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

Suresh K. JOSEPH¹, Darren BOEHNING, Shaila BOKKALA, Richard WATKINS and Johan WIDJAJA

Department of Pathology and Cell Biology, Thomas Jefferson University School of Medicine, Philadelphia, PA 19107, U.S.A.

A prominent labelled polypeptide having the same mobility as type-I inositol trisphosphate receptor (IP₃R) 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 IP₃R antibody. The interaction of calnexin with newly synthesized type-I IP₃R was transient and inhibited by treatment of the cells with dithiothreitol or the glucosidase inhibitor *N*-methyldeoxynojirimycin. In similar experiments, there was no evidence for the binding of type-I IP₃R to calreticulin, an ER luminal chaperone. Calnexin (but not calreticulin) associated with newly synthesized FLAG (DYKDDDDK epitope)-tagged type-III IP₃R expressed in COS-7 cells. In order to further define the mechanism of interaction of nascent IP₃R with chaperones, we have utilized an *in vitro* rabbit

reticulocyte translation assay programmed with RNA templates encoding the six putative transmembrane (TM) domains of IP₃Rs. In accordance with the known preference of calnexin for monoglucosylated 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 IP₃ receptors in the ER.

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

INTRODUCTION

The mobilization of internal stores of Ca²⁺ is associated with cell activation induced by a wide variety of hormones, growth factors and neurotransmitters. This early event in Ca²⁺ signal transduction occurs as a consequence of the production of inositol 1,4,5-trisphosphate (IP₃) and the binding of this small molecule to a family of receptors (IP₃Rs) located in the endoplasmic reticulum (ER) which function as ligand-gated Ca²⁺ 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 IP₃R isoforms have been noted. These include differences in IP₃ binding affinity [6], regulation by Ca²⁺ [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 Ca²⁺ entry across the plasma membrane [10] or apoptosis [11,13]. Overall, the presence of differentially regulated IP₃R isoforms with the ability to form hetero-oligomers provides the cell with considerable flexibility in the regulation of Ca²⁺ signals.

Very little is known regarding the biosynthesis and assembly of IP₃R ion channels. The available evidence suggests that oligomerization of IP₃R 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 IP₃Rs. The present study was undertaken to determine whether the Ca²⁺-binding chaperones calnexin and calreticulin play a role in the biosynthesis of IP₃Rs. Pulse-labelling of endogenous IP₃Rs in cultured WB rat liver epithelial cells or transfected IP₃Rs in COS cells, as well as *in vitro* translation of IP₃R 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 IP₃R isoforms and that this interaction is associated with the processing of N-linked oligosaccharides on the IP₃R molecule. Calnexin interactions with IP₃Rs were not mimicked by the closely related Ca²⁺- and lectin-binding chaperone calreticulin.

EXPERIMENTAL

Materials

T₇ RNA polymerase, DNA ligase, RNasin ribonuclease inhibitor and rabbit reticulocyte lysate were purchased from Promega

Abbreviations used: IP₃, *myo*-inositol 1,4,5-trisphosphate; IP₃R, inositol trisphosphate; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; Ab, antibody; DTT, dithiothreitol; NMDJ, *N*-methyldeoxynojirimycin; FLAG, epitope tag corresponding to DYKDDDDK; TM, transmembrane; N2475S etc., Asn²⁴⁷⁵ → Ser etc.

¹ To whom correspondence should be sent, at the following address: Department of Pathology and Cell Biology, Thomas Jefferson University, Room 230A JAH, 1020 Locust St, Philadelphia PA 19107, U.S.A. (e-mail josephs@jefflin.tju.edu).

(Madison, WI, U.S.A.). *Pfu* polymerase was obtained from Stratagene (La Jolla, CA, U.S.A.). Stabilized acrylamide solution (Protogel) for the preparation of SDS-containing gels was obtained from National Diagnostics (Atlanta, GA, U.S.A.). Tran³⁵S label (a mixture of [³⁵S]methionine and [³⁵S]cysteine) was obtained from ICN Radiochemicals (Irvine, CA, U.S.A.). Protein A-Sepharose, biotinylated concanavalin A, castanospermine and *N*-methyldeoxynojirimycin (NMDJ) were obtained from Sigma (St. Louis, MO, U.S.A.). Digitonin was purchased from Wako Chemical Co. (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.).

Antibodies

The IP₃R isoform-specific antibodies used in these studies were raised against unique C-terminal sequences of IP₃ receptors. The type I and type-III IP₃R antibodies were raised to amino acids 2731–2749 and 2657–2670 respectively of the rat IP₃R isoforms and have been previously characterized [15,16]. Results were confirmed using commercially available type I IP₃R antibody (Ab) (Affinity BioReagents Inc., Golden, CO, U.S.A.) and monoclonal type III IP₃R 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. FLAG-M2 Ab was purchased from Sigma (FLAG is an epitope tag corresponding to DYKDDDDK).

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 μ Ci/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 μ g/ml aprotinin/5 μ g/ml soybean trypsin inhibitor/5 μ g/ml leupeptin]. All lysates were precleared for 30 min by incubation with 20 μ l 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 IP₃R or chaperone Abs at 4 °C together with 50 μ l 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 μ g for every 0.5 mg of cell lysate immunoprecipitated. Under these conditions the IP₃R, 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 IP₃R-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 IP₃R 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 IP₃R cDNA was kindly given by Dr. Graeme Bell (Department of Biochemistry, University of Chicago, Chicago, IL, U.S.A.) [21]. The type-III IP₃R was epitope tagged at the N-terminus by using PCR to amplify the entire coding sequence with rat type-III IP₃R 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 Asn²⁵⁰³ 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 *Bsr*GI/*Eco*RI and ligated into *Bsr*GI/*Eco*RI-digested 1TM.

Cell-free transcription/translation assays

Plasmid DNA (5 μ g) was linearized with *Eco*R1. 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 μ l 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 μ l and

contained 10 μ l of rabbit reticulocyte lysate, 0.5 μ l of RNasin, 0.5 μ l of 1 mM amino acids (minus methionine), 20 μ Ci Tran³⁵S-label, 1.5 μ l of RNA template (1–3 μ g) and 6 μ l of buffer A, pH 7.2, which contained 110 mM potassium acetate, 2 mM magnesium acetate and 20 mM potassium Hepes. Where appropriate, the translation reaction mixtures contained 3 μ l of nuclease-treated canine pancreatic microsomes prepared as described in [23]. For immunoprecipitation, the translation mixture was diluted into 500 μ l of a solubilization buffer containing 150 mM NaCl, 50 mM Tris/HCl, pH 7.8, 1% digitonin, 1 mM EDTA, 0.5 mM PMSF and 5 μ g/ml each of aprotinin, soybean trypsin inhibitor and leupeptin. The samples were pre-cleared with 20 μ l of Pansorbin. After removal of Pansorbin by centrifugation (10000 g; 10 min), IP₃R- or chaperone-specific Ab and 50 μ l 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 autoradiography 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 included a band with a mobility identical with that of newly synthesized type-I IP₃R. This band was not observed in calreticulin 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 immunoprecipitation 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 serum, 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, indicating 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 immunoprecipitating 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 markedly 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 substrate 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 μ l 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 μ l 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 μ l) was added to remove any free IgG and, after centrifugation, 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 $\pm$ 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 IP₃R associated with calnexin by approx. 50% without significantly affecting the basal level of labelled IP₃R (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 IP₃R with calnexin (Figure 3). In our hands castanospermine (1 mM), another commonly used glucosidase inhibitor, was ineffective in WB cells and did not disrupt

Figure 3 Effect of DTT and NMDJ on calnexin-bound nascent IP₃R in WB cells

Confluent WB cells were incubated for 30 min in methionine-free DMEM, pulse-labelled for 30 min with 100 μ Ci/ml of Tran³⁵S-label and chased for 1 h with 20 mM methionine. Where added, the glucosidase inhibitor NMDJ was present at a final concentration of 1 mM during the preincubation, labelling and chase periods. DTT was added at a final concentration of 1 mM during the chase period only, since its presence at any earlier stage of the experiment resulted in a diminished incorporation of label into the IP₃R. Lysates from control and treated cells were divided into two aliquots and immunoprecipitated with either type-I IP₃R Ab (IP₃R) or calnexin Ab (CXN). (A) Shows the result from a representative experiment and (B) shows the quantification of three separate experiments in which the data from each experimental condition were expressed relative to the amount of label immunoprecipitated by type-I IP₃R Ab from untreated cells (A, lane 1). The value to the left of the gels (205) is the molecular mass in kDa. Abbreviation: ip, immunoprecipitating.

interaction of any of the labelled polypeptides immunoprecipitated by calnexin Ab (results not shown).

Calnexin and calreticulin binding to mature IP₃Rs in WB cell lysates

The binding of calnexin or calreticulin to mature (unlabelled) IP₃Rs in WB lysate was investigated in Figure 4. Unlabelled WB cell lysates were immunoprecipitated with non-immune serum, type I IP₃R, type-III IP₃R, 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 IP₃Rs in WB cells, the type-I Ab immunoprecipitated type-III IP₃R (Figure 4B, lane 2) and the type-III Ab immunoprecipitated type-I IP₃R (Figure 4A, lane 3). Calnexin Ab immunoprecipitates contained faint bands corresponding to mature type-I (Figure 4A, lane 4) and type III IP₃R (Figure 4B, lane 4). Calnexin was co-immunoprecipitated by type-I IP₃R Ab (Figure 4C, lane 2), but was not detectable in

Figure 4 Calnexin and calreticulin binding to mature type-I and type-III IP₃R

Cell lysates from unlabelled cells were immunoprecipitated with the following Abs: non-immune serum (lane 1), type-I IP₃R ('IP₃R') Ab (lane 2), type-III IP₃R 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 IP₃R Ab (A). The same immunoblot was then stripped and sequentially immunoblotted with type-III IP₃R Ab (B) and calnexin Ab (C). The values to the left of the gels are molecular masses in kDa.

the type-III Ab immunoprecipitates. The amount of calnexin co-immunoprecipitating with type-I IP₃R was a small fraction of the total calnexin in the lysate (compare with Figure 4C, lane 4).

Immunoblotting with calreticulin Ab on separate samples run on SDS/10%-PAGE showed no calreticulin in the type-I, type-III or calnexin Ab immunoprecipitates (results not shown). Trace amounts of type-III IP₃R (Figure 4B, lane 5) and a ladder of diffuse and weakly-stained type-I IP₃R immunoreactive bands (Figure 4A, lane 5) were immunoprecipitated by calreticulin Ab from unlabelled WB cell lysates. Calnexin was observed in the calreticulin immunoprecipitates (Figure 4C, lane 5), a result which has been previously interpreted as indicating that a fraction of the two chaperones may be complexed together [28]. Overall, the results indicate that a very small proportion of the total pool of mature IP₃Rs is present as stable complexes with calnexin or calreticulin. This is in contrast with the substantial amounts of nascent type-I IP₃R found to be associated with calnexin in WB cells.

Calnexin and calreticulin binding to transfected IP₃Rs in COS cell lysates

WB cells also express the type-III IP₃R isoform which migrates below the type-I IP₃R on SDS/PAGE [5]. However, only a single radioactive band corresponding to the type-I IP₃R is immunoprecipitated by calnexin Ab from pulse-labelled WB cell lysates (Figures 1A, 2A and 4A). The failure to observe significant amounts of an additional radioactive band corresponding to the type-III IP₃R isoform is probably due to the low incorporation of radioactivity into this isoform under the labelling conditions used in the present study. A further complication is that the two isoforms form hetero-oligomers in WB cell lysates [5], making it hard to distinguish selective binding of calnexin to an individual isoform. To circumvent this problem we have chosen to transiently express an epitope-tagged type-III IP₃R isoform in COS cells. These cells contain type-II and type-III IP₃Rs, but have negligible amounts of type-I IP₃R [31]. In separate studies we have found that IP₃R isoforms transfected into COS cells predominantly form homotetramers and have a very limited ability to form hetero-oligomers with the endogenous pool of IP₃Rs (S. K. Joseph, S. Bokkala, D. Boehning and S. Ziegler, unpublished work). Figure 5(A) shows the results of experiments in which COS cells were transfected with type-I or FLAG-tagged type-III IP₃R cDNA and labelled with ³⁵S for 30 min at 48 h

Figure 5 Association of newly synthesized IP₃Rs to calnexin in COS cells transiently transfected with IP₃R cDNA

(A) COS cells grown in 60 mm-diameter dishes were transiently transfected with 6 µg of pcDNA 3.1 vector alone (mock transfection), type-I IP₃R cDNA or FLAG ('Flag')-tagged type-III IP₃R 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. IP₃Rs were immunoprecipitated from one aliquot using type-III IP₃R Ab (lane 1), type-I IP₃R 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 IP₃R 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.

post-transfection. The labelled lysates were immunoprecipitated with either IP₃R, calnexin or calreticulin Ab. As observed in WB cell lysates, calnexin Ab precipitates a band of the same mobility as the newly synthesized type-I IP₃R in COS cells transfected with type-I IP₃R cDNA (Figure 5A, lane 5). Similarly, calnexin Ab also immunoprecipitated FLAG-tagged type-III IP₃R in COS cells transfected with FLAG-III IP₃R cDNA (Figure 5A, lane 8). Calreticulin Ab immunoprecipitated a large number of radiolabelled polypeptides from COS cell lysates, but the pattern of polypeptides was the same in mock-transfected or IP₃R-transfected cells (compare lanes 3, 6 and 9). In particular, there was no band corresponding to the position of the FLAG-III

Figure 6 Calnexin and calreticulin binding to *in vitro*-translated IP₃R 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) IP₃R 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), IP₃R 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.

IP₃R in calreticulin immunoprecipitates (Figure 5, lane 9). Treatment of FLAG-III-transfected COS cells with castanospermine inhibited the association of newly synthesized FLAG-III IP₃R 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 IP₃R with calnexin by 88% (results not shown). These results indicate that the type-III IP₃R 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 IP₃Rs

RNA templates encoding the six TM domains present in the C-terminal portion of type-I and type-III IP₃R 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 IP₃R molecule that are important for such interactions. 1TM and 3TM RNA templates (defined in Figure 6a) were translated in the presence of Tran³⁵S-label. Digitonin lysates were prepared from the microsomes and immunoprecipitated with IP₃R, 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 IP₃R constructs

(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 IP₃R 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 IP₃R and calreticulin Ab, and the samples were processed on 16 cm gels. It should be noted that lower autoradiograph exposures for the IP₃R 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.

Figure 8 Specificity of calnexin association with truncation and glycosylation mutants of 1TM

In vitro translations were programmed with RNA templates encoding 1TM, the C-terminal segment from TM domain 5 to the C-terminus (1TM5,6), the first four TM domains (1TM1-4,tag) or the 1TM construct in which both glycosylation sites had been mutated (1TM-DGM; N2475S,N2503S). The boundaries of each of these constructs are given in Figure 6(a). After 1 h the translation mixtures were lysed in a buffer containing 1% digitonin, split into equal aliquots and immunoprecipitated with IP₃R Ab (I) or calnexin Ab (CXN). Autoradiographs were developed to give approximately equal exposures for the type-I Ab immunoprecipitates. At lower exposures it is evident that 1TM1-4,tag and 1TM-DGM translates as a single radioactive band (results not shown). The values to the left of the gels are molecular masses in kDa. Abbreviation: ip, immunoprecipitating.

time translation was terminated, was $8.0 \pm 1.8\%$ ($n = 7$) and $30.2 \pm 5.8\%$ ($n = 4$) 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 6b, 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 IP₃R 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 IP₃R 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 suggests that this chaperone may recognize a specific 'glycoform' of the IP₃R. 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 (Glc₁Man₆GlcNac₂) 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 IP₃R biosynthesis, and our experimental data show that calnexin interacts with newly synthesized type-I and type-III IP₃R isoforms. 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 IP₃Rs 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 IP₃R was specific for a pool of glycosylated translation products. The inhibitory effects of glucosidase inhibitors, the localization of the calnexin-binding region of the IP₃R to the segment containing the glycosylation sites and the suppression of binding when these sites are mutagenized all point to the importance of the glycosylation sites in calnexin recognition of the type-I IP₃R. The experiments on COS cells, a cell line which contains extremely low levels of endogenous type-I IP₃R [31], confirm that calnexin can also interact directly with the type-III IP₃R 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 IP₃Rs, that are normally resident in the ER.

Surprisingly, calreticulin did not bind to newly synthesized IP₃Rs in lysates from WB cells, transfected COS cells or the *in*

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, vesicular-stomatitis-virus G-protein [38], the CD3 δ 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 μ M) or thapsigargin (10 μ M) treatment in WB cells, and was also not affected by the addition of the calcium ionophore A23187 (1 μ M) 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.

We thank Dr. A. Helenius and Dr. David B. Williams for an initial supply of calnexin Abs, and Dr. Christopher Nicchitta (Department of Cell Biology, Duke University, Durham, NC, U.S.A.) for canine pancreas microsomes. This work was supported by training grant T32-AA07463 (to S.B. and D.F.B.), and grants R01-DK34804 and R01-AA10971 from the National Institutes of Health.

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Received 4 March 1999/30 April 1999; accepted 28 May 1999