropathies related to mutations in PMP22 and P0 involves elimination of misfolded proteins to reduce the severity of the phenotype caused by the mutated proteins [8]. Although recent studies show a critical role for the ER

chaperones in the biology of myelin proteins and the myelination process, the molecular mechanisms underlying interaction between them remains unknown.

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Chapter Three

Cell Surface Targeting of Myelin Oligodendrocyte Glycoprotein (MOG) in the Absence of Endoplasmic Reticulum Molecular Chaperones

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Introduction

Myelin is the multilamellar sheath surrounding large axons in peripheral and central nervous systems. It is essential for fast signal transmission. Myelin formation depends on myelin proteins. Oligodendrocytes are myelin-forming cells in the CNS. Unlike Schwann cells, myelinating cells in the PNS that have a one to one ratio of Schwann cell to axon, a single oligodendrocyte extends into multiple processes (extensions of the cell body), each of which surrounds different axons (Figure 3-1) [1]. Consequently, on the same axon, myelin segments can be formed by different oligodendrocytes (Figure 3-1) [2].

Specific Components of the Oligodendrocytes - CNS myelin is composed of 30% myelin proteins and 70% lipids [1]. Proteins specific for mature myelinating oligodendrocytes include: myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG). MBP and PLP are the major CNS proteins, they constitute 80% of total proteins in the CNS [1]. For MBP, several isoforms have been identified; the molecular masses of major isoforms vary from 21.5 to 14 kDa in mouse and 21.5 to 17.2 kDa in humans [3]. MBP is essential for formation of compact myelin during myelination. Mice with deletion of the MBP gene (shiverer mutant mouse) do not develop a major dense line in myelin, confirming the role of MBP in myelin formation [4, 5].

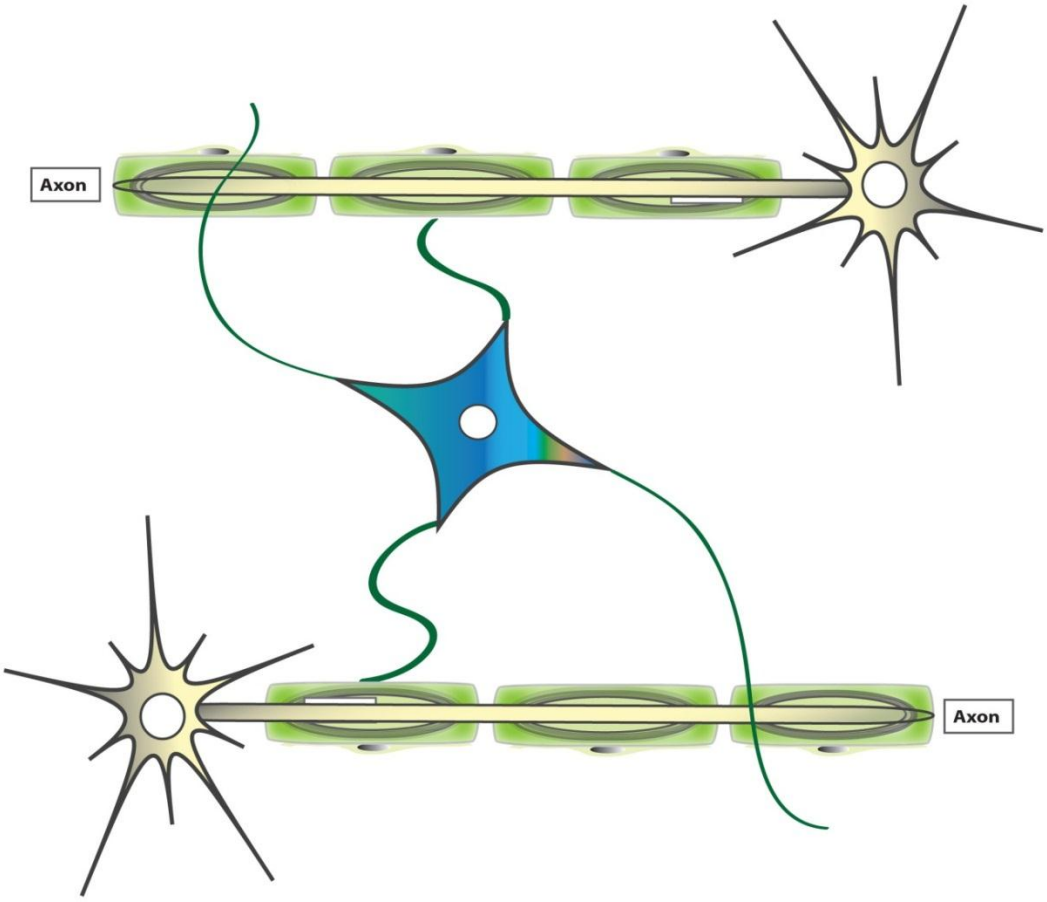

Figure 3-1 Schematic representation of myelinating oligodendrocyte in the central nervous system. Oligodendrocytes are myelin-forming cells in the CNS. A single oligodendrocyte cell with extending processes (in blue) can myelinate many axonal segments depending on the region of the CNS.

PLP corresponds to 50% of CNS myelin proteins. It is a 25-kDa membrane protein with four predicted transmembrane segments, a large extracellular domain between the third and fourth transmembrane domains and short cytosolic N- and C-terminal domains [6-8]. Several naturally occurring mutations in the PLP gene have been associated with dysmyelinating diseases of central nervous system in mice and humans [1, 9]. Many of the mutations occurring in the PLP gene result in ER retention of the mutated protein [10]. Moreover, the ER molecular chaperone, calnexin contributes to ER retention of one of the PLP mutants by binding to the fourth transmembrane domain of the PLP and preventing its degradation [11].

Myelin Oligodendrocyte Glycoprotein (MOG). MOG is a quantitatively minor (0.05%) protein component of the CNS myelin. The protein is expressed within the outermost lamellae of the myelin sheath, the cell body and processes of oligodendrocytes [12]. Interestingly, MOG's expression is delayed 24-48 hours in comparison to other myelin proteins, suggesting that its primary functional role may relate to mature oligodendrocytes [13].

MOG is a type I integral membrane protein with a molecular weight of 28 kDa [14, 15]. MOG consists of a globular N-terminal extracellular domain followed by two hydrophobic domains connected by a cytoplasmic loop and a short C-terminal cytoplasmic domain (Figure 3-2).

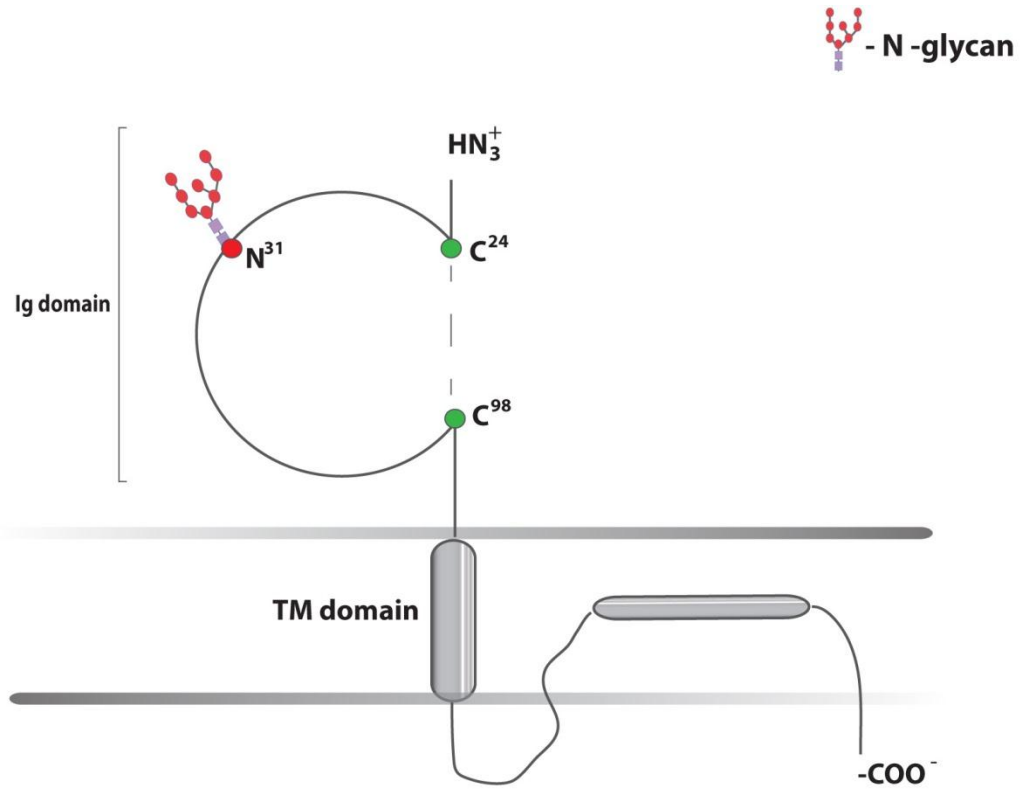

Figure 3-2 Schematic representation of MOG based on the model proposed by Kroepfl et at [16] and crystallographic results for the N-terminal domain. In this model, the extracellular N-domain forms an Ig-like domain with a single glycosylation site at asparagine N^{31} (red circle) and a single disulfide bond formed between cysteines C^{24} and C^{98} (green circles). There are two hydrophobic regions in the sequence of MOG. The first hydrophobic region forms a transmembrane domain, whereas the second is predicted to associate with the membrane but not spanning it. MOG also contains a short cytosolic tail.

The structure of the extracellular domain of MOG has been determined by crystallography [14]. This study confirmed that MOG is a member of the immunoglobulin (Ig) superfamily (Figure 3-2). Crystallography results showed that it adopts a topology of Ig-V domain [14]. The extracellular domain of MOG contains a single N-glycosylation site at N^{31} and one disulfide bond formed between C^{24} and C^{98} (Figure 3-2) [14]. The first hydrophobic domain of MOG forms a single transmembrane domain. In contrast, the second hydrophobic domain is probably juxtaposed to the membrane and not spanning it [12, 16]. The C-terminal cytoplasmic domain of MOG contains targeting signals that presumably account for its localization on the outside surface of oligodendrocytes and myelin sheaths [17, 18].

The amino acid sequence of MOG is highly conserved among animal species, suggesting an important biological function [12]. However, the function(s) of the protein is not fully understood. Its role in cellular adhesion, regulation of oligodendrocyte microtubule stability and activation of the complement cascade have been suggested [12]. The extracellular domain of MOG is suggested to be involved in homophilic (self-association) interactions [14]. A homodimeric form of MOG has been isolated from CNS; its homodimerization has also been confirmed *in situ* [19]. Moreover, the crystal structure of the extracellular domain of MOG demonstrates that it forms a head-to-tail dimer in solution, which would also suggest dimerization and potential adhesive properties [14].

MOG plays an important role in the pathology of multiple sclerosis (MS), a chronic inflammatory disease of the CNS. Interestingly, MOG was first identified as the antigen responsible for demyelination observed in

animals injected with whole CNS homogenate [20]. MOG is thought to influence MS susceptibility as a primary target for autoimmune responses [20]. Anti-MOG antibodies have been found in the sera of patients with MS and their presence is directly associated with myelin damage [21]. Previous studies suggest that encephalitogenic antibodies recognize conformation-dependent epitopes of MOG [22]. However, MOG contribution to MS pathology is still not fully understood. More insights into MOG's role in autoimmune disease come from studies on a MOG-deficient mouse model. Mutant mice lacking a functional *MOG* gene show no clinical or histological abnormalities. Moreover, when inflammatory demyelinating disease of CNS (experimental autoimmune encephalomyelitis (EAE)) is induced in these mice, they show mild symptoms confirming that the anti-MOG response is a major pathogenic component during disease occurrence [23].

As an integral membrane protein, MOG is synthesized in the ER and subsequently targeted to the plasma membrane of oligodendrocytes. These events must be closely monitored by the ER quality control system, which consists of molecular chaperones and folding enzymes. Calnexin and calreticulin are two ER lectin-like chaperones that play a crucial role in the quality control process. The two ER lectin-like chaperones each consist of a globular domain, which contains a glycan-binding site and an extended, proline-rich domain (P-arm) that is known to associate with ERp57, an oxidoreductase that belongs to the PDI family [24, 25]. ERp57 catalyzes formation and isomerization of disulfide bonds in newly synthesized glycoproteins (often calnexin and calreticulin substrates) within the ER. Despite MOG playing a critical role in the pathology of MS,

the role of ER-associated chaperones and folding enzymes in the biology of MOG and consequently in the pathology of multiple sclerosis is not known. Here, we identified calnexin as an ER molecular chaperone for MOG and examined the role of ER quality control in MOG expression, trafficking and activation of the unfolded protein response (UPR). This work provides new important insights into the biology of MOG. We have identified new potential players that may contribute to the folding and conformation of MOG and therefore to its encephalitogenic properties.

Materials and Methods

Vector DNA - The full-length cDNAs encoding mouse MOG were cloned into pEGFP-N1 vector to create vectors encoding MOG with green fluorescent protein (GFP) at its carboxyl terminus, MOG-GFP. The following DNA primers containing an *attB* recombination site (Gateway Cloning, Invitrogen) were used: forward primer 5′- GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TAC CAT GAT GGC CTG TTT GTG GAG CTT CTC reverse primer 5′GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TTA CTT GTA CAG CTC GTC CAT GCC-3′. First, the PCR products were cloned into the pDONR vector (Invitrogen) using LR Clonase Enzyme (Invitrogen). Next, the recombination reaction was carried out using BP Clonase Enzyme Mix (Invitrogen) with the gene of interest, the ubiquitously expressed promoter, EF1α (cellular polypeptide chain elongation factor 1 alpha) and a destination vector $2K7_{bsd}$, containing a blasticidin resistance gene that allows the easy selection of transfected cells [26]. Virus isolation and transduction were carried out as described previously [26]. Site-specific mutagenesis was carried out to generate the expression vector encoding non-glycosylated MOG [MOG(N^{31}A)-GFP]. For site-specific substitution of the N^{31} glycosylation site to Ala on MOG, the following DNA primers were used: forward primer 5'-CTC TCC TGG GAA AGC TGC CAC GGG CAT GGA-3' and reverse primer 5'-TCC ATG CCC GTG GCA GCT TTC CCA GGA GAG-3′. An expression vector encoding MOG-GFP was used as a template. Site-directed mutagenesis was carried out using QuickChange Site-Directed Mutagenesis Kit (Stratagene).

Cell culture, cell lines and immunofluorescence - Wild-type (K41) and calreticulin-deficient (K42) mouse embryonic fibroblasts were described previously [27]. ERp57-deficient mouse embryonic fibroblasts were isolated from *ERp57*$^{-/-}$ embryos and immortalized [27, 28]. Wild-type and calnexin-deficient mouse fibroblasts were isolated from newborn mice and immortalized [29]. Lentiviral expression constructs encoding MOG-GFP were used to create wild-type, calnexin deficient (*cnx*$^{-/-}$), calreticulin deficient (*crt*$^{-/-}$) and *ERp57*$^{-/-}$ deficient cell lines stably expressing recombinant MOG-GFP. Cells were cultured in the presence of 7 μg/ml blasticidine for 14 days. Expression of MOG-GFP protein was monitored by Western blot analysis using anti-GFP antibodies or by immunofluorescence, co-staining with the ER marker Texas Red-Concanavalin A conjugate using confocal microscopy. For immunofluorescence, cells expressing MOG-GFP were grown for 24 hours after seeding in culture media and fixed in 4% paraformaldehyde for 12 min [29]. Images were collected by spinning-disk microscopy (Guelph, Canada) set up on an Olympus IX-81 inverted stand (Markham, Canada). Images were acquired through a 60X objective (N.A. 1.42) with an Electron Multiplying Charge Coupled Device (EMCCD) camera (Hamamatsu, Japan).

Western blot analysis and immunoprecipitation -Brains and cerebella from mice were isolated, crushed in liquid nitrogen and lysed in RIPA buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.5% Nonidet P-40, 1% deoxycholate, 0.1% sodium dodecyl sulfate and protease inhibitors for 15 min on ice [30]. For Western blot analysis, cells expressing MOG-GFP were lysed on ice in RIPA buffer for 15 min [29]. The following antibodies were used: goat anti-GFP 1:10,000, rabbit anti-MOG 1:300, rabbit anti-

calnexin (Stressgene), rabbit anti-calreticulin 1:300, rabbit anti-ERp57 1:1000, and rabbit anti-glyceraldehyde phosphate dehydrogenase (GAPDH) 1:1000.

For immunoprecipitation, cells were grown to 80-90% confluency followed by the addition of 500 μl per 10 cm dish of A lysis buffer containing 50 mM Hepes, pH 7.4, 200 mM NaCl, 2% CHAPS and protease inhibitors [31]. Samples were incubated on ice for 30 min and then spun at 11,600 x *g* for 15 min to remove insoluble material. The supernatant was pre-cleared with a 1/15th aliquot of 10% protein A/G Sepharose bead suspension in an HBS buffer containing 50 mM HEPES, pH 7.4 and 200 mM NaCl. Beads were centrifuged followed by addition of 2 μl of an appropriate antibody. The samples were incubated overnight at 4 ^{o}C with rotation followed by addition of 100 μl of 10% protein A/G Sepharose in HBS buffer and incubated for an additional 4 hrs. Beads were centrifuged, washed 3 times with HBS containing 1% CHAPS, one time with HBS, followed by addition of 30 μl of SDS-PAGE sample buffer [32]. Proteins were separated by SDS-PAGE (10% acrylamide). Cells were also cultured with 1 mM castanospermine for 16 hrs.

Endoglycosidase digestions -Cellular proteins were extracted with RIPA buffer and 20 μg of total protein were incubated with EndoH (endoglycosidase H) or PNGaseF (Peptide: N-glycosidase F) at 37 ^{o}C for 3 hrs according to the manufacturer's protocols. Digested and undigested samples were separated by SDS-PAGE (10% acrylamide) followed by Western blot analysis with rabbit anti-GFP antibodies at 1:10,000.

Cell surface biotinylation - For cell surface biotinylation, cells stably expressing MOG (in 10 cm dishes) were first washed with phosphate-

buffered saline and then with 5 ml of cold borate buffer [33]. Biotinylation was in a buffer containing 10 mM boric acid, pH 9.0, 154 mM NaCl, 7.2 mM KCl, and 1.8 mM $CaCl_2$ followed by incubation with sulfosuccinimido-NHS-biotin (0.5 mg/ml of borate buffer) at 4 ^{o}C for 30 min. The biotinylation reaction was stopped by addition of 5 ml of quenching buffer containing 192 mM glycine, 25 mM Tris, pH 8.3. Cells were carefully washed 3 times with cold quenching buffer followed by lysis for 20 min on ice in 500 μl of lysis buffer containing 10 mM Tris-HCl, pH 7.5, 1% NP40, 5 mM EDTA, 150 mM NaCl, 0.5% deoxycholate and protease inhibitors. Samples were centrifuged at 13,400 x *g* for 20 min. The supernatant was used for the protein assay (250 μl) and for incubation with streptavidin agarose (150 μl). Samples were incubated with streptavidin agarose overnight at 4 ^{o}C. Resin containing biotinylated plasma membrane proteins was spun down at 7500 x *g* for 2 min, followed by 5 washes with lysis buffer without protease inhibitors. The percent of biotinylated proteins was determined using densitometry and normalized to equivalent levels of GAPDH: %biotinylated at cell surface = (total protein – unbound) * 100/ total protein). The percent of biotinylated proteins was determined using densitometry. The percentage of biotinylated, plasma membrane localized MOG, was expressed as a percentage of total MOG content as determined by Western blot analysis. Twenty μg of biotinylated proteins were digested with trypsin at a 1:10 protein/trypsin ratio (w/w) at 37 ^{o}C for 5 or 30 min. Digestion was stopped by the addition of the Laemmli sample buffer [32]. Samples were boiled for 5 minutes and followed by SDS-PAGE (10% acrylamide) and Western blot analysis using rabbit anti-GFP antibodies at 1:10,000 dilution.

Analysis of the UPR - The UPR was measured using the luciferase reporter gene assay [29]. In brief, wild-type, *cnx*$^{-/-}$, *ERp57*$^{-/-}$ and *crt*$^{-/-}$cells stably expressing MOG-GFP were transfected with pRL-XFL vector encoding *Renilla* luciferase and *Firefly* luciferase reporter genes [29, 34]. Briefly, cells were transfected with 1 μg of DNA using Effectene (Qiagen). Cells were treated with 1 μM thapsigargin for 16 hrs, lysed, diluted and assayed with the Dual-Luciferase Assay Kit (Promega) using a luminometer (Berthold-Lumat LB 9501). Relative light units (RLUs) were normalized to an internal control. Average ± SD (n = 3).

Results and Discussion

Calnexin interacts with MOG - In eukaryotic cells, membrane and secreted proteins fold and achieve their native structures in the ER [35]. Calnexin is one of the ER-associated molecular chaperones and together with the oxidoreductase ERp57, assists many glycoproteins to achieve their final conformation [36]. In mice, calnexin deficiency leads to disruption in myelination in the peripheral and central nervous systems (CNS) [37]. MOG is a glycoprotein of CNS myelin, potentially involved in the formation of compact myelin sheaths [14]**.** MOG is a glycoprotein with a single N-glycosylation site and a single disulfide bond, which makes it a potential calnexin and ERp57 substrate. We asked whether calnexin and ERp57 form complexes with newly synthesized MOG. Stable expression of MOG-GFP in wild-type cells was followed by immunoprecipitation with anti-GFP antibodies and Western blot analysis with either anti-ERp57 or anti-calnexin antibodies. Figure 3-3 shows that calnexin formed complexes with MOG, indicating that calnexin may play a role in MOG folding. Attempts to immunoprecipitate MOG with ERp57 failed, indicating that ERp57 may not interact directly with MOG. This result is in agreement with the view that ERp57 does not bind substrates directly, but is thought to be recruited; the *b* and *b'* domains of ERp57 interact with the extended P-domains of both calnexin and calreticulin [38]. It has been suggested that the role of calnexin and calreticulin is to bring ERp57 into close proximity to substrate, resulting in an enhancement of ERp57 activity [39].

To examine if the interaction between calnexin and MOG was mediated by glycan, we treated cells stably expressing MOG-GFP with

castanospermine. This glycosidase inhibitor prevents removal of the first glucose residue and consequently processing of the oligosaccharide chain, enhancing the formation of monoglucosylated substrate that would be recognized by calnexin (Figure 1-2) [40]. Figure 3-3 shows that calnexin-MOG complexes formed even in the presence of castanospermine, an inhibitor of glucosidase I/II, indicating this interaction was sugar-independent. This further supports the "dual binding" model for glycan-dependent and glycan-independent interactions between calnexin and its substrates as has been proposed previously by Williams et al [41].

Here, I have demonstrated that MOG, a type I integral membrane protein localized to central nervous system myelin, interacts with calnexin and that castanospermine treatment did not abolish this interaction, suggesting sugar-independent nature of the complex formation.

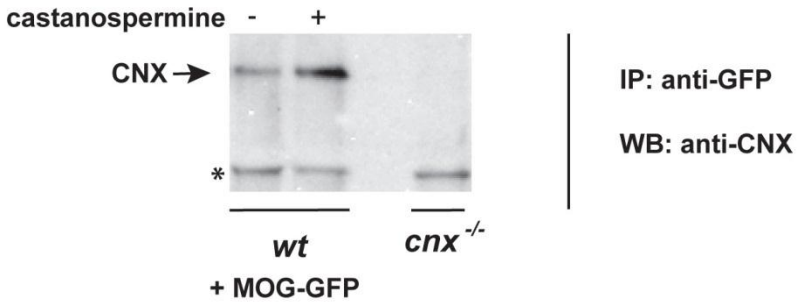

Figure 3-3. Interaction between calnexin and MOG - Cell lysates were collected from wild-type (*wt*) cells stably expressing MOG-GFP followed by addition of the goat anti-GFP antibodies as indicated in the Figure and as described under "Material and Methods". Proteins were separated by SDS-PAGE followed by Western blot analysis with anti-calnexin (*anti-CNX*) antibodies. A representative Western blot of 3 independent experiments is shown. Cells were cultured in the presence of 1 mM castanospermine for 16 hrs followed by immunoprecipitation and Western blot analysis to test for a glycan-dependent interaction between calnexin and MOG-GFP. Calnexin-deficient cells (*cnx*$^{-/-}$) stably expressing MOG-GFP were used as a negative control. The arrow indicates the location of calnexin (CNX) and the asterisk marks a nonspecific band.

Expression of MOG in the absence of ER chaperones – Next, we examined if the expression of MOG is affected by the absence of quality control chaperones. Wild-type, *cnx*$^{-/-}$, *crt*$^{-/-}$ and *ERp57*$^{-/-}$ cells were transfected with an expression vector encoding MOG-GFP followed by Western blot analysis with anti-GFP and anti-MOG antibodies (Figure 3-4). Figures 3-4A and B show that expression of MOG was at comparable levels in chaperone-deficient and wild-type cells, indicating that expression of this myelin protein was not affected by the absence of ER chaperones. Calnexin-deficient mice are viable [37] and therefore permit analysis of MOG expression in mouse tissue. Figure 3-4C shows that similar to the analysis of cultured cells expressing MOG-GFP, the endogenous expression of MOG in wild-type and *cnx*$^{-/-}$ brain and cerebellum of the mouse were not altered. We concluded that the absence of ER chaperones calnexin, calreticulin and folding enzyme ERp57 did not have any effect on the expression of MOG.

Interestingly, even though calnexin interacts with MOG, we have observed that the absence of calnexin, calreticulin or ERp57, crucial ER chaperones, did not interfere with expression levels of the MOG in fibroblasts or in brain or cerebellum of calnexin-deficient mice. These results suggest that it is not the expression level of MOG in calnexin-deficient mice that contributes to changes in the myelin in the CNS [37].

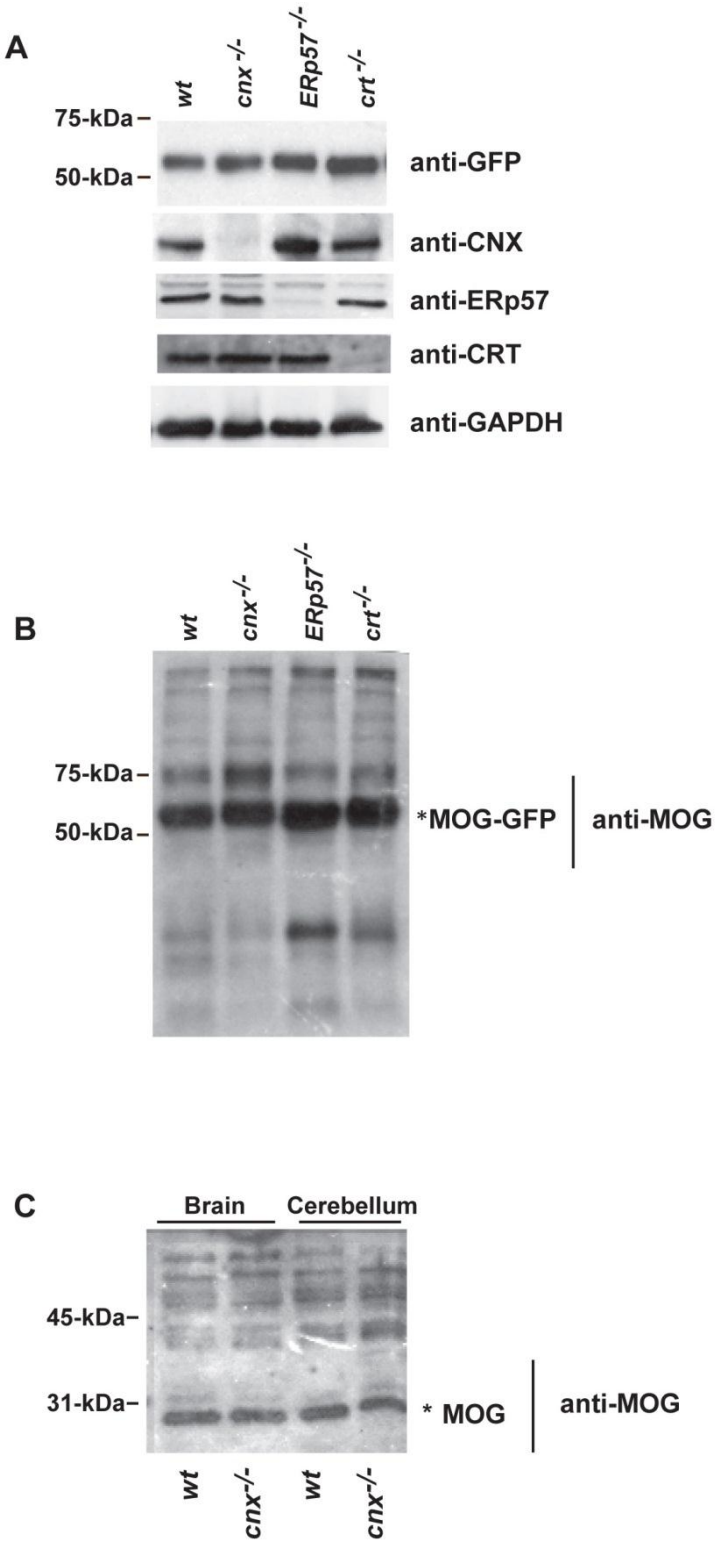

Figure 3-4. Expression of MOG-GFP in *wt* and chaperone-deficient cells - *A-B.* Wild-type (*wt*), calnexin-deficient (*cnx*$^{-/-}$), ERp57-deficient (*ERp57*$^{-/-}$) and calreticulin-deficient (*crt*$^{-/-}$) cells were transfected with expression vectors encoding MOG-GFP as described under "Material and Methods". Western blot analysis was carried out with antibodies as indicated in the Figure. Calreticulin (*CRT*), calnexin (*CNX*), green fluorescent protein (*GFP*) and glyceraldehyde phosphate dehydrogenase (*GAPDH*). **C.** Brain and cerebellum samples from wild-type (*wt*) and calnexin-deficient (*cnx*$^{-/-}$) mice were collected with RIPA buffer followed by Western blot analysis with anti-MOG antibodies. The asterisk indicates the location of MOG.

Plasma membrane localization of MOG in cells deficient in ER chaperones - For myelin proteins to perform their function, they must be localized to the plasma membrane of cells forming myelin sheaths. To test if MOG cell surface targeting was affected in the absence of ER chaperone, we expressed MOG-GFP in chaperone-deficient cells (*cnx*$^{-/-}$, *crt*$^{-/-}$ and *ERp57*$^{-/-}$) and examined the localization of MOG-GFP by confocal microscopy. Figure 3-5 shows that MOG was localized primarily to the plasma membrane in the absence of calnexin, calreticulin or ERp57. We did not observe significant co-localization of MOG with Concanavalin A, which was used as a marker of an ER-like compartment. The weak intracellular GFP signal likely corresponds to ER-localized MOG-GFP that is newly synthesized.

MOG contains a single N-linked glycosylation site at N^{31}. This post-translational modification has been identified to be important during antibody recognition of MOG upon EAE, since pathogenic antibodies recognize glycosylated form of this protein [21]. Calnexin, calreticulin and ERp57 play a key role in the folding of glycosylated proteins [42, 43]. Properly folded, mature plasma membrane-localized MOG contains a single N-glycosylation site within its extracellular domain, and the presence of mature sugar indicates plasma membrane localization. We asked whether the absence of ER quality control chaperones affects the glycosylation state of myelin proteins. The state of maturity of a specific glycoprotein can be deduced from its sensitivity to hydrolysis by specific glycosidases, Endo H or PNGaseF. Digestion with EndoH removes high-mannose, ER-localized, N-linked carbohydrates, whereas digestion with PNGaseF removes both high-mannose and complex carbohydrates. A fully matured glycoprotein will be modified with complex

oligosaccharides, making it resistant to cleavage by EndoH but not PNGaseF [44]. MOG-GFP expressed in wild-type and chaperone-deficient cells was highly sensitive to PNGaseF digestion, indicating the presence of complex, mature carbohydrate (Figure 3-6A). In contrast, MOG expressed in all the cell lines was resistant to EndoH digestion (Figure 3-6A). These results indicate that the absence of an essential ER chaperone did not result in changes to glycosylation of MOG. Furthermore, they support cell surface localization of MOG as revealed by confocal analysis (Figure 3-5). Combined, confocal analysis and glycosidase sensitivity studies demonstrate that the absence of calnexin, calreticulin or ERp57 did not interfere with the cell surface trafficking of MOG-GFP. This indicates that ER quality control components (calnexin, calreticulin and ERp57) did not affect expression and cell surface targeting of MOG.

A single disulfide bond is found in the extracellular domain of MOG between C^{24} and C^{98}, and the disulfide bond on MOG may be essential for its function as it is for myelin protein zero (P0) that contains a single disulfide bond in its Ig-like extracellular domain just like MOG [45]. ERp57 is the oxidoreductase that assists many calnexin substrates in disulfide bond formation and/or isomerization [36]. Therefore, we used SDS-PAGE under reducing and non-reducing conditions to test whether disulfide bond formation in MOG was affected in the absence of the oxidoreductase ERp57.

MOG, like other myelin proteins contains an Ig-like domain [14]. These proteins in order to perform their function must be localized to the plasma membrane of cells forming myelin sheaths. Results obtained with

confocal microscopy show that the absence of ER chaperones did not interfere with correct localization of MOG to plasma membrane.

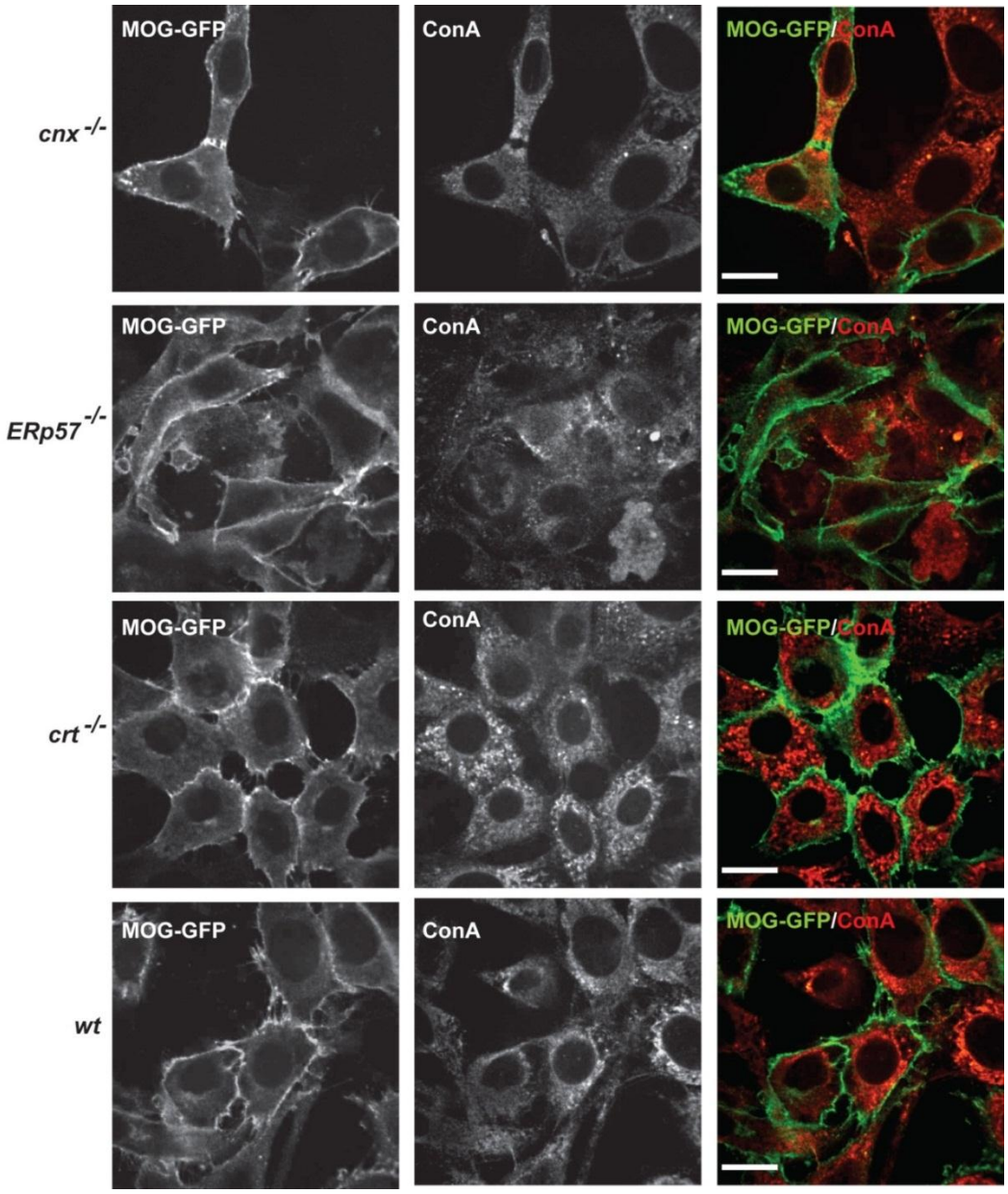

Figure 3-5. Expression and cell surface targeting of MOG-GFP in wild-type and chaperone-deficient cells - Confocal images of cell surface expression of MOG-GFP (green) in calnexin-deficient (*cnx*$^{-/-}$), ERp57-deficient (*ERp57*$^{-/-}$), calreticulin-deficient (*crt*$^{-/-}$) and wild-type (*wt)* cells. Concanavalin-A (red) staining was used as an ER marker. Scale bar=17 μm.

As expected, in the wild-type cells expressing MOG-GFP, the mobility of the protein in SDS-PAGE was affected under reducing and non-reducing conditions (Figure 3-6, *wt*), indicating that MOG-GFP expressed in wild-type cells formed a disulfide bond. Surprisingly, the SDS-PAGE mobility of MOG-GFP expressed in ERp57-deficient cells was also sensitive to reducing and non-reducingd environments, indicating that MOG formed a disulfide bond even in the absence of ERp57 oxidoreductase (Figure. 3-6, *ERp57*$^{-/-}$). This was a surprising result as disulfide bond formation of calnexin substrates in the ER is catalyzed by ERp57 [36]. MOG, like other myelin proteins, contains an Ig domain, and the disulfide bond is an important posttranslational modification of the protein affecting its stability and function [45, 46]. ERp57 has been extensively studied from the perspective of the folding of MHC I molecules, which belong to the same Ig family as MOG [25]. Our findings indicate that the absence of the ERp57 oxidoreductase did not result in the failure to form a disulfide bond in the extracellular domain of MOG, suggesting that disulfide bond formation occurs in the absence of this oxidoreductase ERp57. It is possible that other members of the PDI family of oxidoreductases compensate for the absence of ERp57 and promote disulfide bond formation for this calnexin substrate.

Many integral membrane proteins contain a disulfide bond that stabilizes their final structure. Its formation is a crucial element in protein maturation. The disulfide bond formation in the ER is catalyzed by members of the PDI family. One of them is ERp57, which is known to interact with calnexin and calreticulin and assist in maturation of their substrates [47]. ERp57 has been extensively studied from the perspective

of the folding of MHC I molecules; its role in folding and quality control of many other proteins has also been shown. Myelin protein zero that shares core secondary structural elements with MOG also contains a single disulfide bond in its extracellular domain [46]. It has been proposed that this disulfide bond is essential for myelin protein zero trafficking to plasma membrane [46] as well as for its function [45].

Attempts to immunoprecipitate complexes between ERp57 ER oxidoreductase and MOG failed. However, we have identified that the absence of this oxidoreductase did not result in the failure to form the disulfide bond in the extracellular domain of MOG (Figure 3-6). These data suggest that disulfide bond formation occurs in the absence of ERp57.

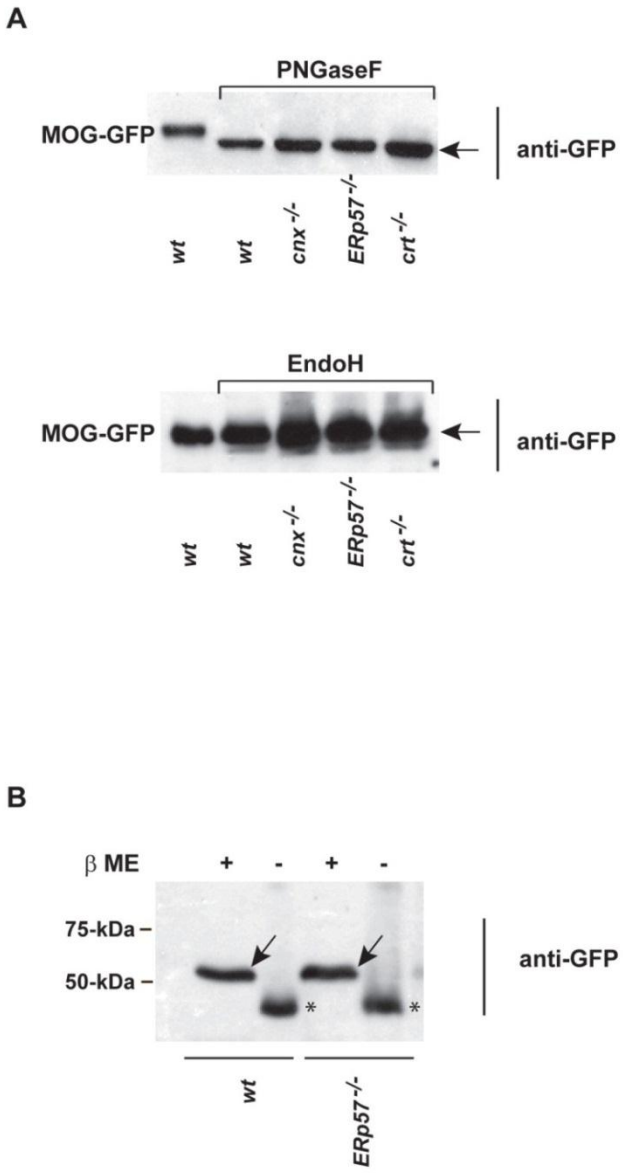

Figure 3-6. Glycosylation and disulfide bond formation in MOG expressed in wild-type and chaperone-deficient cells - *A.* A representative Western blot of endoglycosidase digestion of MOG-GFP expressed in wild-type (wt) and chaperone-deficient cell lines: calnexin-deficient (*cnx*$^{-/-}$), ERp57-deficient (*ERp57*$^{-/-}$) and calreticulin-deficient (*crt*$^{-/-}$). First lane represents untreated sample; *PNGaseF*, N-glycosidase-F digestion; *EndoH*, endoglycosidase H digestion. Blots were probed with anti-GFP antibodies. The arrows show the endoglycosidase-sensitive fraction of the protein. ***B.*** Western blot analysis of MOG-GFP expressed in wild-type (*wt*) or ERp57-deficient (*ERp57*$^{-/-}$) cells under reducing (+β ME) or non-reducing (-β ME) conditions. The location of reduced MOG-GFP is indicated by the arrows and non-reduced MOG-GFP by asterisk.

N-glycosylation of MOG – Glycosylation, as well as the native conformation of MOG are thought to be important for its encephalitogenicity [48]. High concentrations of anti-MOG antibodies are detected in sera of MS patients; however, the antibodies appear to recognize predominantly the glycosylated form of the protein [48, 49]. MOG has one glycosylation site and to further investigate the role of sugar in the biology of MOG, I have created a MOG mutant with substitution of N^{31} to A^{31} to prevent glycosylation of the protein. Mutant MOG was expressed in *wt* and chaperone-deficient cells. Wild-type, *cnx*$^{-/-}$, *crt*$^{-/-}$ and *ERp57*$^{-/-}$ cells were transfected with an expression vector encoding MOG(N^{31}A)GFP followed by Western blot analysis with anti-GFP and anti-MOG antibodies (Figure 3-7). Figure 3-7 shows that the MOG(N^{31}A)GFP protein has a faster mobility in SDS-PAGE when compared to the wtMOG-GFP protein due to the absence of glycan (Figure 3-7A).

Properly folded mature plasma membrane-localized MOG contains a single N-glycosylation site within its extracellular domain. The presence of mature sugar indicates plasma membrane localization. Here, using endoglycosidases, we show that the absence of essential ER chaperones did not result in changes to glycosylation of MOG. These results are the confirmation of plasma membrane localization that we observe with confocal microscope. The single N-glycan has also been suggested to be important for encephalitogenicity of MOG [21]. It has been previously presented that antibody to human MOG binds to the glycosylated form of MOG [21]. Our data show that the absence of crucial ER chaperones has no effect on N-glycosylation of MOG.

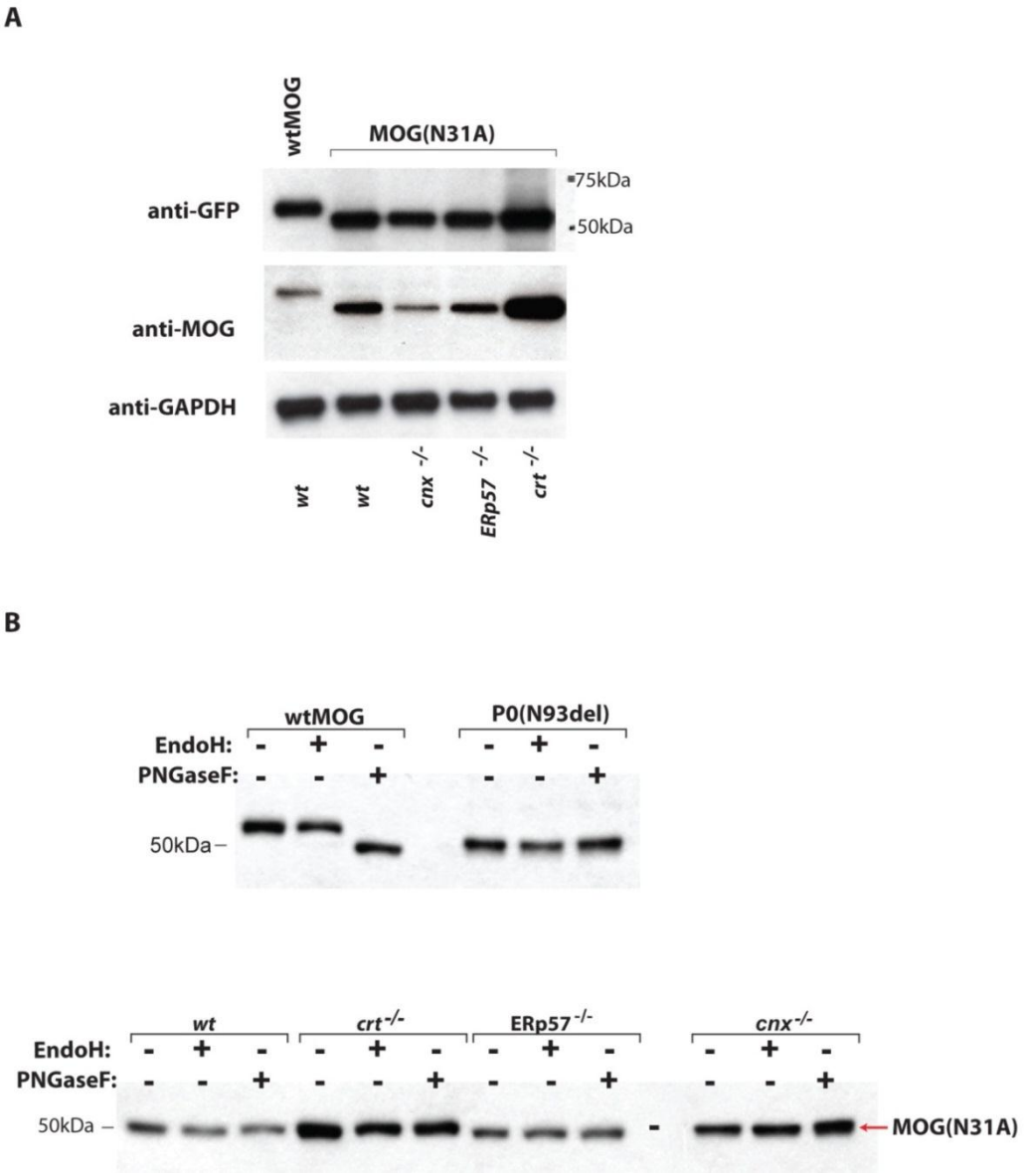

Figure 3-7 Expression of MOG(N^{31}A) mutant in *wt* and chaperone-deficient cells – A. *wt*, *cnx*$^{-/-}$, *ERp57*$^{-/-}$ and *crt*$^{-/-}$ cells were transfected with expression vectors encoding MOG(N^{31}A)GFP as described under "Material and Methods". Western blot analysis was carried out with anti-GFP antibodies, commercially available anti-MOG antibodies as well as with anti-*GAPDH* antibodies. **B**. Endoglycosidase digestion of MOG-GFP and P0(N^{93}del)GFP (top panel) used as positive and negative control, respectively. The lower panel shows endoglycosidase digestion of MOG(N^{31}A)GFP expressed in *wt* or chaperone-deficient cell lines *cnx*$^{-/-}$, *crt*$^{-/-}$ and *ERp57*$^{-/-}$. Immunoblots of cell lysates were treated with EndoH or PNGaseF.

First, we examined if the expression of MOG(N^{31}A)GFP was affected by the absence of quality control chaperones. Figures 3-7A shows that all cells express MOG(N^{31}A)GFP. Interestingly, *crt*$^{-/-}$ cells expressed the MOG mutant protein at higher levels when compared to the *wt* cells, which was surprising since *crt*$^{-/-}$-deficient cells have increased ubiquitin-proteasome activity [50]. In contrast, in *cnx*$^{-/-}$ cells, the expression of MOG(N^{31}A)GFP was decreased, which might be the effect of enhanced proteasomal degradation of the misfolded protein. In conclusion, unlike the expression of wtMOG-GFP, which was at comparable levels in chaperone-deficient and wild-type cells, expression of the mutant protein lacking the glycan was influenced by the absence of the ER chaperones.

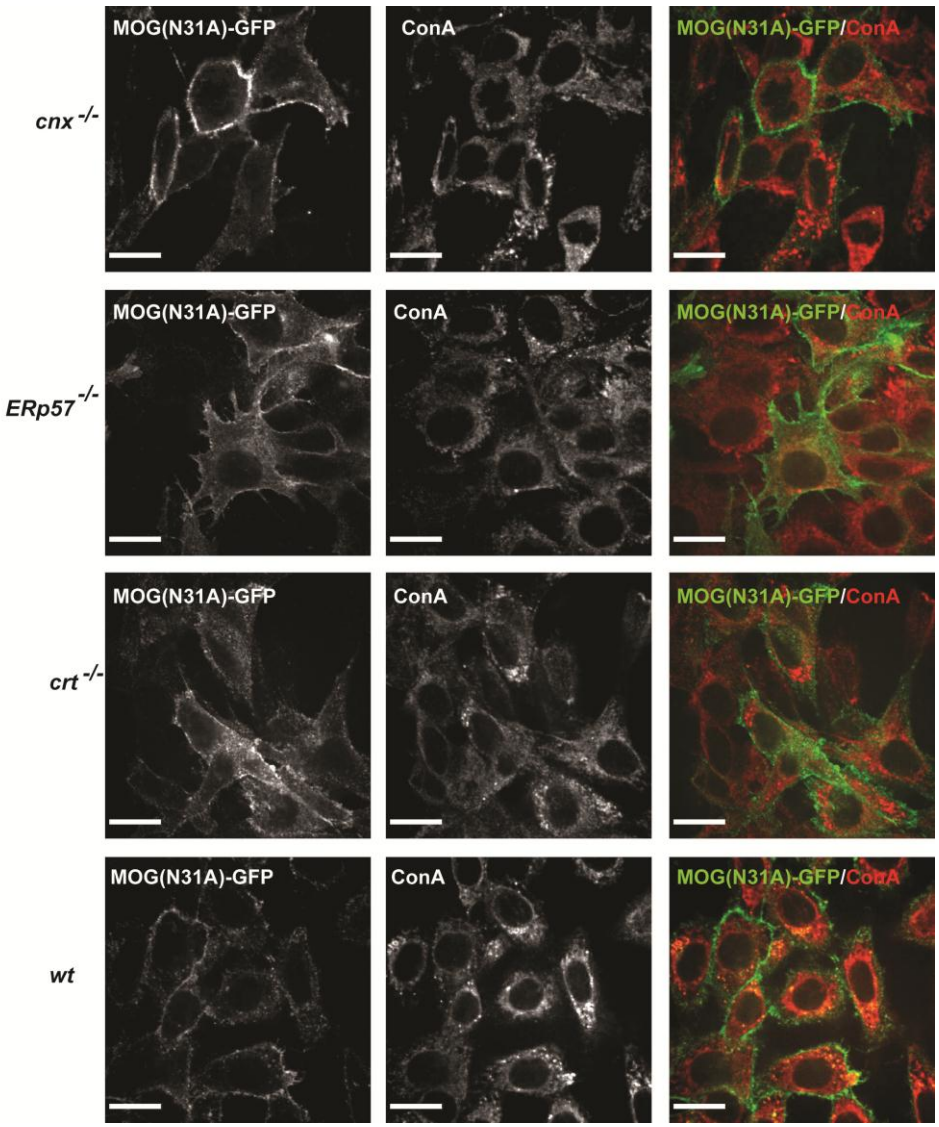

Figure 3-8. Expression and cell surface targeting of MOG(N^{31}A)GFP in wild-type and chaperone-deficient cells - Confocal images of cell surface expression of MOG(N^{31}A)GFP (green) in calnexin-deficient (*cnx*$^{-/-}$), ERp57-deficient (*ERp57*$^{-/-}$), calreticulin-deficient (*crt*$^{-/-}$) and wild-type (*wt)* cells. Concanavalin-A (red) staining was used as an ER marker. Scale bar=17 μm.

Next, to confirm the absence of sugar on MOG(N^{31}A)GFP, we performed a digestion experiment with the specific glycosidases EndoH or PNGaseF (Figure 3-7B); digestion with EndoH removes high-mannose sugar, a carbohydrate characteristic to ER-localized proteins, whereas digestion with PNGaseF removes both high mannose and complexed carbohydrates found in the Golgi and the plasma membrane. Figure 3-7B shows digestion of a positive (glycosylated) control (wtMOG-GFP) and a negative control P0(N^{93}del)GFP, which is fully resistant to glycosidase digestion as described in Chapter 2. As predicted MOG(N^{31}A)GFP expressed in wild-type and chaperone-deficient cells was strongly resistant to both EndoH and PNGaseF digestion, indicating the lack of glycan on the mutated protein (Figure 3-7B lower panel).

We used confocal microscopy to investigate the intracellular localization of MOG(N^{31}A)GFP in cells deficient in ER quality control chaperones. Figure 3-8 shows that MOG(N^{31}A)GFP was primarily localized to the plasma membrane in the absence of calnexin, calreticulin or ERp57. We concluded that the absence of calnexin, calreticulin and ERp57 did not affect cell surface targeting of MOG(N^{31}A)GFP.

Cell surface localization of MOG(N^{31}A)GFP was further investigated using a membrane-impermeant biotin that labels surface proteins. Wild-type and chaperone-deficient cell lines expressing MOG(N^{31}A)GFP were labeled with sulfo-NHS-biotin followed by isolation of the biotinylated proteins using streptavidin-affinity chromatography [33]. Total cellular proteins (Figure 3-9 lane *t*), streptavidin unbound (Figure 3-9 lane *ub*) and streptavidin bound (Figure 3-9 lane *b*) proteins were separated by SDS-PAGE followed by Western blot analysis with

anti-GFP antibodies to identify in which fraction MOG($N^{31}A$)GFP was present. Western blot analysis of GAPDH, a cytoplasmic protein was used as an internal control (Figure 3-9), indicating the cell surface biotinylation protocol identified cell surface proteins only. Figure 3-9 shows that the levels of cell surface, biotinylated MOG($N^{31}A$)GFP in wild-type and chaperone-deficient cells were relatively similar to the cell surface-localized wtMOG-GFP (Figure 3-9A, left panel).

Combined, our results confirmed the lack of glycan on MOG($N^{31}A$)GFP. Interestingly, the absence of N-linked glycan did not interfere with cell surface targeting of the MOG($N^{31}A$)GFP as shown by confocal images and cell surface biotinylation assay and it did not result in increased intracellular retention of the mutant protein.

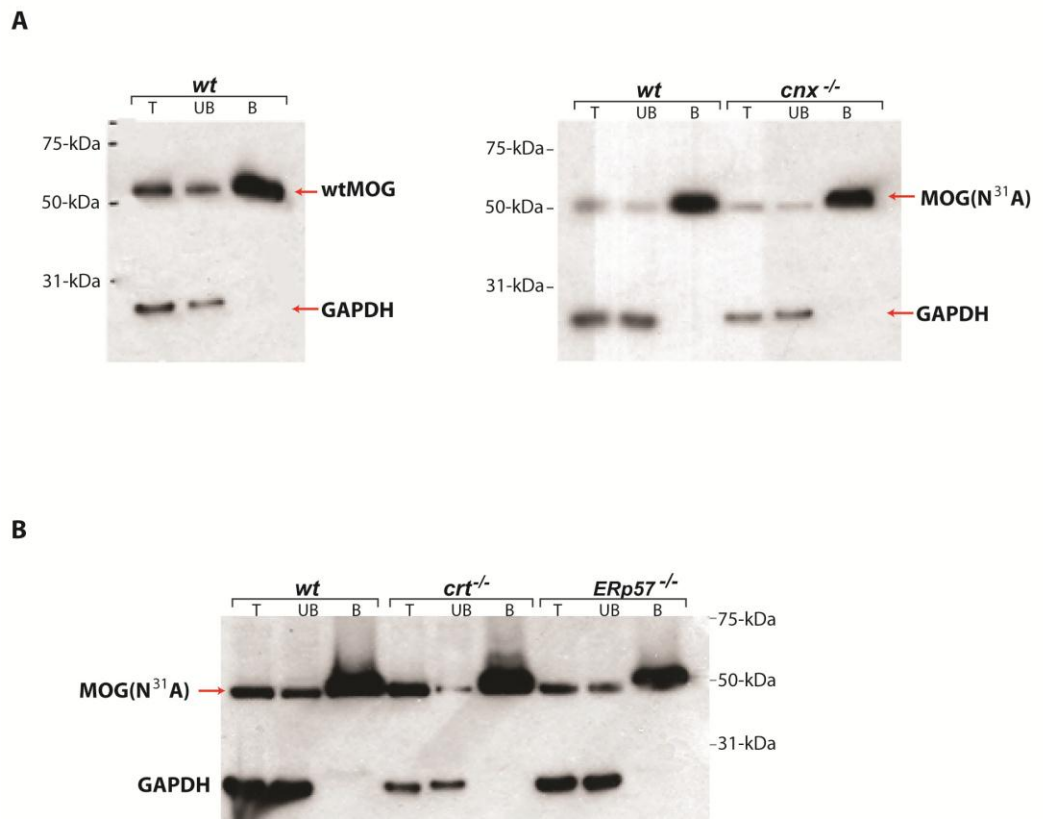

Figure 3-9 Cell surface targeting of MOG-GFP and MOG(N^{31}A)GFP in wild-type and chaperone-deficient cells. Cell surface biotinylation of MOG-GFP, MOG(N^{31}A)GFP expressed in wild-type (*wt*), calreticulin (*crt*$^{-/-}$), calnexin- (*cnx*$^{-/-}$) and ERp57-deficient (*ERp57*$^{-/-}$) cell lines was carried out as described under "Materials and Methods". Western blots were probed with anti-GFP or anti-GAPDH antibodies. *Lanes t*, total cellular extracts; *lanes ub*, material not bound to the streptavidin affinity beads; *lanes b*, streptavidin bound, biotinylated fractions.

Figure 3-3 shows that calnexin interacts with wtMOG in the presence of castanospermine, indicating that the interaction was glycan-independent. This was further confirmed by analysis of MOG($N^{31}A$)GFP. Figure 3-10 shows MOG($N^{31}A$)GFP formed immunoprecipitable complexes with calnexin, confirming previous results that interaction between calnexin and MOG were glycan-independent.

Our results show for the first time that the N-glycosylation of MOG is not essential for its intracellular trafficking. MOG is a crucial target of MS; the antibodies against MOG are found in sera of patients. Moreover, it has been suggested that pathogenic antibodies bind to glycosylated MOG [21]. Therefore, an important question should be asked: would manipulation of N-glycosylation of MOG result in properly localized protein with decreased encephalitogenic properties?

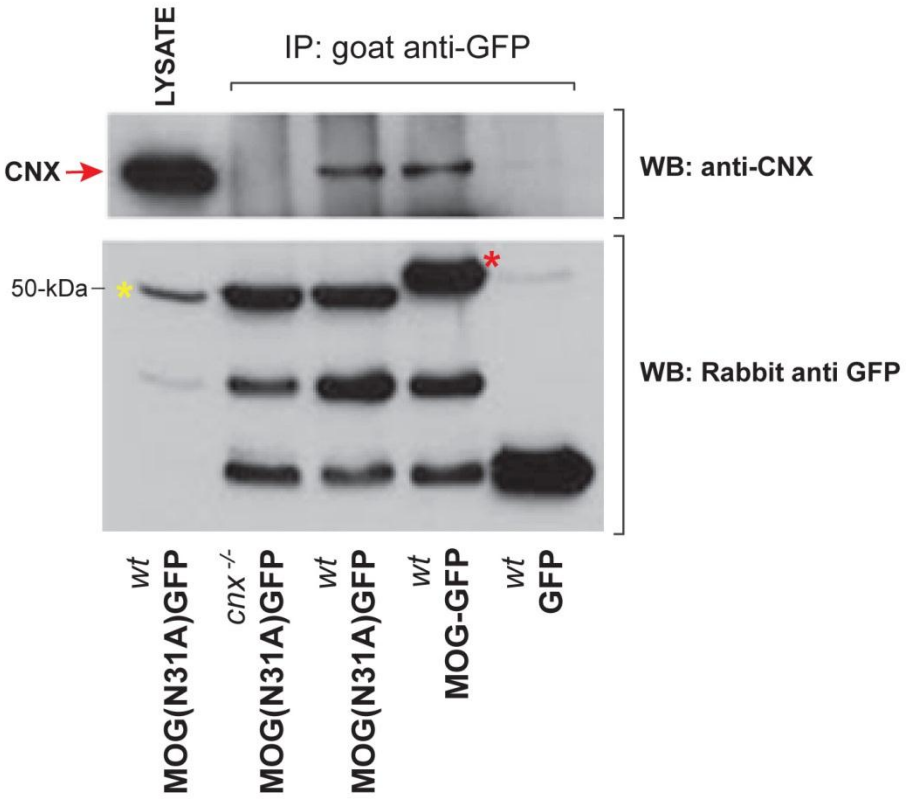

Figure 3-10 Interaction between calnexin and MOG(N^{31}A)GFP. Cell lysates from *wt* cells expressing MOG(N^{31}A)GFP, MOG-GFP or GFP were collected, as well as lysate from calnexin-deficient cells (*cnx*$^{-/-}$) that was used as a control lacking calnexin protein. Next, goat anti-GFP antibodies were added as indicated in the Figure followed by the addition of streptavidin beads and as described under "Materials and Methods". Proteins were than separated by SDS-PAGE followed by Western blot analysis with anti-calnexin (CNX) or rabbit anti-GFP antibodies. The red arrow indicates the size of the immunoprecipitated calnexin. The yellow asterisk indicates MOG(N^{31}A)GFP in the lysate from *wt* cells before the addition of the antibody. The red asterisk indicates the size of the immunoprecipitated wtMOG with anti-GFP antibodies from the *wt* cells.

The UPR in the absence of ER chaperones - Accumulation of misfolded proteins in the ER might result in the activation of the UPR and may lead to pathogenic consequences [51]. To test if expression of MOG in cells deficient in ER chaperones activated the UPR, we used a dual luciferase system to measure the UPR [29, 34]. Thapsigargin induces a high level of ER stress, and was used in these experiments as a positive control (Figure 3-11). Figure 3-11 shows that expression of MOG in the absence of ER chaperones did not result in activation of the UPR and confirmed that MOG was not accumulating in the ER in the absence of the ER quality control chaperones calnexin, calreticulin or ERp57. Although indirectly, these results support our conclusions that MOG-GFP was efficiently targeted to the cell surface in the absence of calnexin, calreticulin or ERp57. High levels of ER stress and induction of UPR signaling can lead to apoptosis [52] and pathological phenotypes. Interestingly, ER stress-related pathways are activated when EAE antibodies bind to MOG [53] and may have a protective effect against EAE-induced oligodendrocyte death and demyelination [54]. The ER stress may therefore have both beneficial and detrimental effects on immune-mediated demyelination and consequently, on the pathology of multiple sclerosis. We show here that MOG is not accumulated in the ER in the absence of ER-associated chaperones and it does not activate ER stress. A more detailed analysis and understanding of the biology of MOG, ER chaperones and ER stress could be critical when considering potential therapeutic approaches for myelin disorders.

When protein folding homeostasis in the ER is disrupted, the UPR is activated. Accumulation of misfolded proteins in the ER results in activation of one or more of the branches of the UPR. Our data show that

when MOG is expressed in the absence of calnexin, calreticulin or ERp57 its intracellular trafficking to plasma membrane is not disturbed and we do not observe accumulation of misfolded proteins in the ER. I confirmed this observation by measuring UPR in cells expressing MOG in the absence of ER chaperones. Here, we show that expression of MOG in the absence of ER chaperones did not result in the induction of the UPR.

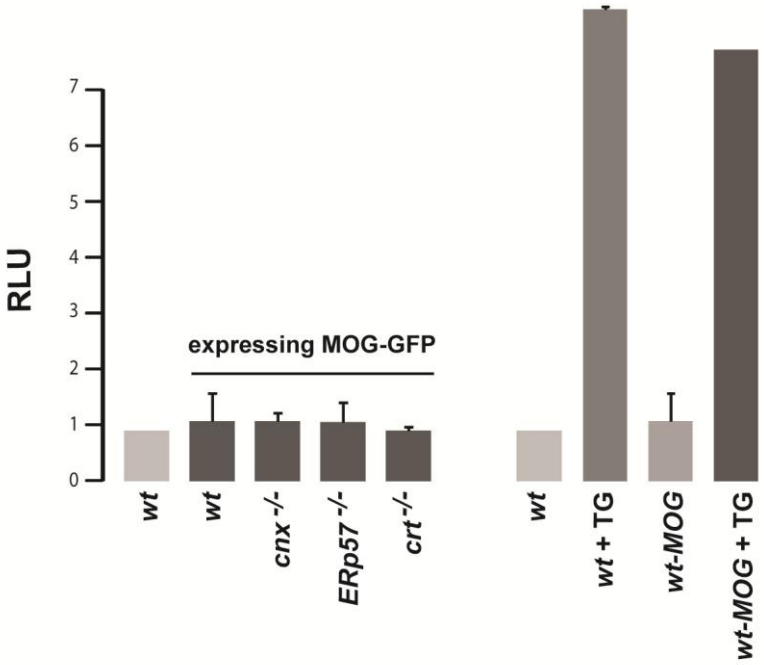

Figure 3-11. Induction of the unfolded protein response in chaperone-deficient cells expressing MOG - Wild-type (*wt*), *cnx*$^{-/-}$, *ERp57*$^{-/-}$; and wild-type, *cnx*$^{-/-}$, *ERp57*$^{-/-}$ stably expressing MOG-GFP were transfected with pRL-XFL vector encoding *Renilla* luciferase and firefly luciferase reporter genes as described previously [29, 34]. As a positive control, cells were treated with 1 μM thapsigargin (TG) for 16 hrs. Cell extracts were collected and assayed for luciferase activity with the Dual-Luciferase Assay Kit (Promega) using a luminometer (Berthold-Lumat LB 9501) [29, 34]. Relative light units (RLU) were normalized to internal control. Average values ± SD (n=3).

Conclusions

Several results suggest the role of MOG as an adhesion molecule involved in the formation and maintenance of myelin sheath [12, 14]. Our preliminary data using the adhesion assay support this notion and show that MOG functions as an adhesion molecule. Most importantly, I discovered that in the absence of calnexin or ERp57, MOG adhesive properties are compromised.

MS is a chronic inflammatory disease of the CNS often affecting young adults. The etiology of the disease is still largely unknown, but there is strong evidence that this is a multifactorial autoimmune disease. Despite many efforts to identify major genes involved in disease susceptibility, not many genes have been identified to be involved. However, the gene encoding MOG has been identified in genome screens of MS families. However, association between *MOG* gene polymorphisms and susceptibility to MS are inconsistent and inconclusive [55]. Polymorphisms or mutations in the *MOG* gene contribute to development and progression of MS by diverse mechanisms [55], including loss of function that may lead to myelin instability and increased sensitivity to immune attack. However, MOG contribution to MS pathology is not fully understood. We have identified a chaperone for MOG that potentially can influence its folding. Our data show a link between MOG and the ER, an organelle of protein synthesis. This work may lead to a better understanding of the physiological function of this protein and its role in human pathology. Changes in conformation may expose different peptides and buried ones and therefore change encephalitogenic properties of MOG.

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Chapter Four

General Discussion

The objective of my thesis was to determine the role of the ER chaperones, key components of the quality control system in the biology of major glycoproteins of peripheral (PNS) and central (CNS) nervous system myelin. Major findings from my work include:

- A deficiency in the ER molecular chaperones does not interfere with cellular localization of myelin glycoproteins.
- Calnexin transiently interacts with P0, the most abundant glycoprotein of the PNS.
- Interactions between calnexin and P0 are glycan-independent.
- Calnexin and ERp57 are essential for the folding and function of myelin glycoprotein P0.
- Calnexin is essential for the folding and function of PMP22.
- Calnexin associates with MOG, a CNS myelin glycoprotein.
- N-linked glycan is not essential for the interaction between calnexin and MOG.
- A deficiency in the ER molecular chaperones does not affect plasma membrane localization or the glycosylation pattern of MOG.
- N-glycan-deficient MOG localizes to the plasma membrane in wild-type and chaperone deficient cells.
- Expression of MOG in the absence of the ER molecular chaperones does not result in activation of the UPR.

I investigated major glycoproteins of the PNS and CNS to try to reveal a molecular explanation of the neuropathy-like phenotype of the calnexin-deficient mouse model, as well as to provide insights into the biology of these proteins. The involvement of the ER molecular chaperones in the cellular trafficking, folding and function of P0, PMP22

and MOG were determined. These studies contributed significant knowledge about the players involved in the maturation of myelin glycoproteins and provided likely molecular explanations for the phenotype of the calnexin-deficient mouse by providing important insights into the biology of two major glycoproteins of peripheral nervous system myelin.

Previous results from our laboratory showed that calnexin is highly expressed early on in mouse embryonic development in cartilage and within neuronal tissues, with later expression in the liver and little to no expression observed in the heart [1]. This suggested that calnexin expression may be restricted to specific tissues, especially the nervous system during embryonic development [1]. Therefore, it was not surprising that calnexin deficiency had an impact on the nervous system [2]. The calnexin-deficient mouse model is viable but displays a severely ataxic phenotype [2]. These mice are 30-50% smaller than their wild-type littermates and exhibit severe gait disturbances [2, 3]. Electron micrographs show severe dysmyelination of the spinal cord and sciatic nerve, resulting in reduced nerve conduction velocity. The result showed that calnexin deficiency led to a peripheral neuropathy [3]. We observed that peripheral nervous system abnormalities displayed in calnexin-deficient mice are similar to the symptoms exhibited by patients with hereditary neuropathies.

Inherited neuropathies are the most common inherited diseases of the nervous system. They affect approximately 1 in 2,500 people [4, 5]. They have been classified into several categories based on clinical, neurophysiological and neuropathological features. Charcot-Marie-Tooth (CMT) disease belongs to a group of hereditary motor and sensory neuropathies (HMSNs), and it was first characterized as a slowly progressive syndrome that begins in childhood [6]. Clinical CMTs can have an early childhood or early adulthood onset; the symptoms, among others, include sensory loss, distal weakness and absent reflexes. However, diverse disease severities and onset have been described among patients. Recent discoveries in molecular genetics allowed for the division

of CMT neuropathies into three main subgroups related to genetic, clinical and neuropathological criteria [7]. Patients are diagnosed with CMT1 if carrying an autosomal dominant demyelinating neuropathy. CMT2 is primarily related to axonal disorders. CMT4 neuropathy is recessive and CMTX is related to X-chromosome-linked neuropathy [8]. CMT1 can be further divided into several subgroups based on genetic factors.

Most patients diagnosed with CMT1 have a benign disease course with a slowly progressive muscle weakness and sensory loss. However, some patients develop a much more severe phenotype. Factors that account for the variability of symptoms are not well characterized.

CMT1A is related to genetic abnormalities within the *pmp22* gene and it is the most common type of CMT. In approximately 60% of patients diagnosed with CMT1A, there is a 1.5 megabase duplication of the gene encoding PMP22 protein [9]. This duplication results in overexpressed PMP22 protein in nerves of CMT1A patients [8]. It has been suggested the overexpression of PMP22 might affect the physiology of Schwann cells. Moreover, many point mutations within the *pmp22* gene have been identified to cause CMT1A. PMP22 is a relatively minor (2-5%) component of the myelin, surrounding large axons in the PNS. PMP22 plays a role as an adhesive molecule in the formation of myelin sheaths, as well as in the communication between Schwann cells and the extracellular matrix [10, 11]. PMP22 is essential for proper myelination and maintenance of peripheral myelin. Results obtained from experiments with transgenic mice have established that alterations within the *pmp22* gene are a direct cause of CMT1A. However, the relationship between genetic abnormalities within the *pmp22* gene and the severity of the disease phenotype remains largely unknown.

CMT1B accounts for a much smaller percent of CMT1 cases, and is related to various mutations within the *P0* gene. P0 is the most abundant protein component of the PNS myelin and it is involved in formation of compact myelin sheaths in the PNS. Patients diagnosed with CMT1B show very diverse severities of disease syndromes, from very severe, early onset to benign disease occurrence in late adulthood [12]. The molecular mechanisms underlying this diversity within CMT1B are unclear. The type and location of the point mutation within the *P0* gene are factors that determine the severity of the disease.

Although, detailed molecular mechanisms contributing to symptoms and severity of CMT1 remain unclear, general mechanisms underlying the pathogenicity of this syndrome involve the intracellular accumulation of mutant protein within the ER and cytosol (PMP22 in patients diagnosed with CMT1A, P0 in patients diagnosed with CMT1B). This ER retention leads to the diverse toxic effects. There is significant experimental evidence that ER and ER molecular chaperones are critical modulators of mechanisms underlying the pathogenesis of neuropathies.

The complexity of hereditary neuropathies originates from the mechanism that different mutations can cause different phenotypes. My results identified the molecular chaperones calnexin and ERp57, to be essential for the production of functional, properly folded, wild-type P0 and PMP22 (calnexin only). Therefore, they provide new potential targets for therapeutic approaches in the treatment of peripheral neuropathies.

Calnexin Interacts With Peripheral Myelin Glycoproteins

Work by several research groups demonstrates that PMP22 transiently associates with calnexin during its folding in the ER [13, 14]. Moreover, it has been shown that the interactions between wild-type PMP22 and calnexin are mediated through the N-linked glycan at position N^{41} [14]. Interestingly, the $N^{41}Q$ mutant lacking the N-linked glycan is not retained in the ER and is correctly localized to the plasma membrane [15], suggesting that N-linked glycan is not essential for plasma membrane trafficking of PMP22 and that it does not result in intracellular retention [15]. In contrast, calnexin interacts with the $L^{16}P$ mutant of PMP22 in a glycan-independent manner and contributes to its intracellular retention [13]. Moreover, the first transmembrane segment of the wild-type or mutant PMP22 proteins have been identified to be involved in the glycan-independent interactions with calnexin [13].

Glycan-independent interaction between calnexin and its glycosylated substrates are quite common. Another myelin protein that has been identified as a calnexin substrate is non-glycosylated PLP, which is involved in myelin formation in the CNS. As with PMP22, calnexin associates with mutant PLP through its transmembrane segment [16]. Neurotransmitter GAT1 (γ-aminobutyric acid transporter 1) is another example of a protein that interacts with calnexin in a glycan-independent manner. Moreover, as with PMP22 and PLP, calnexin is involved in ER retention of GAT1 mutant proteins by interaction with their transmembrane domains [17].

My studies revealed that P0 is a new calnexin substrate. Figures 2-10 and 2-12 show that calnexin transiently interacts with P0. This is the first report of the interactions between these two proteins. Moreover, this

study also showed that the presence of glycan at position N^{93} is not essential for the interaction between calnexin and P0 (Figure 2-12).

Additionally, in this study, I have identified that the folding and function of P0 and PMP22 are specifically dependent on calnexin, but not on calreticulin, a structural homolog of calnexin. Although calnexin and calreticulin are homologous lectin chaperones that both reside in the lumen of ER, the substrate specificity of each chaperone has been well described [18]. The difference in substrate specificity between calnexin and calreticulin may be related to one lectin being soluble (calreticulin) and the other being membrane bound (calnexin) [19]. In fact, when the soluble N-terminal domain of calnexin is expressed alone, the pattern of substrate binding is very similar to that of calreticulin; however, soluble calnexin cannot replace calreticulin in all cases [19-21]. Moreover, although calnexin and calreticulin may bind the same substrate, they often bind different oligosaccharides or glycans at different stages of the maturation process [19]. Another possible explanation is that calnexin's transmembrane domain mediates its substrate specificity, either by promoting association of calnexin with membrane proteins by placing it at the ER membrane or directly binding the transmembrane segments of substrate proteins [22].

Plasma membrane localization of P0 and PMP22 in the absence of calnexin

I have identified that calnexin but not calreticulin is essential for the folding of PMP22 and P0 (Figure 2-8). The increased trypsin sensitivity of cell surface-localized P0 and PMP22 in cells deficient in calnexin is strong evidence for the crucial role of the ER chaperone in the process of maturation of myelin proteins. Misfolded cell surface P0 and PMP22

might be a result of premature release from the ER in the absence of calnexin. Although, how misfolded P0, PMP22 and possibly other substrate proteins escape rapid degradation in cells deficient in calnexin remains unclear. Despite the increased activation of the UPR observed in the calnexin-deficient cells, there is no increase in overall cell death, which would suggest that there is no significant accumulation of misfolded proteins that could lead to apoptosis [23].Some cases of chronic stress (for example, various neurodegenerative disorders caused by protein aggregation) can lead to the development of a cellular mechanism that helps to alleviate cellular stress and minimize cell death [24]. It appears that calnexin-deficient cells have evolved a mechanism that allows them to avoid accumulation of misfolded proteins in the ER. This mechanism might involve the accelerated exit of proteins from the ER. Moreover, calnexin-deficient cells have increased proteasomal activity, an indication of increased ER-associated degradation (ERAD). It is possible that constitutively up-regulated ERAD is a mechanism developed by calnexin-deficient cells to deal with misfolded proteins in the ER.

My results show that plasma membrane localized P0 and PMP22 are at comparable levels in wild-type and calnexin-deficient cells (Figure 2-5A, B). The key question is: what are the molecular mechanisms that select which misfolded substrate is degraded and which is secreted? Both processes of protein secretion and of degradation might be affected by a number of factors, and may compete for specific substrates [25, 26]. Lately, a new adaptable model for protein transport has been proposed. In this model, mutated proteins are degraded by ERAD if they are less stable than wild-type and sent for secretion if they are more stable than wild-type [27]. Protein secretion and degradation are complex processes that

closely dependent on each other. Calnexin deficiency results in incorrectly folded calnexin substrates that might not be efficiently recognized by the ERAD machinery, manage to escape quality control and proceed to their correct cellular localization.

The Functions of PMP22 and P0 depend on Calnexin

Dysmyelination is a consequence of the instability of myelin sheaths and a hallmark of inherited neuropathies. Both major PNS myelin glycoproteins P0 and PMP22 are engaged in homophilic and possibly heterophilic interactions, which are critical for proper myelination. Thus, neuropathy-causing mutations in P0 and PMP22 often impair their adhesion properties and, consequently myelin compaction. Mutations in P0 and PMP22 can be clustered into two groups. First, mutations that cause very severe structure disruptions result in ER retention of the mutant protein and failure to reach the plasma membrane. These mutations are related to severe, early onset neuropathies. The second group is the mutations that do not affect plasma membrane trafficking, are incorporated into myelin, but disrupt its structure. These mutations are the cause of late-onset disease, often characterized by mild demyelination. Studies involving cell lines expressing mutants that reach the plasma membrane but lead to packing defects of the myelin showed that their adhesive properties are severely impaired [28]. Interestingly, expression of wild-type P0 or PMP22 in cells deficient in calnexin had a similar result as expression of various mutants of these proteins in wild-type cells. As with mutants producing late onset neuropathies, wild-type P0 and PMP22 expressed in the absence of calnexin are correctly localized to the plasma membrane but cannot perform their function. These observations provide

a molecular explanation for peripheral neuropathy in the calnexin-deficient mouse model. Mutants of P0 that exhibit a similar phenotype to the one observed in calnexin-deficient cells include: $I^{130}T$, $S^{44}F$, $S^{63}F$, $D^{75}V$, $T^{142}M$, $R^{227}S$ [29]. Missense mutations in PMP22 that lead to a similar phenotype, include $V^{30}M$, $N^{41}A$ and $G^{93}R$; however, most known PMP22 mutants are retained in the ER [30].

Treatments with various chemical chaperones have been shown to improve folding and trafficking of some misfolded glycoproteins [31]. It would be important to test whether chemical chaperones would be able to rescue P0's function when expressed in the absence of calnexin. In order to investigate the potential effects of chemical chaperones in folding and function of P0, we could use sodium 4-phenylbutyrate (PBA), a compound previously identified to restore secretion of mutant myocilin protein from the ER [32]. PBA has been shown to reduce the amount of myocilin aggregates by diminishing its interaction with calreticulin [32]. Using the assays previously described in this study (adhesion assay used to measure function of P0), we could assess the potential role of PBA in enhancing the folding of myelin glycoprotein in calnexin-deficient cells lines. By growing calnexin-deficient cells stably expressing P0 in the presence of PBA, we could test its effect on P0 folding by performing the adhesion assay used in our previous studies. PBA acts on various components in the ER. Moreover, it might have an effect on P0 stability and a direct effect on the folding state of the mutant protein. It would be important to see if it would improve the folding state of P0 protein expressed in the absence of calnexin. These studies would allow us to identify potential chemical chaperones that could be used to improve the function of P0 in patients with CMT1B.

ERp57 and P0

Disulfide bond formation in the ER is an essential part of the maturation process for many proteins that enter the secretory pathway. This process involves the oxidation of the cysteines catalyzed by the group of ER residents that belong to the PDI family. ERp57 is one of the oxidoreductases in the ER with specificity towards glycoproteins [33]. ERp57 is a part of the calnexin-calreticulin cycle and catalyzes disulfide bond formation in many of the substrate glycoproteins that enter the cycle. My work showed that ERp57 is critical for folding and function of P0, which contains a single disulfide bond within its extracellular domain. I was not able to detect complexes between ERp57 and P0, which would indicate that there is no direct interaction between these two proteins. However, calnexin's interaction with P0 may bring it in close proximity with ERp57 for the oxidoreductase to function.

Similarl to the absence of calnexin, deficiency in ERp57 resulted in expression of misfolded P0 (showed by increased sensitivity of P0 to trypsin digestion, Figure 2-8) that correctly localized to the plasma membrane. The adhesion assay revealed that P0 expressed in the absence of ERp57 has severely impaired function. Interestingly, I found that even in the absence of ERp57, the disulfide bond in P0 was still present (Figure 2-9), suggesting that other members of the PDI family might compensate for the absence of ERp57. It has been previously reported that ERp72 can catalyze the formation of a disulfide bond within SFV (Semliki Forest Virus) E1 and p62 proteins, a known ERp57 substrates, in the cells deficient in ERp57 [34]. This result indicates that ERp57 might share substrate specificity with another oxidoreductase, potentially ERp72.

Although, I observed the correct formation of a disulfide bond in P0, its folding and function were impaired, suggesting that ERp57 serves a specialized role for maturation of P0 but not for disulfide bond formation. A similar observation was previously reported for MHC class I, where ERp57 had a significant effect in maturation of MHC class I molecules that was not shared with ERp72 [35].

My studies identified ERp57 as an essential component in the folding and subsequent function of P0. I believe that differential roles of ERp57 in the folding and function of P0 and PMP22 are related to the presence of the disulfide bond in the P0 extracellular domain.

Unfolded Protein Responses and Neuropathy

The ER is the primary protein folding compartment of the cell and, as such, during cell growth, differentiation and in response to environmental stimuli, it has to deal with various protein folding demands [36]. The ER has developed efficient mechanisms to enhance protein folding in response to developmental and various physiological requirements [36]. However, if the ER continues to be overwhelmed by the protein load, there is activation of the UPR (Figure 1-6) [37]. Experimental evidence suggests that long-lasting ER stress results in prolonged activation of UPR and this can contribute to the pathogenesis of many diseases [36]. Activation of apoptotic pathways are an integral part of the UPR and the main mediators include IRE1, caspase-12 and PERK/CHOP [36]. It has been reported that the UPR is active in the absence of calnexin and the absence of ERp57 [1, 38]. However, calnexin deficiency does not lead to increased ER-stress induced apoptosis [23].

There are two types of ER stress: (1) acute stress that is transient in nature, such as chemical insult and (2) chronic stress that is long lasting and could be the result of a genetic mutation to a component of the quality control pathway [24]. Acute stress requires cells to tolerate a short period of perturbation that is followed by recovery while chronic stress requires long term adjustments to cellular function to restore ER homeostasis [24]. Calnexin-deficiency accounts for a chronic stress, where a component of the quality control pathway is absent. Calnexin-deficient cells survive in cell culture and show no significant increase in cell death, suggesting that these cells have evolved an adaptive response that allows them to survive and avoid apoptosis [1, 23, 24]. Interestingly, calnexin-deficient cells have increased proteasomal activity, suggesting an increase in ER-associated degradation ERAD [1].

As with calnexin-deficient cells, ERp57-deficient cells also showed an increase in the UPR that did not result in the activation of the apoptosis pathway. ERp57-deficient cells, like the calnexin-deficient cells might have adapted to chronic, prolonged stress. These results are similar to the deficiency in UGGT1, another component of the quality control pathway, which has evolved an adaptive response to a chronic stress [39].

A large group of mutations in P0 and PMP22 result in ER retention of a misfolded protein. For some of them, like the S^{34}del mutation in the extracellular domain, the molecular mechanisms underlying their pathogenesis have been revealed [40, 41]. This mutation causes ER accumulation of P0 and activation of the UPR. This results in toxic consequences to the Schwann cells.

My work showed that expression of P0 or PMP22 in the calnexin-deficient cells did not result in ER accumulation of misfolded proteins. I

did not observe the increased UPR when measured by Xbp1 mRNA splicing. This observation confirmed previous results that expression of myelin glycoproteins in the calnexin-deficient cells results in their correct trafficking to the plasma membrane. Consequently, the lack of calnexin produced a phenotype similar to the one caused by mutations in P0 or PMP22 that do not disturb plasma membrane trafficking, but severely impaired myelin glycoproteins adhesiveness.

Schwann cells upon myelination produce large amounts of plasma membrane and plasma membrane proteins; this makes them incredibly sensitive to the disruption of ER homeostasis and the secretory pathway. Recent studies suggest that ER stress and the UPR are important in the pathogenesis of many myelin disorders including CMT, highlighting the importance of better understanding of these pathways for the design of new therapeutic strategies to treat these disorders.

There is considerable evidence that disease-causing mutations in P0 lead to ER stress [41]. A mouse model of CMT1B expressing the human disease-causing mutant of P0 (S^{34}del) has been generated. Further studies revealed that this mutation disrupts the alignment of hydrophobic residues in the β strand of P0. The P0 S^{34}del mutation results in the ER retention of the protein and leads to activation of the UPR. More detailed studies showed increased levels of phosphorylated eIF2α, activated ATF4, increased expression of CHOP (enhancer binding protein homologous protein) and enhanced splicing of XBP-1 in Schwann cells of mice expressing mutated P0 [5]. All these results indicate the induction of the PERK and IRE-1 branches of the URP (Figure 1-6). CHOP, a transcription factor downstream of the PERK pathway, is important in the induction of UPR-initiated apoptosis, and its deletion protects various cell types

against ER stress-induced apoptosis [42]. Interestingly, CHOP is absent in normal Schwann cells; however, detectable levels are present in myelinating cells expressing the S^{34}del mutant of P0 [41]. To investigate the role of CHOP in the pathogenic mechanism related to CMT1B caused by the P0 S^{34}del mutant, P0 S^{24}del mice were crossed with *Chop*$^{-/-}$ mice [41]. In the absence of CHOP, the motor abnormalities of P0 S^{34}del mice were rescued and demyelination was significantly reduced [41]. However, inactivation of CHOP did not decrease the number of apoptotic Schwann cells [41].

Mutations in the gene encoding the PMP22 protein are linked to CMT1A neuropathy. PMP22 is a highly hydrophobic protein, prone to forming aggregates [43]. Folding of PMP22 is an inefficient process; in Schwann cells, a large fraction of wild-type PMP22 is rapidly degraded [44]. Although the mechanisms involved in the pathology of CMT1 caused by mutants of PMP22 are not fully understood, the formation of aggregates and impaired intracellular trafficking have been proposed to play roles [45-48]. The accumulation of aggregates formed by misfolded protein may lead to decreased proteasome activity [49]. In humans, many cases of CMT1A are caused by duplication of the region of chromosome 17 that contains the *pmp22* gene, leading to increased expression of this myelin protein [7]. This human disorder has been modeled in mice by enforced over-expression of PMP22 [50]. Further studies showed that the mechanism underlying myelin defects caused by over-expression of PMP22, includes gain-of-function and toxic effects on myelinating Schwann cells [51]. The spontaneous mutations in the mouse *pmp22* gene Trembler and Trembler-J have been identified and described to result in CMT1A-like symptoms. It has been demonstrated that the Tr and Tr-J

mutated forms of PMP22 accumulate in the ER [47]. Importantly, calnexin has been identified to be involved in their ER retention in Schwann cells. Several lines of evidence suggest that the Tr and Tr-J PMP22 mutants accumulate in the ER and induce ER stress, resulting in Schwann cell apoptosis and myelin abnormalities [49, 52]. Interestingly, further studies showed that there is no activation of ER stress or UPR markers in cells expressing PMP22 mutants, suggesting that the role of UPR in pathogenesis of CMT1A needs to be examined in more details and might involve other players.

Calnexin has been shown to associate with PMP22 mutant proteins [14]. It also was found to co-localize with the P0 S^{34}del and other P0 mutants that show ER retention, leading to the induction of the UPR and ER stress-induced apoptosis of myelinating cells [5]. In order to investigate in detail the role of calnexin in the ER retention of the disease-related mutants of myelin glycoproteins, it would be important to examine the activation of the UPR by myelin glycoprotein mutants in calnexin-deficient cells. In order to carry out this experiment, we would create wild-type or ER chaperone-deficient cell lines stably expressing various PMP22 or P0 mutants. We could measure the induction of the UPR by analysis of XBP1 mRNA spicing using the luciferase reporter system developed by Kaufman's group [53]. It would be most interesting to see the effects of human disease-related mutants like P0 S^{34}del. We could test if the absence of calnexin, ERp57 or calreticulin would result in an attenuated UPR when compared to wild-type cells expressing P0 or PMP22 mutants. Moreover, we could examine if intracellular trafficking of mutant proteins changes in the absence of ER molecular chaperones. With

these experiments, we could address questions concerning the physiological importance of ER molecular chaperones in myelin disorders.

The Quality Control of MOG

Calnexin-deficient mice show dysmyelination within the CNS. Myelin abnormalities include thinner, wavy, decompacted myelin around large axons in the spinal cord, clearly showing that the lack of calnexin is affecting myelination of the central nervous system myelin [3].

Myelin glycoproteins are essential for the formation of functional myelin. My results identified an association between calnexin and the central nervous system myelin glycoprotein MOG. These findings suggest that the defects in the CNS may be related to incorrect folding of MOG in the absence of calnexin. However, glycosylation and cellular localization of MOG were unaltered in the absence of this ER chaperone. Moreover, expression of MOG in the calnexin-deficient cells does not result in intracellular aggregation, retention or induced ER stress (Figure 3-5). In addition, MOG-deficient mice do not develop any pathological characteristic, suggesting that the presence of MOG is not essential for myelin development [54]. These results suggest that if the absence of calnexin results in misfolded MOG, it is unlikely the factor contributing to the central myelin defects in calnexin-deficient mice.

MOG is localized to the outermost surface of the myelin sheath. This localization makes it a perfect target for antibody-mediated demyelination. Interestingly, in patients with MS, an inflammatory disease of the CNS, anti-MOG antibodies have been detected, indicating that they are important for the pathology and progression of the disease [55, 56]. Analysis of pathogenic monoclonal antibodies against MOG

showed that in many cases they recognized strictly conformation-dependent epitopes [57].

MOG-specific antibodies play an important role in the immunopathogenesis of MS. Experimental autoimmune encephalomyelitis (EAE) is the most commonly used animal model for MS [58]. EAE induction includes the stimulation of an immune response directed against CNS components that become the antigens. Current EAE protocols often use recombinant MOG as a primary immunogen. Clinical signs of MOG-induced EAE include activation of the T- and B-cells and inflammation mainly localized to the spinal cord [59, 60]. EAE allows the study of many pathogenic aspects of human MS.

Although, MOG is a minor component of CNS myelin, it is highly encephalitogenic in many species [61]. The encephalitogenic properties of MOG are known to be dependent on its folding [62]. My results show that calnexin is a potential molecular chaperone of MOG. Therefore, it would be interesting to test the encephalitogenic properties of MOG when expressed in the absence of this ER molecular chaperone.

To investigate if MOG is misfolded and therefore its encephalitogenic properties are altered when expressed in the absence of calnexin, we could use serum from EAE mice (this serum is known to contain anti MOG antibodies) in FACs analysis. We would test if the antibodies produced against wild-type MOG (upon EAE induction) recognize MOG expressed in the absence of calnexin. This result would show if MOG expressed in the ER chaperone-deficient cells is less or more immunoreactive. This experiment would not only confirm the essential role of calnexin in the folding of MOG.

Calnexin plays an important role in the immune system. It is involved in the folding and maturation of MHC class I molecules, associates with cell surface CD3 complexes and regulates T-cell receptor assembly [63-65]. Surprisingly, calnexin-deficient mice show no abnormalities within the immune system, suggesting that this chaperone is not essential for its development [3]. Calnexin-deficient mice are viable and give great opportunity to test the role of calnexin upon induction of diverse diseases. Although the immune system in the calnexin-deficient mouse is intact, it would be important to investigate immune responses upon inflammation. Challenging the immune system of calnexin-deficient mice might show deficiencies caused by the absence of calnexin that are not seen under normal conditions. It would be interesting to induce EAE in the calnexin-deficient mouse using MOG as a primary antigen. We could compare the clinical severities of EAE in wild-type and calnexin-deficient mice. Induction of EAE in calnexin-deficient mice would answer two important questions: (i) how does the calnexin-deficient mouse immune system react when stimulated; (ii) is MOG as autoimmunogenic as when expressed in the wild-type cells.

Conclusions

Considerable progress has been made towards better understanding of the involvement of the ER and ER-related pathways in the pathology of human neuropathies. Moreover, recent studies indicate that manipulating these pathways has therapeutic potential [7]. Therapies with chemical chaperones, such as 4-phenylobutyric acid, taurine-conjugated urosdeoxycholic acid or resveratrol or compounds that act on the UPR pathways and prevent ER stress-induced apoptosis are promising. For

example, curcumin has been shown to promote mutant PMP22 transport from the ER to the plasma membrane [66]. Yet, there is still no effective treatment available for patients with disorders that involve myelinating cells.

The selective manipulation of the activity of molecular chaperones of the ER without causing severe side effects is definitely challenging. However, it is important to gain more detail about the mechanisms involved in the pathogenesis of human myelin diseases in order to design effective therapeutic strategies.

In this study, I examined the function of the ER proteins calnexin, ERp57 and calreticulin in the quality control of major myelin glycoproteins. I studied the intracellular localization, function, folding and induction of the UPR by these glycoproteins in the absence of these chaperones.

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