# Calnexin can interact with *N*-linked glycans located close to the endoplasmic reticulum membrane

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**Abstract Calnexin is a central component of the 'quality control' system in the endoplasmic reticulum (ER). Calnexin binds to monoglycosylated oligosaccharides on incompletely folded soluble and membrane proteins in the lumen of the ER and prevents exit from the organelle. We have previously found that the oligosaccharide transferase enzyme can add glycosyl moieties to a membrane protein when the acceptor site is as close as 12–13 residues away from the nearest transmembrane segment (J. Biol. Chem. 268, 5798). We now show that calnexin can bind to oligosaccharides located this close to the membrane, suggesting that its binding site is held at a similar distance from the membrane as is the active site of the oligosaccharide transferase. We further show that calnexin can bind efficiently to glycosylated but not to non-glycosylated forms of a bacterial inner membrane protein, suggesting that it does not have a general affinity for non-glycosylated proteins.**

*Key words:* Calnexin; Glycosylation; Oligosaccharide

## 1. Introduction

Calnexin is a type I membrane protein believed to be involved in the folding of both soluble and membrane proteins in the endoplasmic reticulum (ER) (for a review see [1]). Calnexin interacts with incompletely folded proteins through their partially deglycosylated, *N*-linked oligosaccharides [2,3]. Although calnexin preferentially interacts with monoglycosylated oligosaccharides, the fact that some unglycosylated proteins can also interact with calnexin shows that *N*-linked glycans are not the only site of interaction [4–6]. Recent findings suggest that the oligosaccharide interaction is essential for the initial formation of the calnexin/glycoprotein complex, but that once this complex has formed the glycan is dispensable and binding occurs through the polypeptide chain [7,8]. In addition, incorrectly folded, non-glycosylated proteins can form high-molecular-weight aggregates including calnexin in the ER [18].

We have previously shown that integral membrane proteins can only be glycosylated on asparagine residues located at least 12–13 residues away from the nearest transmembrane segment, suggesting that the active site of the oligosaccharyl transferase enzyme is positioned a certain distance above the lumenal surface of the ER membrane [12]. Since calnexin is also an integral membrane protein of the ER, we wanted to determine whether its glycan binding site was located as close to the membrane as the oligosaccharyl transferase active site, or whether *N*-glycans located too near a transmembrane segment would not be able to bind to calnexin.

For this study, the *E. coli* inner membrane protein leader peptidase (Lep) [9] was chosen as a model protein (Fig. 1). Lep has previously been expressed in an in vitro transcription/translation system and was shown to be correctly inserted into dog pancreas microsomes and glycosylated [10]. Lep has also been expressed in BHK cells where the P2 domain is translocated into the ER lumen and properly folded as assayed by disulphide bridge formation and glycosylation at a naturally occurring acceptor site for *N*-linked glycosylation (our unpublished data; cf. [17]). Lep thus behaves like an endogenous eukaryotic ER membrane protein when expressed in BHK cells. Further, since Lep is a prokaryotic protein, it has not evolved specific binding sites for calnexin and is thus an ideal model protein for studying the interaction between calnexin and proteins either with or without *N*-linked glycans.

Our results show that calnexin can bind glycosylated wild type Lep but not a non-glycosylated mutant ($Asn^{214} \rightarrow Gln$) in which the acceptor site for *N*-linked glycosylation has been destroyed. Another mutant with its only glycosylation site placed 13 amino acids from the H2 transmembrane domain can still interact with calnexin. This demonstrates that calnexin can bind to *N*-linked glycans located very near the ER membrane and further that calnexin cannot bind a non-glycosylated prokaryotic membrane protein.

## 2. Materials and methods

### 2.1. Cell culture

Baby hamster kidney 21 (BHK) cells were grown in Glasgow minimal essential medium (Gibco, BRL, Glasgow MEM (BHK-21), with L-glutamine, without tryptose phosphate broth) supplemented with 10% tryptose phosphate broth, 5% fetal calf serum, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid pH 7.3) and 2 mM glutamine. Penicillin (100 U/ml) and streptomycin (100 μg/ml) were also added to media for passage of cells. Cells were incubated at 37°C and 5% $CO_2$. Cells were grown in 35-mm dishes (about $1 \times 10^6$ cells).

### 2.2. DNA constructs

Wild-type Lep and the Lep mutants used were originally cloned into pGEM1 and have been described in detail elsewhere [12,13]. Cloning of the Lep constructs into the Semliki Forest Virus expression vector (SFV1) was carried out by PCR using the pGEM1 plasmids as templates. PCR primers were designed to create restriction sites flanking the Lep gene.

The 5'-end primer GGGCGGATCCGGCGAATTCCACCAGCCAGGGTTC and the 3'-end primer GCCTATCCCGGGCAAACGTGAACGAAGATGG created a *Bam*HI and *Sma*I site, respectively. The amplified Lep gene fragment was digested with *Bam*HI and *Sma*I and inserted into the polylinker region of SFV1 [14].

### 2.3. Transfection of cells

Transcription of RNA from plasmid DNA was done in a 50 μl mixture as described in [15]. 20 μl of the transcription reaction was used to transfect $5 \times 10^6$ BHK cells by electroporation in a 0.4-cm cuvette (850 V/25 μF) [15]. Efficient transfection was achieved by

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thoroughly suspending cells beforehand by trypsinisation and gentle mixing. After transfection the cells were diluted 20-fold with BHK medium, distributed into 35-mm plates and incubated at 37°C in a 5% $CO_2$ atmosphere.

*2.4. Metabolic labelling of proteins*

For the labelling of one dish of cells approximately 50 μCi of [$^{35}$S]methionine (Amersham, specific activity >37 TBq/mM, >1000 Ci/mM) was used. Labelling was carried out for different times in 500 μl of MEM lacking methionine (Gibco, BRL, MEM with Earle's salts, without L-glutamine and L-methionine). The incubation of cells in this medium was started 30 min before labelling, in order to pre-starve cells of methionine. Labelling was terminated by changing the medium to MEM containing 10 times concentrated, unlabelled methionine. Chase in this medium was continued for different times. Transfected cells were labelled 7 h after electroporation.

*2.5. Preparation of cell lysates and media*

After pulse chase, cells were put on ice, the medium was removed, and the cells were washed twice with cold PBS and solubilised with 300 μl of HBS buffer (200 mM NaCl, 50 mM HEPES, pH 7.6) containing CHAPS (2%). PMSF (phenylmethylsulphonyl fluoride; 1 mM), aprotinin (5 μg/ml), and leupeptin (5 μg/ml) were added to prevent protein degradation. The lysate was incubated for 30 min at 4°C and centrifuged at 11 000×*g* for 10 min at 4°C. The supernatant was transferred to a fresh tube and diluted once with HBS buffer. Pansorbin (50 μl) was added and the lysate was incubated for 30 min at 4°C and then centrifuged at 11 000×*g* for 5 min.

*2.6. Immunoprecipitation and SDS-PAGE*

The supernatant was split into two and used for protein A-mediated immunoprecipitation using 2 μl of either anti-Lep or anti-calnexin antibody. Final precipitates were solubilised in 20 μl SDS gel sample buffer and applied onto a 10% acrylamide gel (Hoefer Mighty Small gel system).

## 3. Results and discussion

To analyse the interaction between calnexin and glycosylated, wild-type Lep, BHK cells expressing Lep were pulsed with [$^{35}$S]Met for 2 min and chased with non-radioactive Met for 2, 5, 30, 60 or 90 min. The cells were then lysed in CHAPS and precipitated with either a Lep or a calnexin polyclonal antibody. Lep could be precipitated with the calnexin antibody and the interaction was strongest after a 5-min chase (Fig. 2, bottom panel). At longer chase times, the intensity of the signal decreased, indicating a transient interaction between Lep and calnexin, as has also been observed for other glycoproteins [16]. The dissociation was not due to protein degradation, since the intensity of the Lep signal in a Lep antibody precipitation was equally strong throughout the chase period (Fig. 2, top panel). The glycosylated protein remained sensitive to EndoH throughout a 3-h chase period and gave an ER-like staining in immunofluorescence (data not shown), suggesting that it did not reach the medial Golgi compartment.

As a control, the same experiment was carried out with a Lep mutant where Asn$^{214}$ was changed to Gln to prevent glycosylation. This mutant could not be precipitated with the calnexin antibody (Fig. 3). Further, when cell lysis was carried out by addition of the detergent NP-40 (1% final concentration) that is more disruptive to protein-protein interactions than CHAPS [19], wild-type Lep could be efficiently precipitated by Lep antiserum but not by calnexin antiserum

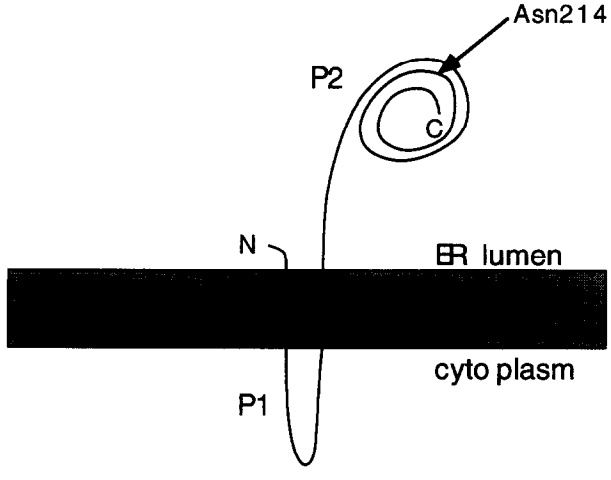


Fig. 1. Orientation of Lep in the ER membrane. The two hydrophobic transmembrane segments H1 and H2, the cytoplasmic loop P1, the large lumenal domain P2, and the Asn$^{214}$ glycosylation site are indicated. In the Lep mutant analyzed in Fig. 4, the H2 region (residues 59–80) was replaced by the sequence $IK_4L_{14}VQ_3P$, Asn$^{214}$ was changed to Gln, and an Asn-Ser-Thr acceptor site for *N*-linked glycosylation was inserted 13 residues downstream of the H2 transmembrane segment [13].

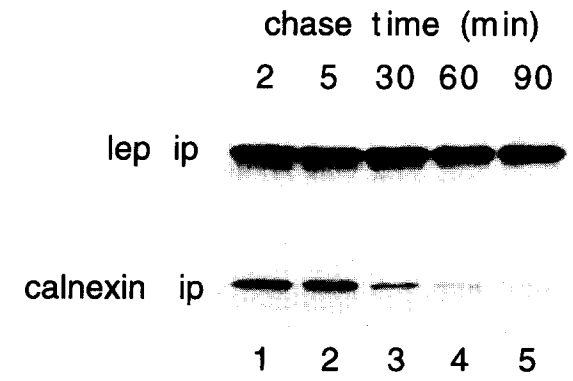


Fig. 2. Interaction between calnexin and wild-type Lep. Lep was expressed in BHK cells and metabolically labelled for 2 min with [$^{35}$S]methionine. After a chase of 2, 5, 30, 60, or 90 min cells were lysed in CHAPS lysis buffer and the lysates were immunoprecipitated with either a Lep (top panel) or a calnexin (bottom panel) polyclonal antibody, and the samples were analysed by SDS-PAGE and autoradiography.

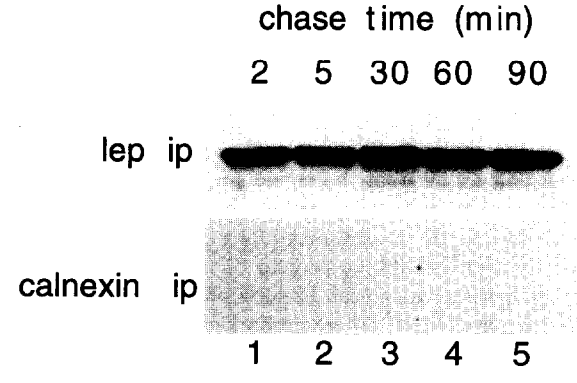


Fig. 3. A non-glyosylated Lep mutant is not precipitated by calnexin antibody. Cell lysates were immunoprecipitated with either a Lep (top panel) or a calnexin (bottom panel) polyclonal antibody. See Fig. 2 for details.

(data not shown), demonstrating that Lep does not bind to calnexin during the immunoprecipitation step.

Having shown that calnexin can only interact with glycosylated Lep, it was of interest to determine whether calnexin can bind to a Lep mutant with its sole acceptor site for *N*-linked glycosylation located only 13 amino acids away from the H2 transmembrane segment, i.e. at the minimal distance from the membrane required for efficient glycosylation by oligosaccharyl transferase [12,13]. For this experiment, we chose a mutant where the H2 segment is composed of a stretch of 14 leucine residues and one valine (see legend to Fig. 1), as the minimal glycosylation distance has been carefully determined for this construct [13]. As shown in Fig. 4, this mutant could also be precipitated with the calnexin antibody. In this case, the Lep-calnexin interaction persisted throughout the chase period, suggesting that the glycosylation may prevent proper folding of Lep.

Misfolding of the mutant molecules caused by the introduction of the glycosylation site might expose new segments of the protein for binding to calnexin, bypassing the need for a glycan moiety. To rule this out, cells expressing the mutant were pretreated with castanospermine and pulse-chased in the presence of the drug. Castanospermine is a glucosidase inhibitor and prevents the trimming of glucose residues from the core glycan [11]. This treatment inhibits the binding of calnexin to *N*-linked glycans since calnexin, at least preferentially, binds to monoglucosylated oligosaccharides [2,3,18]. As shown in Fig. 5 (top panel), the amount of mutant protein that could be precipitated with the calnexin antibody in castanospermine-treated cells decreased drastically, indicating that the interaction between calnexin and the mutant protein was exclusively mediated by the glycosyl moiety. The same was true for wild-type Lep (Fig. 5, bottom panel). In both cases, the castanospermine-treated molecules had a slightly lower mobility, consistent with the block of glucose trimming caused by the drug. Similar result was obtain using tunicamycin, an inhibitor of *N*-linked glycosylations (data not shown).

In conclusion, our results show that two glycosylated forms of the bacterial inner membrane protein Lep can bind to calnexin in the ER, whereas a non-glycosylated form cannot. Thus, calnexin has no or little affinity for a non-glycosylated prokaryotic protein. Further, calnexin can bind to *N*-linked sugars located very close to a transmembrane segment. It thus seems that calnexin and the oligosaccharyl transferase have co-evolved in such a way that calnexin can bind glycan moieties located as near to the ER membrane as the oligosaccharyl transferase can put them.

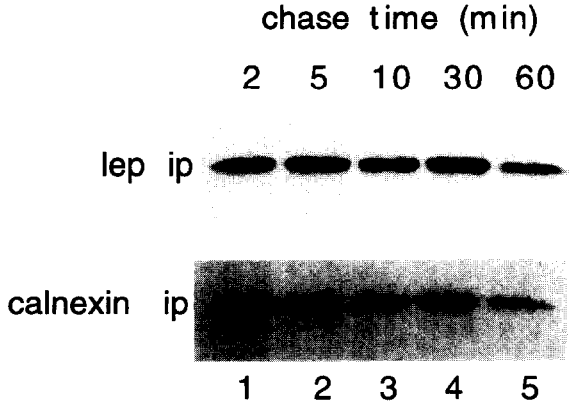


Fig. 4. Calnexin interacts with a Lep mutant with its only glycosylation site located 13 residues downstream of H2. The interaction between calnexin and Lep was probed as decribed in Fig. 2, except that the cells were chased for 2, 5, 10, 30, or 60 min.

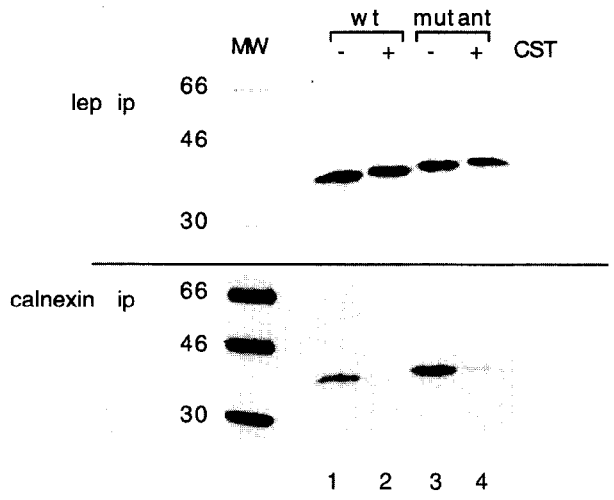


Fig. 5. The calnexin-Lep interaction is inhibited by castanospermine. Cells expressing wild-type or mutant Lep were pretreated for 150 min with 1 mM castanospermine (CST), radiolabelled for 10 min, and chased for 5 min in the presence of the inhibitor. Untreated cells were used as a control (lanes 1 and 3). After cell lysis, the lysates were precipitated with either a Lep (top) or a calnexin (bottom) antibody. The slower mobility of the mutant compared to the wild-type protein is caused by the additional residues in and around the H2 segment in the mutant.

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