**Biosynthesis of inositol trisphosphate receptors: selective association with the molecular chaperone calnexin**

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A prominent labelled polypeptide having the same mobility as type-I inositol trisphosphate receptor (IP$_3$R) was immunoprecipitated from WB-cell lysates by antibodies to calnexin, an ER integral membrane chaperone. The identity of this polypeptide was confirmed by re-immunoprecipitation of the radioactive polypeptides released from calnexin–antibody immunoprecipitates with type-I IP$_3$R antibody. The interaction of calnexin with newly synthesized type-I IP$_3$R was transient and inhibited by treatment of the cells with dithiothreitol or the glucosidase inhibitor N-methyldoexynojirimycin. In similar experiments, there was no evidence for the binding of type-I IP$_3$R to calreticulin, an ER luminal chaperone. Calnexin (but not calreticulin) associated with newly synthesized FLAG (DYKDDDDK epitope)-tagged type-III IP$_3$R expressed in COS-7 cells. In order to further define the mechanism of interaction of nascent IP$_3$R with chaperones, we have utilized an in vitro rabbit reticulocyte translation assay programmed with RNA templates encoding the six putative transmembrane (TM) domains of IP$_3$Rs. In accordance with the known preference of calnexin for monoglycosylated oligosaccharide chains, calnexin antibody preferentially immunoprecipitated a proportion of glycosylated type-I translation product. Addition of glucosidase inhibitors prevented the association of calnexin with in vitro translated type-I TM construct. Using truncated RNA templates we found that calnexin did not associate with the first four TM domains but retained affinity for the construct encoding TM domains 5 and 6, which contains the glycosylation sites. We propose that calnexin is a key chaperone involved in the folding, assembly and oligomerization of newly synthesized IP$_3$ receptors in the ER.

**Key words:** calcium, calreticulin, inositol phosphates, endoplasmic reticulum.

**EXPERIMENTAL**

Materials

T$_7$, RNA polymerase, DNA ligase, RNasin ribonuclease inhibitor and rabbit reticulocyte lysate were purchased from Promega

Very little is known regarding the biosynthesis and assembly of IP$_3$R ion channels. The available evidence suggests that oligomerization of IP$_3$R subunits requires specific regions within the C-terminal transmembrane (TM) domains and occurs in ER membranes [14,15]. In common with other multimeric integral membrane proteins, it would be anticipated that specific ER chaperones would be involved in the early stages of membrane insertion, folding and assembly of IP$_3$Rs. The present study was undertaken to determine whether the Ca$^{2+}$-binding chaperones calnexin and calreticulin play a role in the biosynthesis of IP$_3$Rs. Pulse-labelling of endogenous IP$_3$Rs in cultured WB rat liver epithelial cells or transfected IP$_3$Rs in COS cells, as well as in vitro translation of IP$_3$R RNA templates, were used as experimental systems in the present study. Our data suggest that calnexin is an important chaperone involved in the biosynthesis of IP$_3$R isoforms and that this interaction is associated with the processing of N-linked oligosaccharides on the IP$_3$R molecule. Calnexin interactions with IP$_3$Rs were not mimicked by the closely related Ca$^{2+}$- and lectin-binding chaperone calreticulin.

**INTRODUCTION**

The mobilization of internal stores of Ca$^{2+}$ is associated with cell activation induced by a wide variety of hormones, growth factors and neurotransmitters. This early event in Ca$^{2+}$ signal transduction occurs as a consequence of the production of inositol 1,4,5-trisphosphate (IP$_3$) and the binding of this small molecule to a family of receptors (IP$_3$Rs) located in the endoplasmic reticulum (ER) which function as ligand-gated Ca$^{2+}$ channels (reviewed in [1,2]).

Full-length sequences of three members of this receptor family have been identified. Each channel is believed to be tetrameric, and both homo- and hetero-tetramers are found in cells expressing more than one isoform [3–5]. A number of differences in the properties of individual IP$_3$R isoforms have been noted. These include differences in IP$_3$ binding affinity [6], regulation by Ca$^{2+}$ [7] and ability to bind calmodulin [8]. The primary sequences also show differences in consensus phosphorylation and N-glycosylation sites [9]. In addition, it has been suggested that individual isoforms may be localized to discrete regions of the cell [10–12] or that specific isoforms may regulate distinct cellular processes such as Ca$^{2+}$ entry across the plasma membrane [10] or apoptosis [11,13]. Overall, the presence of differentially regulated IP$_3$Rs with the ability to form hetero-oligomers provides the cell with considerable flexibility in the regulation of Ca$^{2+}$ signals.

Abbreviations used: IP$_3$, myo-inositol 1,4,5-trisphosphate; IP$_3$R, inositol trisphosphate; DMEM, Dulbecco’s modified Eagle’s medium; ER, endoplasmic reticulum; Ab, antibody; DTT, dithiothreitol; NMDJ, N-methyldoexynojirimycin; FLAG, epitope tag corresponding to DYKDDDDK; TM, transmembrane; N2475S etc., Asn$^{2475}$→Ser etc.

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Antibodies

The IP, R isofrom-specific antibodies used in these studies were raised against unique C-terminal sequences of IP, receptors. The type I and type-III IP, R antibodies were raised to amino acids 2731–2749 and 2657–2670 respectively of the rat IP, R isoforms and have been previously characterized [15,16]. Results were confirmed using commercially available type I IP, R antibody (Ab) (Affinity BioReagents Inc., Golden, CO, U.S.A.) and monoclonal type III IP, R Ab (Transduction Laboratories, Lexington, KY, U.S.A.). Calnexin Ab was raised against a peptide comprising the C-terminal 15 amino acids of canine calnexin [17]. The peptide was synthesized with an additional N-terminal cysteine residue, which was used for conjugation to keyhole-limpet haemocyanin [18]. The Ab was raised in rabbits by Cocalico Biologicals (Reamstown, PA, U.S.A.) and was affinity-purified using the peptide coupled to Ultralink Iodoacetyl beads as described by the manufacturer (Pierce Chemical Co., Rockville, IL, U.S.A.). Initial experiments were performed with batches of calnexin Ab supplied by Dr. Ari Helenius (Department of Cell Biology, Yale University, New Haven, CT, U.S.A.) and Dr. David B. Williams (Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada). Additional Abs to calnexin were purchased from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada). A polyclonal Ab to calreticulin was obtained from Affinity Bioreagents Inc. (Richmond, VA, U.S.A.). Richter’s modified minimal essential medium was from Irvine Scientific Co. (Santa Ana, CA, U.S.A.). Normal and Ultra enhanced chemiluminescence reagents were from Pierce Chemical Co. (Rockville, IL, U.S.A.).

Cell culture, metabolic labelling and immunoprecipitation

WB rat liver epithelial cells [19] were grown to confluence in 100 mm-diameter dishes in Richter’s minimal essential medium containing 5% fetal-bovine serum. The cells were incubated for 1 h in methionine-free Dulbecco’s modified Eagle’s medium (DMEM; Gibco–BRL, Gaithersburg, MD, U.S.A.) and then incubated for 30 min in the same medium containing 100 μCi/ml of Tran35S-label. At the end of the labelling period, the medium was removed and the plates were washed twice in ice-cold PBS and solubilized in 0.5 ml of digitonin lysis buffer [150 mM NaCl/50 mM Tris/HCl (pH 7.8)/1% (w/v) digitonin/1 mM EDTA/5 mM iodoacetamide/0.5 mM PMSF/5 μg/ml aprotinin/5 μg/ml soybean trypsin inhibitor/5 μg/ml leupeptin]. All lysates were preclarced for 30 min by incubation with 20 μl of a 50% (v/v) slurry of Staphylococcus aureus cell wall (Pansorbin; Calbiochem). Insoluble material was removed by centrifugation for 10 min at 25 000 g. Unless otherwise stated, equal amounts of lysate protein were incubated overnight with IP, R or chaperone Abs at 4 °C together with 50 μl of Protein A-Sepharose (20%, v/v). Immune complexes were isolated by centrifugation, washed three times in lysis buffer containing 0.1% digitonin and analysed by SDS/PAGE. Routinely, the amount of Ab used was 1–2.5 μg for every 0.5 mg of cell lysate immunoprecipitated. Under these conditions the IP, R, calnexin and calreticulin Abs optimally immunoprecipitated their cognate antigens (results not shown). In some experiments, the polypeptides in the gel were transferred to nitrocellulose, which was autoradiographed and then immunoblotted with IP, R-isoform-specific Abs to locate the receptor. Repeated immunoblotting of the same nitrocellulose sheet was carried out after treating blots for 30 min at 60 °C in a stripping buffer containing 65 mM Tris/HCl, pH 6.8, 2% SDS and 100 mM β-mercaptoethanol.

Plasmids and transfection experiments

The rat type-I IP, R cDNA was kindly given by Dr. Thomas Sudhof (Department of Molecular Genetics, Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.) [20]. The rat type-III IP, R cDNA was kindly given by Dr. Graeme Bell (Department of Biochemistry, University of Chicago, Chicago, IL, U.S.A.) [21]. The type-III IP, R was epitope tagged at the N-terminus by using PCR to amplify the entire coding sequence with rat type-III IP, R cDNA as template. The forward primer encoded an NheI restriction site and the FLAG epitope. The reverse primer encoded an EcoRI site. The reaction was carried out using the Expand long-template PCR system according to the manufacturer’s instructions (Boehringer-Mannheim). The 8037 bp PCR product was digested with NheI/EcoRI, purified and ligated into NheI/EcoRI-digested expression plasmid pcDNA3.1(+) (Invitrogen). All plasmids used for transfection were purified by CsCl banding [22]. COS-7 cells were grown to approx. 70% confluence in DMEM supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 5% fetal-bovine serum. Cells were transfected with Lipofectamine (Gibco–BRL) using the procedure recommended by the manufacturer.

Mutagenesis of glycosylation sites

The preparation of the 1TM, 1TM1-4,tag, 1TM5,6 and 3TM cDNA constructs encoding different segments of the transmembrane (TM) domain of the type-I and type-III receptor have been described previously [15]. To permit immunoprecipitation, the truncation mutants were engineered to contain the C-terminal Ab epitopes. Mutagenesis of both the glycosylation sites in the type-I construct was carried out using the Quick-change mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). The 1TM5,6 construct was used as template and oligonucleotide primers were designed to mutate the glycosylation sites at Asn2475 and Asn2903 to Ser residues. The presence of the double glycosylation mutant (N2475S,N2503S) was confirmed by automated DNA sequencing. The cassette containing the double glycosylation mutant was excised from 1TM5,6 using BsrGI/EcoRI and ligated into BsrGI/EcoRI-digested 1TM.

Cell-free transcription/translation assays

Plasmid DNA (5μg) was linearized with EcoRI. Capped transcripts were synthesized with T7 RNA polymerase using an mRNA synthesis kit (Ambion, Austin, TX, U.S.A.). Transcribed templates were purified by phenol/chloroform extraction and ethanol precipitation. The sample was resuspended in 20 μl of 0.05% diethyl pyrocarbonate-treated water (to inactivate RNases) and stored at −80 °C. Routinely, cell-free translations were carried out for 1 h at 30 °C in a final volume of 25 μl and
contained 10 μl of rabbit reticulocyte lysate, 0.5 μl of RNAsin, 0.5 μl of 1 mM amino acids (minus methionine), 20 μCi 
Tran35S-label, 1.5 μl of RNA template (1–3 μg) and 6 μl of buffer 
A, pH 7.2, which contained 110 mM potassium acetate, 2 mM 
magnesium acetate and 20 mM potassium Hepes. Where appro-
priate, the reaction mixture was incubated for 4 h at 4 
°C to dissociate immune complexes and then diluted to 1 ml with WB solubilization buffer. Additional Protein A–Sepharose (50 
μl) was added to remove any free IgG and, after 
digestion with 20 μl of Pansorbin. After removal of Pansorbin by cen-
trifugation (10000 g; 10 min), IP3R- or calnexin-specific Ab 
and 50 μl of a 20% (v/v) slurry of Protein A–Sepharose was 
added and the sample was incubated for 4 h at 4 °C. Immune 
complexes were isolated by centrifugation, washed three times in 
lysis buffer containing 0.1%, digitonin and analysed by SDS/ 
PAGE. The gels were fixed, dried and subjected to auto-
radiography as described previously [15].

RESULTS
Calnexin and calreticulin binding to newly synthesized IP3Rs in 
WB cell lysates
In order to examine the binding of nascent type-I IP3R to 
chaperones, a short 30 min pulse of Tran35S-label was used to 
label newly synthesized proteins in WB cells. Lysates were 
immunoprecipitated with Abs specific for type-I IP3R, calnexin 
or calreticulin (Figure 1A). As observed previously [24], the type-
I IP3R Ab immunoprecipitated a predominant labelled band of 
235 kDa corresponding to newly synthesized type-I IP3R (Figure 
1A, lane 1). In agreement with the established role of calnexin 
and calreticulin as molecular chaperones, a large number of 
newly synthesized polypeptides were immunoprecipitated from 
WB cell lysates by calnexin or calreticulin Ab (Figure 1A, lanes 
2 and 3). The pattern of labelled polypeptides bound to calnexin 
and calreticulin were different. In the case of calnexin this 
was added with a mobility identical with that of newly 
synthesized type-I IP3R. This band was not observed in cal-
reticulin Ab immunoprecipitates (Figure 1A, lane 3). In order to 
determine whether the labelled band immunoprecipitated by 
calnexin Ab corresponded to type-I IP3R, a sequential immuno-
precipitation was performed. Lysates from pulse-labelled WB 
cells were first immunoprecipitated with calnexin Ab and the 
immune complexes were disrupted by incubation of the Protein 
A–Sepharose beads at 55 °C in a buffer containing 1% SDS and 
1 mM dithiothreitol (DTT). The SDS and DTT were diluted 10-
fold with additional buffer and three equal aliquots were subjected 
to a second round of immunoprecipitation with non-immune 
system, IP3R Ab or calnexin Ab (Figure 1B). In control 
experiments with unlabelled lysate it was apparent that the 
conditions used to maximally disrupt the immune complexes also 
greatly decreased the efficiency of IP3R immunoprecipitation. 
Consequently a 10-fold greater amount of lysate was used for 
calnexin Ab immunoprecipitation than used in Figure 1(A). The 
results show that a 35S-labelled band of 235 kDa corresponding 
to IP3R was immunoprecipitated by IP3R Ab from the mixture 
of polypeptides released from the calnexin immunoprecipitates 
(Figure 1B, lane 2) This band was absent when the second round 
of immunoprecipitation was carried out with non-immune serum or 
with calnexin Ab (Figure 1B, lanes 1 and 3). When the second 
immunoprecipitation was carried out with calnexin Ab, only a 
90 kDa band corresponding to calnexin was prominent, indi-
cating that the treatment conditions used to disrupt the immune 
complexes also disrupted calnexin–IP3R interactions.

In most instances it has been demonstrated that calnexin 
dissociates from nascent polypeptide targets as assembly and 
oligomerization proceeds to completion [25]. In order to examine 
the time course of calnexin interaction with newly synthesized 
type-I IP3R, WB cells were pulse-labelled for 30 min with 
Tran35S-label and then chased with 20 mM methionine. Figure 
2(A) shows the result of immunoprecipitatsing lysates with type-
I IP3R Ab or calnexin Ab after chase periods of 1 and 2.5 h. 
Previous pulse–chase experiments in WB cells have estimated the 
turnover rate of the type-I IP3R to be approx. 12 h [24]. The 
radioactivity associated with type-I IP3R did not decline mark-
edly over the 2.5 h chase period used in these experiments. By 
contrast the pool of IP3R co-precipitated by calnexin Ab showed a 
progressive decline in radioactivity during the chase. At 2.5 h the 
radioactivity associated with calnexin-bound IP3R was only 
25% of that present after the 30 min pulse (Figure 2B). These 
results are consistent with a transient association of calnexin with 
newly synthesized type-I IP3R. It should be noted that the 
radioactivity associated with the IP3R actually increased at the 
1 h chase time point (Figure 2A, lane 3, and Figure 2B). This 
observation, seen previously for the IP3R [24] and in pulse–chase 
experiments with other proteins [26,27], has usually been 
attributed to completion of nascent radiolabelled polypeptides 
initiated during the pulse period. The absence of a similar 
increase in radioactivity at the 1 h chase time point in the 
calnexin-bound IP3R suggests that calnexin may bind to a very 
specific pool of newly synthesized IP3R.

A number of pharmacological interventions known to interfere 
with the ability of calnexin to bind to substrates proteins were

Figure 1 Calnexin binding to newly synthesized type-I IP3R

(A) WB cells were pulse-labelled with Tran35S-label for 30 min as described in the Experimental 
section. Cell lysates (0.5 mg of protein) were immunoprecipitated with Abs specific for type-
I IP3R (IP3R) (lane 1), calnexin (CXN, lane 2) and calreticulin (CRT, lane 3). The 
immunoprecipitates were analysed by SDS/5%-PAGE and autoradiography. (B) WB cells were 
pulse-labelled with Tran35S-label for 30 min. A 6 mg portion of cell lysate protein was 
immunoprecipitated for 4 h with calnexin Ab and 75 μl of Protein A–Sepharose (20%, v/v). 
The immunoprecipitate was washed twice in WB solubilization buffer and divided into three 
equal aliquots. Each aliquot was incubated in 100 μl of release buffer [150 mM NaCl/20 mM 
Tris/HCl (pH 7.8)/1 mM DTT/1 mM EDTA/1% SDS]. The samples were heated for 10 min at 
55 °C to dissociate immune complexes and then diluted to 1 ml with WB solubilization buffer. 
Additional Protein A–Sepharose (50 μl) was added to remove any free IgG and, after 
digestion, the samples were subjected to a second round of immunoprecipitation overnight 
with non-immune serum (lane 1), type-I IP3R Ab (lane 2) and calnexin Ab (lane 3). The 
immunoprecipitates were resolved on SDS/5%-PAGE and autoradiographed. The values to the 
left of the gels are molecular masses in kDa. Abbreviation: ip, immunoprecipitating.
Figure 2 Kinetics of association of calnexin with nascent type-I IP₃R

(A) WB cells were pulse-labelled for 30 min with Tran³⁵S-label and then chased for 1 and 2.5 h with 20 mM methionine. Cell lysates were prepared at each time point and immunoprecipitated with type-I IP₃R Ab (I) or calnexin Ab (C). The immunoprecipitates were analysed by SDS/5%-PAGE, followed by autoradiography. The values to the left of the gels are molecular masses in kDa. Abbreviation: ip, immunoprecipitating. (B) The data from three separate experiments as shown in (A) were quantified by laser scanning densitometry. The total radioactivity in the IP₃R band in IP₃R–Ab and calnexin–Ab immunoprecipitates at each time point was expressed relative to the control shown in (A), lane 1. Results are means ± S.E.M.

examined in WB cells (Figure 3). The thiol reductant DTT has been shown to inhibit association of several nascent proteins with calnexin [29,30]. Treatment of WB cells with 1 mM DTT during a 1 h chase period decreased the amount of type-I IP₃₆ associated with calnexin by approx. 50% without significantly affecting the basal level of labelled IP₃₆ (Figure 3B). The binding of calnexin to substrate proteins has been shown to require the presence of a monoglucosylated oligosaccharide side chain and to be inhibited by glucosidase inhibitors that prevent the trimming of glucose residues. The addition of NMDJ, an inhibitor of glucosidases I and II, during the 1 h chase period inhibited the association of type-I IP₃₆ with calnexin (Figure 3). In our hands castanospermine (1 mM), another commonly used glucosidase inhibitor, was ineffective in WB cells and did not disrupt interaction of any of the labelled polypeptides immunoprecipitated by calnexin Ab (results not shown).

Calnexin and calreticulin binding to mature IP₃₆ in WB cell lysates

The binding of calnexin or calreticulin to mature (unlabelled) IP₃₆ in WB lysate was investigated in Figure 4. Unlabelled WB cell lysates were immunoprecipitated with non-immune serum, type I IP₃₆, type-III IP₃₆, calnexin or calreticulin Abs, and the five immunoprecipitates were sequentially probed by immunoblotting with type-I (Figure 4A), type-III (Figure 4B) or calnexin (Figure 4C) Abs. In agreement with our previous studies on hetero-oligomerization of IP₃₆ in WB cells, the type-I Ab immunoprecipitated type-III IP₃₆ (Figure 4B, lane 2) and the type-III Ab immunoprecipitated type-I IP₃₆ (Figure 4A, lane 3). Calnexin Ab immunoprecipitates contained faint bands corresponding to mature type-I (Figure 4A, lane 4) and type III IP₃₆ (Figure 4B, lane 4). Calnexin was co-immunoprecipitated by type-I IP₃₆ Ab (Figure 4C, lane 2), but was not detectable in...
Figure 4 Calnexin and calreticulin binding to mature type-I and type-III IP$_3$R

Cell lysates from unlabelled cells were immunoprecipitated with the following Abs: non-immune serum (lane 1), type-I IP$_3$R (‘IP3R’) Ab (lane 2), type-III IP$_3$R Ab (lane 3), calnexin Ab (lane 4) and calreticulin Ab (lane 5). The immunoprecipitates were analysed by SDS/5%-PAGE, transferred to nitrocellulose and immunoblotted with type-I IP$_3$R Ab (A). The same immunoblot was then stripped and sequentially immunoblotted with type-III IP$_3$R Ab (B) and calnexin Ab (C). The values to the left of the gels are molecular masses in kDa.

Figure 5 Association of newly synthesized IP$_3$Rs to calnexin in COS cells transiently transfected with IP$_3$R cDNA

(A) COS cells grown in 60 mm-diameter dishes were transiently transfected with 6 μg of pcDNA3.1 vector alone (mock transfection), type-I IP$_3$R cDNA or FLAG (‘Flag’)-tagged type-III IP$_3$R cDNA. At 48 h after transfection the cells were incubated for 30 min in methionine-free medium and then labelled for a further 30 min with Tran35S-label (100 μCi/ml). Lysates were prepared from the transfected cells and divided into three equal aliquots. IP$_3$Rs were immunoprecipitated from one aliquot using type-III IP$_3$R Ab (lane 1), type-I IP$_3$R Ab (lane 4) and FLAG Ab (lane 7). The remaining two aliquots were immunoprecipitated with calnexin Ab (CXN, lanes 2, 5 and 8) and calreticulin Ab (CRT, lanes 3, 6 and 9). The immunoprecipitates were analysed by SDS/5%-PAGE and autoradiography. The values to the left of the gels are molecular masses in kDa. Abbreviation: ip, immunoprecipitating. (B) COS cells were transfected with the FLAG (‘Flag’) tagged type-III IP$_3$R construct. The cells were incubated in methionine-free medium for 30 min, labelled with Tran35S-label for 30 min and chased with 20 mM methionine for 1 h. Where added, castanospermine (1 mM) was present during all three periods. Lysates were prepared from control and inhibitor-treated cells and equal aliquots of protein were immunoprecipitated with either FLAG Ab or calnexin Ab. The immunoprecipitates were analysed by SDS/5%-PAGE and autoradiography. The inset shows representative gels, and the histogram shows the densitometric quantification for three such experiments. All values were normalized to the label immunoprecipitated by FLAG Ab from untreated cell lysates.
Figure 6 Calnexin and calreticulin binding to in vitro-translated IP$_3$R constructs

(a) The amino acid boundaries of the TM domain constructs used in this and subsequent in vitro-translation experiments are shown schematically. The location of putative TM domains (open boxes), pore-forming domain (hatched box), glycosylation sites (filled circles) and isoform-specific C-terminal Ab epitopes (oval region) are as indicated. (b) RNA templates encoding the C-terminal portion of type-I (1TM, lanes 1–4) or type-III (3TM, lanes 5–8) IP$_3$R were translated in a rabbit reticulocyte translation system in the presence of canine pancreatic microsomes as described in the Experimental section. After 1 h, the translation mixture was lysed in a buffer containing 1% digitonin and split into four equal aliquots. These were immunoprecipitated with non-immune serum (lanes 1 and 5), IP$_3$R Ab (type-I, lane 2, and type-III, lane 6), calnexin Ab (CXN, lanes 3 and 7) and calreticulin Ab (CRT, lanes 4 and 8). The immunoprecipitates were analysed by SDS/10%-PAGE followed by autoradiography (b). The values to the left of the gels are molecular masses in kDa. The apparent molecular mass difference of the 3TM translation product in lane 8 is a gel artifact caused by the presence of unlabelled calreticulin in the immunoprecipitates (see the text). Abbreviation: ip, immunoprecipitating.

IP$_3$R in calreticulin immunoprecipitates (Figure 5, lane 9). Treatment of FLAG-III-transfected COS cells with castanospermine inhibited the association of newly synthesized FLAG-III IP$_3$R with calnexin by 70% (Figure 5B). In two experiments the addition of DTT as described in Figure 3 also inhibited the association of FLAG-III IP$_3$R with calnexin by 88% (results not shown). These results indicate that the type-III IP$_3$R isoform can also interact with calnexin (but not calreticulin) and that this interaction is sensitive to glucosidase inhibitors and DTT in the same manner as calnexin interactions with the type-I isoform.

Calnexin and calreticulin binding to in vitro-translated IP$_3$Rs

RNA templates encoding the six TM domains present in the C-terminal portion of type-I and type-III IP$_3$R isoforms have been shown to translate, integrate and assemble into homo- and hetero-oligomers in an in vitro translation system consisting of rabbit reticulocyte lysates and canine pancreas microsomes [15]. This experimental system has previously been used to study interactions between nascent proteins and various chaperones, including calnexin and calreticulin [29,32–34]. We have utilized this system to obtain additional information about the specificity of calnexin–calreticulin interactions and to define the structural determinants on the IP$_3$R molecule that are important for such interactions. 1TM and 3TM RNA templates (defined in Figure 6a) were translated in the presence of Tran™S-label. Digitonin lysates were prepared from the microsomes and immunoprecipitated with IP$_3$R, calnexin or calreticulin Ab (Figure 6). It was previously shown that, in the presence of canine pancreas microsomes, almost all of the 1TM and 3TM translation products were integrated into microsomal membranes with a proportion undergoing glycosylation [15]. The resulting translation products appear as doublets, in which the upper bands correspond to the glycosylated polypeptide ([15]; Figure 6b, lanes 2 and 6). Calnexin Ab preferentially immunoprecipitated the upper glycosylated band from both 1TM and 3TM translations. On the basis of quantification of the autoradiographs, it was estimated that the pool of 1TM and 3TM associated with calnexin, at the

![Figure 7 Effect of glucosidase inhibitors on calnexin and calreticulin binding to IP$_3$R constructs](https://example.com/figure7.png)

(A) Translation reaction mixtures containing all components except RNA were preincubated for 5 min at 30 °C with no additions, 1 mM castanospermine (CST) or 1 mM NMDJ (DNJ). Translation was initiated by addition of 1TM RNA. After 1 h, each translation mixture was lysed in a buffer containing 1% digitonin and split into two equal aliquots. One aliquot was immunoprecipitated with type-I IP$_3$R Ab (lanes 1–3) and the other aliquot was immunoprecipitated with calnexin Ab (lanes 4–6). The immunoprecipitates were analysed by SDS/10%-PAGE, followed by autoradiography. (B) The same procedure was followed as in (A), except the RNA template was 3TM, the immunoprecipitation of the lysates was carried out with type-III IP$_3$R and calreticulin Ab, and the samples were processed on 16 cm gels. It should be noted that lower autoradiograph exposures for the IP$_3$R Ab immunoprecipitates were selected to more clearly show the separation between glycosylated and non-glycosylated bands. The values to the left of the gels are molecular masses in kDa.
time translation was terminated, was 8.0 ± 1.8 % (n = 7) and 30.2 ± 5.8 % (n = 4) of the total translation product respectively. In agreement with our observation in WB-cell lysates, there was no evidence that calreticulin interacted with the 1TM translation product (Figure 8, lane 4). By contrast, calreticulin Ab did immunoprecipitate the 3TM translation product, although the preference in this instance was for the lower (non-glycosylated) translation product (Figure 6b, lane 8). The apparent difference in molecular mass between the 35S-labelled 3TM translation products immunoprecipitated by type-III and calreticulin Abs is a gel artifact due to the presence of a large amount of calreticulin in the latter immunoprecipitates, since both calreticulin and 3TM have similar molecular masses (60 kDa). The effect is minimized when the samples are run on larger gels (see Figure 7B).

In order to determine if the observed interactions of calnexin and calreticulin with the in vitro-translated IP_R constructs were related to the lectin-like properties of these chaperones, we tested the effects of adding the glucosidase inhibitors castanospermine and NMDJ (Figure 7). The presence of either inhibitor resulted in an increased separation between the two bands of the 1TM or the 3TM translation doublet (Figure 7A and B, lanes 1–3). The result is consistent with an inhibition of the glucose-trimming reactions producing a glycosylated translation product of higher molecular mass. The presence of the inhibitors resulted in a marked decrease in the amount of calnexin associated with the 1TM translation product (Figure 7A, lanes 4–6). In contrast, calreticulin Ab immunoprecipitated both bands of the 3TM translation doublet, and the interaction between calreticulin and 3TM was not affected by the glucosidase inhibitors (Figure 7B, lanes 4–6).

We have previously shown that the portion of the type-I IP_R from TM5 to the C-terminus is critical for oligomerization [15]. This segment contains the glycosylation sites and the presumed pore domain of the channel. In order to investigate which portion of 1TM is required for calnexin interaction, we tested the calnexin-binding ability of constructs encoding the first four TM domains (1TM1-4,tag) or the remaining portion of the molecule (1TM5,6) in the in vitro translation assay (Figure 8). The results show that 1TM1-4,tag does not bind to calnexin (Figure 8, lane 6), whereas calnexin Ab selectively immunoprecipitates a sharp band from the 1TM5,6 translation product (Figure 8, lane 4). As observed previously, 1TM5,6 translates as a broad smear which at lower exposures appears as a series of bands which we believe originates from heterogeneity in the occupation of the two glycosylation sites in this construct [15]. The presence of a single band immunoprecipitated by calnexin from the 1TM5,6 translation structure suggests that this chaperone may recognize a specific ‘glycoform’ of the IP_R. Additional evidence for the importance of the glycosylation sites was obtained from experiments in which both the sites in 1TM were mutagenized from Asn to Ser (N2475S,N2503S). As expected, this construct was not immunoprecipitated by calnexin Ab (Figure 8, lane 8).

**DISCUSSION**

Calnexin and calreticulin are two Ca²⁺-binding ER chaperones that share many structural and functional features [25,35]. The mode of action of both molecules as chaperones is believed to involve the specific recognition of a monoglucosylated intermediate (Glc³₄Man₆,GlcNac₇) formed during trimming of the N-linked core oligosaccharide attached to the nascent polypeptide chain. The removal of the terminal glucose by glucosidase-II is thought to facilitate the detachment of the nascent protein from the chaperone. If at this stage the substrate protein remains in an unfolded state, the enzyme UDP-glucose/glycoprotein glucosyltransferase can catalyse the reattachment of the terminal glucose, thereby re-initiating chaperone attachment. A model for an ER quality-control cycle has been advanced in which newly synthesized glycoproteins are continuously bound and released from calnexin/calreticulin, with escape from this cycle occurring when the fully folded substrate fails to be recognized by the glucosyltransferase. Experimental support for this model has been obtained from the study of a number of substrate proteins [25,36,37].

The present study is the first to examine the role of chaperones in IP_R biosynthesis, and our experimental data show that calnexin interacts with newly synthesized type-I and type-III IP_R isomers. Evidence for this interaction was based on co-immunoprecipitation experiments carried out in labelled lysates from three independent experimental systems, namely WB cells, transfected COS cells and in vitro translation reaction mixtures. The experiments in WB-cell lysates showed that newly synthesized type-I IP_Rs interact transiently with calnexin, and that this interaction could be inhibited by pharmacological agents known to interfere with calnexin binding, notably, the glucosidase inhibitor NMDJ. The experiments using the in vitro translation system confirmed these findings and showed that the interaction between calnexin and the type-I IP_R was specific for a pool of glycosylated translation products. The inhibitory effects of glucosidase inhibitors, the localization of the calnexin-binding region of the IP_R to the segment containing the glycosylation sites and the suppression of binding when these sites are mutated all point to the importance of the glycosylation sites in calnexin recognition of the type-I IP_R. The experiments on COS cells, a cell line which contains extremely low levels of endogenous type-I IP_R [31], confirm that calnexin can also interact directly with the type-III IP_R isoform. Calnexin has been shown to associate transiently with a large number of proteins that are destined to be secreted from the cell or inserted into the plasma membrane [25]. The present data suggest that calnexin may also function as part of the normal quality-control system that ensures proper folding and assembly of proteins, such as IP_Rs, that are normally resident in the ER.

Surprisingly, calreticulin did not bind to newly synthesized IP_Rs in lysates from WB cells, transfected COS cells or the in...
**vitro** translation system. A major difference between calnexin and calreticulin is that calnexin is an integral membrane protein, whereas calreticulin is soluble in the ER lumen. Examples of differences between these two chaperones in the selection of substrate proteins have been documented previously. For example, vescicular-stomatitis-virus G-protein [38], the CD3δ and ε chains of the T-cell receptor [39], or the α-subunit of the nicotinic acetylcholine receptor [40] associate with calnexin, but not calreticulin, during their biosynthesis. Spatial considerations, in which calnexin preferentially associates with membrane-proximal glycans, or differences in the recognition properties between the two chaperones, have been suggested to underlie their different behaviour [41]. The **in vitro** translation experiments indicated that calreticulin could bind to the type-III translation product (Figures 6 and 7), although there was no evidence for such interactions in the COS cells transfected with epitope-tagged type-III IP₃R. In this instance this discrepancy is likely to reflect a lack of specificity in the **in vitro** translation system, since the interaction between calreticulin and the 3TM construct showed no preference for the glycosylated product and was not affected by glucosidase inhibitors. A glycan-independent association of calnexin with aggregated forms of the vesicular-stomatitis-virus G-protein [42] has been observed in **in vitro** translation systems.

Although we observed calnexin binding to newly synthesized IP₃Rs in pulse-labelled cell lysates, the binding of calnexin (or calreticulin) to non-radioactive, mature IP₃Rs was minimal. This is in contrast with suggestions that calreticulin may bind to IP₃Rs and directly modulate their function [43–45]. Ca²⁺ within the lumen of the ER has also been proposed to regulate IP₃R function [2], and binding sites for Ca²⁺ have been identified in the intraluminal loop between TM domains 5 and 6 in the type-I IP₃R [46]. However, the association of calnexin with newly synthesized IP₃Rs was insensitive to Ca²⁺ mobilization induced by angiotensin (1 µM) or thapsigargin (10 µM) treatment in WB cells, and was also not affected by the addition of the calcium ionophore A23187 (1 µM) to the **in vitro** translation reaction mixtures (results not shown). Thus the role (if any) of the Ca²⁺-binding sites present in calnexin, calreticulin or the IP₃Rs in mediating chaperone interactions remains unclear.

The exact mechanism by which IP₃Rs are assembled into functional Ca²⁺ channels in the ER is unknown. There are several stages at which calnexin can be envisaged to be binding to newly synthesized IP₃R protein. These include steps at which the nascent chain is being translocated and inserted into the ER membrane, attachment to a pool of newly synthesized IP₃R monomers or binding to tetrameric IP₃Rs that are incompletely folded. Previous attempts to identify assembly intermediates of IP₃R monomers using Sephacryl S-200 column chromatography of pulse-labelled WB cell lysates have not been successful [24]. Therefore, our current data would favour a hypothesis in which calnexin binds to the IP₃R as soon as appropriately trimmed oligosaccharides are generated and continues to undergo cycles of binding and release from nascent IP₃R tetramers until the molecule achieves its fully folded and mature state. Our experiments do not distinguish between a direct calnexin–IP₃R interaction or an association that involves intermediary proteins.

IP₃Rs can be degraded in the ER under conditions where chronic agonist production leads to a sustained generation of IP₃ [47,48]. IP₃R degradation in WB cells treated with angiotensin-II is mediated by the ubiquitin–proteasome pathway [48]. Several ER membrane substrates of the ubiquitin–proteasome pathway have also been shown to interact with calnexin during their biosynthesis, e.g. cystic-fibrosis transmembrane conductance regulator (‘CFTR’) protein [49] and MHC class I molecules [50].

Disruption of the gene for calnexin inhibits the ER degradation of pre-pro-α-factor in a yeast ER **in vitro** degradation system [51]. Recently, it has been shown that the ER proteasomal degradation of the unassembled α-subunit of the nicotinic acetylcholine receptor is enhanced when calnexin association is inhibited [40]. Thus, there is evidence in the literature that calnexin may also play a role in ER protein degradation. The relatively narrow aperture of the proteasome would presumably dictate that the oligomeric complex of the IP₃R would have to be disassembled and fully unfolded prior to degradation. The possibility that chaperones, such as calnexin, also participate in the degradation of IP₃Rs remains to be investigated.

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Calnexin association with inositol trisphosphate receptor


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