Calnexin is an integral membrane protein that acts as a chaperone during glycoprotein folding in the endoplasmic reticulum. Cross-linking studies were carried out with the aim of investigating the interactions of calnexin with glycoproteins in vitro. A truncated version of the integral membrane glycoprotein Glut 1 (GT155) was synthesized in a rabbit reticulocyte translation system in the presence of canine pancreatic microsomes. Following immunoprecipitation with an anti-calnexin antiserum, a cross-linker-independent association was observed between GT155 and calnexin. In addition, the anti-calnexin antiserum immunoprecipitated a UV-dependent cross-linking product consisting of GT155 and a protein of approximately 60 kDa designated CAP-60 (calnexin-associated protein of 60 kDa).

Both the GT155-calnexin and the GT155-CAP-60 interactions were dependent on the presence of a correctly modified oligosaccharide group on GT155, a characteristic of many calnexin interactions. A GT155 mutant that was not glycosylated (AGGT155) did not associate with calnexin or CAP-60.

Calreticulin, the soluble homologue of calnexin, was also shown to interact with GT155 only when the protein bore a correctly modified oligosaccharide group. Thus, our data show that both calnexin and calreticulin with Glut 1 in a glycosylation-dependent manner.

Membrane and secretory proteins are co-translationally translocated into the endoplasmic reticulum (ER)* membrane or lumen in an unfolded or partially folded state (1). To ensure that these proteins reach their subcellular destination in the correct conformation a variety of chaperones are required (2). ER chaperones, such as BiP and calnexin, bind transiently to polypeptide chains during the folding and assembly of oligomeric complexes. These interactions prevent inappropriate folding or assembly, and once a certain folding state, or stage of complex assembly, has been reached the chaperone is no longer needed and dissociates. This dissociation often correlates with the acquisition of “transport competence” by the newly synthesized protein, allowing it to exit the ER and be transported to later compartments of the secretory pathway. In this way chaperones such as calnexin and BiP function to ensure “quality control” of proteins during transit through the ER (3, 4).

Calnexin is an integral membrane protein of the ER (reviewed in Ref. 5). Transient associations have been observed between calnexin and many newly synthesized proteins in the ER. When misfolding is induced by the incorporation of a proline analogue (azetidine-2-carboxylic acid) into polypeptide chains, the association of newly synthesized proteins with calnexin is prolonged (6). A similar effect can be seen by induction of a temperature-dependent folding defect in the G protein of ts045 vesicular stomatitis virus (7).

Calnexin is also involved in the assembly of protein complexes such as the T-cell receptor complex (8–10), the MHC class I complex (11–13), and the MHC class II complex (14, 15). Calnexin binds to subunits that are incompletely or incorrectly folded or assembled and retains them in the ER.

A key factor in the function of calnexin in the quality control of protein synthesis at the ER is the glycosylation state of the target protein. Calnexin predominantly interacts with proteins possessing asparagine-linked carbohydrate (6). Furthermore, trimming of the terminal glucose residues of the oligosaccharide chains present on these proteins appears to be necessary for the action of calnexin. Inhibition of the glucose-trimming enzymes glucosidase I and glucosidase II by castanospermine or 1-deoxynojirimycin treatment prevents calnexin binding (6, 10, 16, 17). Three glucose residues are present on the oligosaccharide side chain when it is added to the emerging polypeptide chain by the oligosaccharyl transferase (18). The first two are rapidly removed by glucosidases I and II, and it is the resulting monoglucosylated form that associates tightly with calnexin (17). The final glucose residue is slowly removed by glucosidase II, but reglucosylation can occur catalyzed by UDP-glucose-glycoprotein glucosyltransferase. This enzyme only acts on unfolded or misfolded proteins (19, 20); hence, it is thought to be part of the quality control mechanism of the ER. In this hypothesis, unfolded proteins are reglucosylated and bind to calnexin, whereas properly folded proteins are not reglucosylated and therefore are not good substrates for calnexin binding (18).

The specificity of calnexin may be more complex than implied above since, in certain cases, proteins that are not glycosylated can associate with calnexin. These include the CD3ε subunit of the T-cell receptor complex and thyroglobulin (9, 21). The significance of such cases is not clear.

BiP (heavy chain binding protein) is an ER luminal chaperone, which shares some substrates, for example vesicular stomatitis virus G protein (22) and thyroglobulin (21), with calnexin. In the case of vesicular stomatitis virus G protein it has been shown that BiP binds to early folding intermediates and...
calnexin binds to later folding intermediates. The opposite sequence of binding, first calnexin and then BiP, occurs for thyroglobulin. This indicates that calnexin is part of an integrated chaperone system in the ER involving other proteins such as BiP and protein disulfide isomerase.

In this study we have investigated the interaction of calnexin with a truncated form of the human glucose transporter (Glut 1), a multispanning membrane protein. Our results show that both calnexin and calreticulin interact with the truncated Glut 1 polypeptide.

**EXPERIMENTAL PROCEDURES**

**Materials—** Restriction endonucleases and endoglycosidase H (Endo H) were purchased from New England Biolabs (Herts, United Kingdom). T7 RNA polymerase, transcription buffers, and rabbit reticulocyte lysate were supplied by Promega (Southampton, UK). Cytochrome c, proteinase K, phenylmethylsulfonyl fluoride, and 1-deoxy-d-mannojirimycin were purchased from Sigma (Dorset, UK). The cross-linking reagents 4-4′-diazidodiphenyl ether (DADE) and bis(β-(4-azidosalicylamido)ethyl) disulfide (BASED) were purchased from Procherm, Inc. (Rockford, Ill.). 1-Deoxy-d-mannojirimycin was supplied by Oxford Glycosystems (Oxford, UK). Protein A-Sepharose was supplied by Pharmacia Biotech Inc. (Herts, UK). L-[35S]-Methionine was purchased from Amersham International (Bucks, UK). Polyonal rabbit anti-calreticulin serum was from Affinity BioReagents (Neshanic Station, NJ). Anti-calnexin serum raised against the carbonyl terminus of calnexin was a gift from Dr. Ari Helenius (Department of Cell Biology, Yale University, New Haven, CT). DNA encoding the human glucose transporter, Glut 1, as a BamH/HindIII fragment in pGEM11Zf(+) was kindly provided by Dr. Steve Baldwin (Department of Molecular Biology, University of Leeds, UK). The pH 9-washed microsomes were kindly provided by Dr. Neil Bulleid (Division of Biochemistry, University of Manchester, UK).

**Transcription and Translation—** The Glut 1 plasmid was linearized with Apal, which degrades the DNA within the coding sequence. This linearized plasmid was used as a template in a T7 RNA polymerase transcription system according to the manufacturer's instructions (Promega). The resulting mRNA was translated for 30 min in a rabbit reticulocyte lysate translation system according to the manufacturer's instructions (Promega) or a wheat germ lysate translation system as described previously (23) in the presence of L-[35S]-Methionine to label the translation products. Canine pancreatic microsomes were added prior to commencement of translation. Translation was terminated after 30 min by the addition of 2.5 mM cycloheximide and subsequent incubation on ice.

Following translation the membrane-associated fraction was isolated by centrifugation through a high salt/sucrose cushion (250 mM sucrose, 500 mM potassium acetate, 5 mM magnesium acetate, 50 mM Hepes/KOH, pH 7.9) at 140,000 g for 10 min at 4°C. The membrane pellet was resuspended in a low salt/sucrose cushion (250 mM sucrose, 100 mM potassium acetate, 5 mM magnesium acetate, 50 mM Hepes/KOH, pH 7.9).

**Cross-linking—** Following translation of GT155 in the wheat germ lysate system, samples were treated by the addition of either reticulocyte lysate prior to centrifugation or DADE or BASED subsequent to centrifugation through a high salt/sucrose cushion. Following translation of GT155 in the reticulocyte lysate system no additions were made. UV irradiation was carried out using a Spectroline model B-100/F black light lamp equipped with a 100-W mercury bulb and a 365-nm filter. Irradiation was for 10 min (Fig. 2) or 30 min (Figs. 3A, 4, and 5) at 100 W/m2. DADE and BASED are homobifunctional cross-linking agents that contain azido groups and generate reactive nitrates when irradiated with UV. BASED is a thiol-deactivable reagent. Cross-linking with DADE and BASED involved the addition of a stock solution of the reagent (at 50 mM in dimethyl sulfoxide) to the resuspended membrane pellet to 1 mM final concentration followed by irradiation with UV for 30 min where indicated.

Following cross-linking the samples were subjected to either trichloroacetic acid precipitation to yield the total membrane-associated fraction or immunoprecipitation.

**Immunoprecipitation—** For “native” immunoprecipitation the resuspended membrane pellet was diluted with 4 volumes of IP buffer (100 mM Hepes/KOH, pH 7.6, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100). For “denaturing” immunoprecipitation 1% SDS was added to the samples followed by heating to 95°C for 5 min and dilution with 4 volumes of IP buffer as above. Methionine was added to 1 mM, and the protease inhibitor phenylmethylsulfonyl fluoride was added to 0.2 mM. The samples were incubated on ice for 30 min and then centrifuged at 16,000 × g for 5 min. To aliquots of the resulting supernatant 1 μl of control (preimmune or nonrelated) serum or antisem was added as indicated. The samples were incubated overnight at 4°C with mixing. Protein A-Sepharose, which had been preincubated with 20% bovine serum albumin for 30 min and then washed 5 times with IP buffer, was added to the samples, and the incubation continued for 2 h. The samples were washed 5 times with IP buffer.

**RESULTS**

Association of GT155 and Calnexin—With the aim of studying the interactions of newly synthesized glycoproteins in the ER, we chose the Glut 1 glucose transporter as a model membrane glycoprotein. Full-length Glut 1 has 12 membrane-spanning domains; however, following preliminary work, we decided to use a truncated version encoding only the amino-terminal 155 residues (GT155). This construct contains a site for N-glycosylation and should be sufficiently long to allow the first two membrane-spanning domains to insert into the microsomal membrane. In addition, due to the absence of a stop codon we were able to study the interactions of GT155 while it was still attached to the ribosome (24).

GT155 was translated in the absence and presence of microsomes (Fig. 1A). A shift in mobility occurred equivalent to an increase of approximately 4 kDa upon addition of microsomes (Fig. 1A, lane 2 compared with lane 1). Endo H digestion of the larger species confirmed that it was due to the addition of an oligosaccharide side chain to GT155, this process being almost 100% efficient (Fig. 1A, lane 3). Glut 1 has a single site for glycosylation at asparagine residue 45. The observation that this residue was glycosylated in the in vitro system indicated that it was correctly located on the luminal side of the membrane. Further evidence that membrane insertion of GT155 occurred in this system was provided by the resistance of the polypeptide to protease digestion (Fig. 1A, lane 4 compared with lane 5). A model of the putative structure of the translation intermediate GT155 is given in Fig. 1B.

An “aglyco” mutant of GT155, AGGT155, in which asparagine 45 had been mutated to a threonine with the aim of preventing glycosylation (25), was translated in this system (Fig. 1A, lane 6). AGGT155 was not glycosylated and migrated with an identical mobility to GT155 following incubation in the absence of microsomes (Fig. 1A, lane 1). AGGT155 was also protease-resistant and therefore membrane-inserted (data not shown).

The membrane chaperone calnexin interacts with many glycoproteins in the ER (6), so we tested whether GT155 associated with this chaperone. Following translation of GT155 and centrifugation through a sucrose cushion to isolate the membrane-associated fraction, immunoprecipitation was carried out with the anti-calnexin antibody. Glycosylated GT155 was found to be co-immunoprecipitated by the calnexin antibody, suggesting that there was an interaction between calnexin and GT155 (Fig. 2, lane 2).

A further product, which migrated at approximately 25 kDa, was also co-immunoprecipitated by calnexin antisera (Fig. 2, band X). This product was sensitive to Endo H treatment (data not shown). We believe this product to be the result of a post-translational modification to glycosylated GT155 causing a change in mobility.

Preliminary experiments had demonstrated a difference between in vitro translation systems with regard to the products immunoprecipitated by the anti-calnexin antibody. This was investigated further using the wheat germ lysate translation system or immunoprecipitation.
Glut 1-Calnexin and -Calreticulin Interactions

A product of approximately 110 kDa, which was barely visible in the absence of additions, was immunoprecipitated by the anti-calnexin antibody following the post-translational addition of reticulocyte lysate (Fig. 2, lane 6). UV irradiation of the reticulocyte lysate-treated samples led to the appearance of an additional product of approximately 80 kDa (Fig. 2, lane 8). These findings indicated that reticulocyte lysate could promote the formation of adducts between GT155 (approximately 20 kDa) and two different species of approximately 90 and 60 kDa.

The effect of reticulocyte lysate in this system could be duplicated by using two homobifunctional photoactivable crosslinking reagents, DADE and BASED. Both compounds were able to mimic the effect of reticulocyte lysate addition to GT155 translated in the wheat germ system (Fig. 2, lanes 9–16). This evidence supports the existence of an endogenous cross-linking factor present in reticulocyte lysate and reinforces our observation that GT155 interacts with two species, of approximately 90 and 60 kDa, both products being immunoprecipitated with anti-calnexin antibodies.

Calnexin migrates with an apparent molecular weight of 90 kDa in the SDS-PAGE system, suggesting that the 110-kDa product was a complex of GT155 and calnexin. Treatment of the membrane fraction with 1% SDS and heating to 95°C prior to immunoprecipitation with anti-calnexin confirmed that the

110-kDa product was recognized under these denaturing conditions and therefore was a calnexin-derived complex (data not shown, and Fig. 5, lane 5). The 80-kDa product was not immunoprecipitated under these denaturing conditions, indicating that the protein involved was not calnexin but remained associated with calnexin under “native” conditions. This protein was designated a calnexin-associated protein of 60 kDa, CAP-60.

UV irradiation of the membrane fraction was not required in order to obtain the calnexin adduct (Fig. 2, lane 6 compared with lane 8). Thus the 110-kDa product is not a true cross-linking product but does represent an SDS-stable complex or adduct formed between calnexin and GT155. A comparison of samples solubilized in sample buffer with and without dithiothreitol was performed, and N-ethylmaleimide was used to treat samples after solubilization in the presence of dithiothreitol. In all cases a similar amount of the 110-kDa product was observed, indicating that the product is not the result of aberrant disulfide bond formation (data not shown). The CAP-60-GT155 product was observed only after UV irradiation.

The Presence of a Glucose-trimmed Oligosaccharide Side Chain Is Essential for Calnexin Binding to GT155—Previous studies have demonstrated that calnexin requires glycosylation of the substrate protein for binding and also that calnexin interacts with the glucose-trimmed monoglucosylated form of proteins such as influenza hemagglutinin and not the triglucosylated form of the protein (17). The effect of glycosylation and glucose-trimming on the interactions between GT155 and calnexin and CAP-60 was investigated.

First, the GT155 product immunoprecipitated by anti-calnexin were treated with Endo H to confirm that the glycosylated form of GT155 was present in the products. The apparent molecular weight of the GT155-calnexin adduct and the GT155-CAP-60 cross-link decreased following Endo H treatment (Fig. 3A, lane 3 compared with lane 2). This indicated the presence of at least one glycosylated side chain in each product. Since calnexin is not glycosylated, the GT155 in the GT155-calnexin adduct must be glycosylated.
The need for glycosylation and glucose trimming, already present while calreticulin (a luminal protein) was greatly decreased in comparison (data not shown).

Calreticulin shows strong sequence similarity to calnexin and has been implicated as a potential chaperone in glycoprotein folding (28, 29). Since calreticulin has an apparent mobility of 60 kDa on SDS-PAGE it was an obvious candidate for being CAP-60. This theory was tested by immunoprecipitation using an anti-calreticulin antibody. Under "native" immunoprecipitation conditions the anti-calreticulin antibody recognized a band of identical mobility to GT155-CAP-60 (Fig. 5, lane 9). This result suggested that CAP-60 may be calreticulin. Denaturing immunoprecipitation with this antibody failed to detect the GT155-CAP-60 cross-linking product. However, this may be due to the overall low efficiency of immunoprecipitation under these conditions (compare GT155-calnexin in Fig. 5, lanes 8 and 11).

The need for glycosylation and glucose trimming, already
Glut 1-Calnexin and -Calreticulin Interactions

Fig. 5. Immunoprecipitation with calreticulin antibody. GT155 was translated in a reticulocyte lysate system, and the membrane-associated products were isolated. The samples were subjected to UV irradiation where indicated. Immunoprecipitation (native) was carried out with control serum (lanes 1 and 7), anti-calnexin (lanes 2 and 8), and anti-calreticulin (lanes 3 and 9). Immunoprecipitation following treatment with 1% SDS at 95 °C for 5 min (denaturing) was carried out with control serum (lanes 4 and 10), anti-calnexin (lanes 5 and 11), or anti-calreticulin (lanes 6 and 12). The samples were analyzed on a 10% polyacrylamide gel.

Fig. 6. Association of CAP-60 with GT155 requires glycosylation and glucose trimming. Translations were set up in a reticulocyte lysate system as follows: an "aglyco" mutant of GT155, which cannot be glycosylated (AGGT155); GT155; GT155 + 5 mM 1-deoxynojirimycin (GT155 + dNM); GT155 + 5 mM 1-deoxymannojirimycin (GT155 + dMM). The membrane-associated products were isolated and irradiated with UV. Aliquots were taken for trichloroacetic acid precipitation (membrane associated, lanes 1–4). The remaining samples were subjected to the following: immunoprecipitation with control serum (lanes 5, 8, 11, and 14); immunoprecipitation with anti-calnexin (lanes 6, 9, 12, and 15); immunoprecipitation with anti-calreticulin (lanes 7, 10, 13, and 16). The samples were analyzed on a 12% polyacrylamide gel.

Demonstrated for calnexin association, was investigated for the interaction of GT155 with CAP-60. The UV-dependent 80-kDa product was absent when the total membrane-associated fraction was analyzed in the aglyco mutant (Fig. 6, lane 1). As previously observed, following immunoprecipitation with anti-calnexin, the calnexin adduct and CAP-60 cross-link observed in the wild type (Fig. 6, lane 9) were both absent in the aglyco mutant (Fig. 6, lane 6). Similarly, immunoprecipitation with calreticulin antibodies resulted in a cross-linking product between wild type GT155 and calreticulin (Fig. 6, lane 10) that was absent in the aglyco mutant (Fig. 6, lane 7), confirming that glycosylation is essential for this interaction.

Inhibition of glucose-trimming by dMM treatment followed by analysis of the total products in the membrane-associated fraction showed that the 80-kDa product was absent following dMM treatment (Fig. 6, lane 3). Likewise, an 80-kDa cross-linking product was no longer immunoprecipitated by calreticulin antibodies following dNM treatment (Fig. 6, lane 13). In addition, the co-immunoprecipitation of glycosylated GT155 with calreticulin was greatly decreased following dNM treatment. Thus, the inhibition of glucose trimming was sufficient to prevent the association of GT155 with calreticulin. As a control, the mannosidase inhibitor 1-deoxymannojirimycin was also tested. No effect on calnexin, CAP-60, or calreticulin interactions was observed with this drug (Fig. 6, lanes 4, 15, and 16).

These data suggest that the CAP-60 component closely associated with calnexin is either calreticulin or a novel protein of identical mobility that binds specifically to glycoproteins with trimmed, N-linked, carbohydrate side chains.

DISCUSSION

The interaction between calnexin and nascent glycoproteins has been studied in various ways. To date, the majority of studies have analyzed the interaction between newly synthesized glycoproteins and calnexin by co-immunoprecipitation from metabolically labeled cells (6), from virally infected and metabolically labeled cells (16), and after in vitro translation with pancreatic microsomes (17). Stable complexes formed between calnexin and nascent proteins have been identified by these approaches. In this study we have used a cross-linking approach to stabilize interactions prior to solubilization and immunoprecipitation. Calnexin (p88) had previously been shown to cross-link to the transmembrane region of the heavy chain of the class I MHC complex (11, 30).

A glycosylated, truncated translocation intermediate of the multispansing membrane protein Glut 1 was chosen as a target protein for analyzing calnexin interactions. Immunoprecipitation with calnexin antibodies co-immunoprecipitated the truncated, glycosylated, GT155 polypeptide, together with two high molecular weight products that were candidates for calnexin-containing cross-linking products. Further investigation revealed one product to be a calnexin-GT155 complex, which was recognized by the calnexin antibody even following SDS denaturation.

The calnexin-GT155 interaction required glycosylation and glucose trimming, a feature consistent with many calnexin interactions (6, 10, 16, 17). A model for calnexin action has developed in which calnexin acts as a lectin, binding to the oligosaccharide side chains of glycoproteins (18). This model involves cycling of the glycosylation state of the protein involving the action of glucosidase I and glucosidase II, which remove glucose residues, and UDPglucose-glycoprotein glucosyltransferase, which adds a single glucose residue to a nonglycosylated oligosaccharide side chain. The folding sensor in this cycle seems to be the glucosyl transferase, which only acts on incompletely folded proteins (19, 20). Hence, the only escape from this cycle, and therefore from calnexin binding, is correct folding.

Evidence has been presented by Ware et al. (31) indicating that endoglycosidase H treatment of calnexin-glycoprotein

endoglycosidase H treatment of calnexin-glycoprotein
complexes does not dissociate the complex as would be expected if calnexin associations were solely via interaction with the carbohydrate moiety of the glycoprotein. Our data confirm the requirement for a correctly modified oligosaccharide side chain on GT155 in order to see calnexin binding; the specific molecular details remain to be established.

Co-immunoprecipitated with the calnexin-GT155 product, a smaller complex was observed. This complex, of approximately 80 kDa, behaves as a genuine cross-linking product mediated by UV-activation of an unidentified reticulocyte lysate factor. The 20-kDa GT155 was cross-linked to a 60-kDa protein (CAP-60), an interaction confirmed in a wheat germ lysate system, using two homobifunctional photoactivatable cross-linking agents, DADE and BASED.

The co-precipitation of the CAP-60-GT155 cross-linking product with anti-calnexin is very efficient, suggesting CAP-60 and calnexin may be in a complex. Indeed, a high molecular weight product (~190 kDa) was seen in some experiments (for example Fig. 6, lanes 9 and 15), which may represent cross-linking between all the components of such a ternary complex. At this point we cannot exclude the possibility that the co-precipitation of calnexin and CAP-60 is due to an indirect interaction, via either GT155 or an as yet unidentified molecule.

Further investigation using pH 9-washed microsomes demonstrated CAP-60 to be an ER luminal protein. A good candidate for CAP-60 was calreticulin, and we found that anti-calreticulin antibodies do recognize a product of identical size to the GT155-CAP-60 complex under native conditions, although not under denaturing conditions. Thus CAP-60 is either calreticulin or a protein of identical mobility with similar properties. A chaperone function for calreticulin has been proposed by other groups (28, 29). Calreticulin is a 46-kDa soluble ER calcium-binding protein, which has an apparent mobility on SDS-PAGE close to 60 kDa (reviewed in Refs. 32 and 33). Considerable sequence homology is observed between calreticulin and several luminal domains of calnexin (34, 35).

We found that the binding of calreticulin to GT155 also requires both glycosylation and processing of that glycosylation (see also Ref. 29). In the absence of these events the 80-kDa cross-linking product is absent from both total products and after immunoprecipitation with anti-calnexin and anti-calreticulin. In all cases the cross-linking between GT155 and calreticulin can occur from any region of GT155 since the photoactivatable reagent has not been biosynthetically incorporated (i.e. during translation) and does not have restricted reactivity for only certain amino acid side chains. We are therefore unable to address the question of exactly which region of GT155 is cross-linked to calreticulin. Since calreticulin is a luminal ER protein and the interaction is sensitive to the glycosylation of the luminal domain of GT155, we assume calreticulin is cross-linked somewhere within this portion of GT155.

Our analysis with pH 9-washed microsomes showed that GT155 glycosylated can interact with calnexin in the absence of most luminal components. Thus, the presence of calreticulin and/or CAP-60 is not essential for the interaction per se. However, our results do suggest that calnexin and CAP-60 may function as a protein complex that specifically binds to nascent glycoproteins. CAP-60 could influence the activity of calnexin or have a distinct role in modulating glycoprotein folding. In the latter case such a complex would facilitate a coordinated transfer of glycoprotein folding intermediates between subunits (cf. Ref. 36). Our current aim is to establish the identity of CAP-60 conclusively.

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Note Added in Proof—We have recently shown that CAP-60 is distinct from calreticulin and may represent a new glycoprotein-specific ER component (J. D. Oliver, N. J. Bulleid, and S. High, manuscript in preparation).

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