Calnexin Interaction with N-Glycosylation Mutants of a Polytopic Membrane Glycoprotein, the Human Erythrocyte Anion Exchanger 1 (Band 3)*

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The interaction of the endoplasmic reticulum chaperone calnexin with N-glycosylation mutants of a polytopic membrane glycoprotein, the human erythrocyte anion exchanger (AE1), was characterized by cell-free translation and in transfection HEK293 cells, followed by co-immunoprecipitation using anti-calnexin antibody. AE1 contains 12–14 transmembrane segments and has a single site of N-glycosylation at Asn-642 in the fourth extracytosolic loop. This site was mutated (N642D) to create a nonglycosylated protein. Calnexin showed a preferential interaction with N-glycosylated AE1 relative to nonglycosylated AE1 both in vitro and in vivo. This interaction could be blocked by inhibition of glucosidases I and II with castanospermine. Calnexin had access to novel N-glycosylated sites created in other extracytosolic loops in AE1 by site-directed or insertional mutagenesis. The interaction with AE1 was enhanced when multiple sites were introduced into the same loop or into two different loops. An association of calnexin with truncated versions of N-glycosylated AE1 was detected after release of the nascent chains from ribosomes with puromycin. The results show that the interaction of calnexin with the polytopic membrane glycoprotein AE1 was dependent on the presence but not the location of the oligosaccharide. Furthermore, calnexin was associated with AE1 after release of AE1 from the translocation machinery.

Calnexin, an integral membrane protein, and its soluble homolog, calreticulin, are endoplasmic reticulum (ER)* chaperones that bind transiently to newly synthesized glycoproteins, thereby helping their folding and oligomerization (1–4). These chaperones also display a prolonged interaction with misfolded and aggregated proteins, leading to their retention in the ER (5–7). Nascent polypeptides are targeted to the ER membrane by a signal recognition particle-dependent mechanism. The subsequent translocation of the polypeptide into the ER lumen and the integration of transmembrane segments into the ER membrane are mediated by a protein channel that forms the translocon (8). Calnexin is part of a multimeric protein complex, including calreticulin, BiP, GRP94, and Erp57, that “proofreads” the protein folding process in the ER (7, 9–11).

N-Glycosylation is a co-translational event involving the en bloc transfer of a Glc3Man9GlcNAc2 oligosaccharide from a dolichol donor to a lumenal acceptor site (Asn-X-Ser/Thr) in the nascent chain by the ER oligosaccharyl transferase (12–14). Co-translational removal of the terminal glucose residue occurs rapidly by the action of α-glucosidase I, followed by the successive removal of the second and third glucose residues by α-glucosidase II (15–19). The monoglucosylated oligosaccharide can be regenerated by a glucosyltransferase that acts posttranslationally on unfolded proteins (20). Calnexin and calreticulin are lectins that interact with the Glc3Man9GlcNAc2 oligosaccharide (21–26), and calnexin-bound monoglucosyl glycoproteins are protected from α-glucosidase II removal of the final glucose (25). Inhibition of α-glucosidase I and II with castanospermine (CST) prevents calnexin binding (27, 28), and calnexin associations are absent in glucosidase-deficient cell lines (29). The presence of a single oligosaccharide moiety on a protein is sufficient for the interaction with calnexin; however, the interaction is enhanced when multiple N-glycosylated sites are present (30, 31).

It has been proposed that the interaction of calnexin with glycoproteins may proceed in two stages; the first involves lectin binding to the oligosaccharide, and the second involves a protein-protein interaction (23, 32, 34). Calnexin can be co-immunoprecipitated with nonglycosylated proteins (6, 35–39). This oligosaccharide-independent interaction may, however, be the result of calnexin binding to aggregated protein (38).

Most studies of calnexin interactions have focused on secreted proteins or membrane proteins that contain a single transmembrane (TM) segment. Calnexin and calreticulin can interact with both soluble luminal glycoproteins and intrinsic membrane glycoproteins, although some glycoproteins show a preferential interaction (10, 22, 39–41). Calnexin is a type I membrane protein with a luminal lectin domain (42) that can readily interact with membrane proteins that contain N-glycosylated sites including those positioned close to the membrane surface (31). Calnexin is known to bind to newly synthesized, single-span membrane glycoproteins, such as MHC class I and II (23, 37), hemagglutinin (43), and vesicular stomatitis virus glycoprotein (38), facilitating their folding and cell-surface expression (2, 34). Calnexin is also involved in the folding and maturation of polytopic membrane glycoproteins, such as P-glycoprotein (35), cystic fibrosis conductance transmembrane regulator (44), the T cell receptor (39), and the acetylcholine receptor (45, 46). Because most polytopic membrane proteins

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†The abbreviations used are: ER, endoplasmic reticulum; AE, anion exchanger; AE1md, membrane domain of AE1; CST, castanospermine; EC, extracytosolic; PBS, phosphate-buffered saline; TM, transmembrane; ins, insertion; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
are N-glycosylated on a single EC loop, greater than 30 residues in size (47), these proteins are likely to interact with calnexin. However, the position and number of N-glycosylated sites in polytopic membrane proteins varies. We therefore determined whether calnexin was able to interact with oligosaccharide chains located at different positions in a polytopic membrane protein and whether this interaction occurred while the protein was held within the translocation machinery.

In this study, the interaction of calnexin with N-glycosylated mutants of the human erythrocyte anion exchanger, AE1 (band 3) was studied using an in vitro translation system and in transfected cells. AE1 contains two structural domains: a large amino-terminal domain located in the cytoplasm and a carboxy-terminal, membrane-spanning domain responsible for the anion exchange function (Fig. 1) (48). Our recent topology model of AE1 predicts 12 TM segments, with a single site of N-linked glycosylation at Asn-642, located in the fourth EC loop (Fig. 1) (49). We show that calnexin interacts with AE1 in a glycosylation-dependent fashion and that this interaction is not dependent on the position of the oligosaccharide within this polytopic membrane protein. Truncated constructs of AE1 that retain tRNA on their nascent chain were tested for co-translational interaction with calnexin. The calnexin interaction was detected by co-immunoprecipitation when the translationally arrested polypeptide was released by puromycin. This shows that the predominant calnexin-AE1 interaction occurs posttranslationally.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following is a list of products and their sources: Modulus™ in vitro transcription system and canine pancreatic microsomes (MBI Fermentas), Flexi rabbit reticulocyte lysate and RNAin (Promega), [35S]Met (Promega), [35S]Met and [35S]Met (Amersham Pharmacia Biotech), digitonin and Triton X-100 (Roche Molecular Biochemicals), Brij 96 (Sigma), digitonin with 20–30 µm filter unit (Wheaton). Polyclonal rabbit anti-calnexin antibodies directed against a carboxy-terminal peptide (antibody C) corresponding to the last 14 residues of canine calnexin (6) or against ER luminal region of calnexin (antibody N) expressed and purified from Sf9 cells (residues 1–461) as described in Ref. 26 were kind gifts from Dr. David Williams (University of Toronto). Polyclonal rabbit anti-AE1 antibody directed against a carboxy-terminal peptide corresponding to the last 12 residues of human AE1 (50) was employed to detect AE1.

**Oligonucleotide-directed Mutagenesis**—The cDNA of human Band 3 in BlueScript vector BSSK™ was a kind gift from Drs. A. M. Garcia and H. Lodish (Whitehead Institute). The endogenous N-glycosylation site at Asn-642 was mutated to Asp (N642D) in the membrane domain construct of AE1-AE1md (residues 386–911, as described (51). The Full-length AE1 and the AE1md were subcloned into pcDNA3 vector as described (50). A series of unique N-glycosylation acceptor sites were created in the AE1md or in the N642D construct (Fig. 1) using oligonucleotide-directed mutagenesis and the double-stranded mutagenesis system from CLONTECH. A series of mutants were made containing unique restriction enzyme sites that cut at amino acid positions 696 (NcoI), 714 (NotI), 754 (XbaI), 785 (XbaI), and 820 (XbaI) in the AE1md construct.

**Insertion of EC Loop 4 into EC Loops 1, 2, and 7**—Short EC loops in TM were expanded by insertion of a 35-amino acid sequence corresponding to residues 626–650 of EC loop 4, containing the endogenous or mutated (N642D) N-glycosylation acceptor site, as described (49, 52). The insertion mutants are illustrated in Fig. 1. In insertion mutants ins.EC1 and ins.EC7, the sequence of EC loop 4 containing the endogenous acceptor site, Asn-642, was inserted into EC loop 1 and EC loop 7, respectively, whereas the endogenous site was mutated to N642D. Insertion constructs ins.EC2a contained an EC loop 4 insertion in EC loop 1, ins.EC1a in EC loop 2, ins.EC2b in EC loop 1, and ins.EC7b in EC loop 7. For each of these mutants, 50 residues was replaced by two unique restriction enzyme sites that cut at amino acid positions 696 (NcoI), 714 (NotI), 754 (XbaI), 785 (XbaI), and 820 (XbaI) in the AE1md construct.

**In Vitro Transcription and Translation**—Modulus™ in vitro transcription and translation system and plasmid DNA (0.5 µg) in a final volume of 10 µl were used to synthesize mRNA. The transcription mixture (3 µl) was used for in vitro translation using Flexi rabbit reticulocyte lysate as described by the supplier; the lysate was supplemented with 2 units of canine pancreatic microsomes in a final volume of 30 µl. Membrane proteins were separated by SDS-PAGE and proteins were detected by including samples (10 µl) with 100 µl of ice-cold 0.1 M Na2CO3, pH 11.5, followed by recovery of thestriped membranes by centrifugation (16,000 x g for 20 min). Alternatively, 35 µM Ac-NYT-NH2 (to block the N-glycosylation of AE1) or CST (to inhibit α-glucosidase 1 and II) was included in the translation mixture when indicated. For co-translational interaction, the cDNA corresponding to the AE1md or AE1N642D was linearized at amino acid position 696 (NcoI), 714 (NotI), 754 (XbaI), 785 (XbaI), 820 (XbaI), and 854 (NaeI, the endogenous unique restriction site). The insertion mutant ins.EC1 (Fig. 1) was linearized at position 854 (NaeI). The translation reaction was performed at 27 °C for 30 min. Following the translation reaction, two separate additions were made: (i) 1.5 mM puromycin, followed by an additional 10-min incubation at 27 °C, or (ii) 35 µM Ac-NYT-NH2, 1 mM CST, followed by incubation on ice for 5 min. The stability of attached oligosaccharides was time- and temperature-dependent. Lower temperatures (25 °C) and shorter translation times (30 min) favored the stability of the complex but significantly reduced protein biosynthesis (54).

**Immunoprecipitation**—For co-immunoprecipitation, 20 µl of the translation reaction was solubilized in 600 µl of PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) containing 1.0% (w/v) digitonin, 1.0% Triton X-100, 0.1% deoxycholic acid, 1.0 µM apotinin, 1.0 µM leupeptin, 1.0 µM peptatin A. Alternatively, 1.0% Triton X-100, Brij 96, or CHAPS was used as indicated. After 3–4 h of incubation at 4 °C with anti-calnexin antibody (5 µl), 20 µl of protein G-Sepharose beads were washed, followed by incubation with 1% SDS (w/v) at 30 °C for 15 min. Beads were washed with PBS buffer containing 0.2% digitonin and bound proteins were solubilized in 20 µl of 2× SDS sample buffer (4× SDS), at room temperature.

**Expression of AE1 in Transfected Cells**—HEK293 cells were grown in Dulbecco’s modified Eagle’s medium and transfected with 5 µg of DNA/10-cm dish, using the DEAE-dextran method as described in Ref. 50. 20–24 h following transfection, cells were labeled with [35S]Met (0.1 µCi/ml) for 20–25 min. Labeled cells were washed once with PBS buffer, and cells were solubilized in PBS containing 1% (w/v) digitonin and bound proteins were solubilized in 20 µl of 2× SDS sample buffer (4× SDS), at room temperature.

The radiolabeled proteins were examined by SDS-gel electrophoresis followed by autoradiography. The efficiency of N-glycosylation was determined by scanning autoradiographs of SDS gels using a ScanMaker IIHR (Microtek) scanner and the Adobe Photoshop™ program, version 3.0. The pixel density was determined using NIH Image software. The percentage of N-glycosylation was calculated from the areas under the two peaks corresponding to the N-glycosylated and nonglycosylated products. The fraction of protein that co-immunoprecipitated with calnexin was estimated by determining the intensity of the band interacting with calnexin in the immunoprecipitation fraction divided by the total protein integrated into the microsomes.

**RESULTS**

**AE1 Associates with Calnexin in a Glycosylation-dependent Manner**—The interaction of calnexin with human AE1 was studied in a cell-free translation system, using rabbit reticulocyte lysate supplemented with canine pancreatic microsomal membranes. This in vitro system has been used extensively to study the biosynthesis of polytopic membrane proteins, including AE1 (49, 51, 52, 55). Due to its lower protein mass, the AE1md construct (51) was used to more readily distinguish the N-glycosylated and nonglycosylated forms of the protein on SDS gels. The AE1md construct contains the entire transmembrane domain of AE1, encompassing residues 386–911 (Fig. 1). AE1md is sufficient to carry out the anion exchange function
endogenous N-glycosylation acceptor site, was not glycosylated but was also integrated into the membrane (Fig. 2, lane 4).

The association of AE1md with calnexin was determined by solubilization of microsomes with digitonin and immunoprecipitation with the anti-carboxyl-terminal calnexin antibody. The presence of both N-glycosylated and nonglycosylated products in the translation mixture allowed us to determine the glycosylation dependence of the interaction with calnexin in the same sample. The results of co-immunoprecipitation experiments are shown in Fig. 2, lanes 7–14. The N-glycosylated form of the AE1md (Fig. 2, lane 1 versus lane 7, closed circle) was readily co-immunoprecipitated with the anti-calnexin antibody. In contrast, the lower, nonglycosylated form (Fig. 2, lane 7, open circle) that was present in the same translation mixture was poorly immunoprecipitated. In a second immunoprecipitation of the same sample, only minor amounts of either form were present, indicating a quantitative recovery of calnexin in the initial immunoprecipitation. Very little of the nonglycosylated construct, N642D, was co-immunoprecipitated with the anti-calnexin antibody (Fig. 2, lane 4 versus lane 11).

The interaction of N-glycosylated AE1md with calnexin could be inhibited if translation was carried out in the presence of 1 mM CST, an inhibitor of α-glucosidases I and II (Fig. 2, lane 9), whereas posttranslational addition of CST did not prevent the interaction (Fig. 2, lane 10). It has been shown that low concentrations of deoxymycin stabilizes the calnexin-protein complex by slowing down the trimming of the glucose residues (59). We were not able to detect any increase in the number of calnexin/AE1md complexes in the presence of 1 mM CST (Fig. 2, lane 9). Dose dependence experiments showed that 50 μM CST was sufficient to inhibit the interaction of calnexin with AE1 (data not shown). The weak calnexin interaction with the nonglycosylated form of the protein was unaffected by the presence of CST (Fig. 2, lane 9 versus lane 13).

In addition to the full-length AE1md, lower molecular weight products were observed in cell-free translations of the AE1md (Fig. 2, diamonds). We have shown that these products are the result of nonspecific translation initiation at a cluster of Met residues positioned at the end of putative TM segment 6 (51). In the resulting protein, TM7 acts as a signal sequence and the rest of the protein is stably integrated into microsomal mem-

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![Diagram of the AE1md protein structure](image-url)
Calnexin Has Access to Various EC Regions of AE1md—The single site of N-linked glycosylation (Asn-642) in the AE1md is located in the fourth EC loop (Fig. 1). To test whether calnexin can bind to oligosaccharide chains located on other EC loops in AE1md, a series of glycosylation mutants that possess a single N-glycosylation site in other loops were made using the N642D mutant. Novel N-glycosylation sites in EC loop 3 at position 556 and in the loop structure at position 743 were introduced into AE1md (Fig. 1). This truncated AE1md7–12 was glycosylated at Asn-642 (Fig. 2, lane 1, filled diamond) and was efficiently co-immunoprecipitated with the anti-calnexin antibody (Fig. 2, lane 7). CST prevented this interaction (Fig. 2, lane 9). The truncated nonglycosylated mutant was not co-immunoprecipitated (Fig. 2, lane 11, open diamond). The results indicate that calnexin binds to AE1md or truncated AE1md7–12 in a N-glycosylation-dependent manner and that the interaction requires a glucose trimmed carbohydrate structure.

Quantification of the percentages of co-immunoprecipitation of AE1md from at least three separate translations is summarized in Table I. On average, 46% of the glycosylated form of the AE1md (Asn-642) was co-immunoprecipitated with anti-calnexin antibody, whereas 20% of the nonglycosylated form was co-immunoprecipitated. Only 12% of the total translated protein of N642D construct was co-immunoprecipitated with calnexin. Similar results were obtained when detergents 1% CHAPS or 1% BRIJ 96 were used instead of digitonin. Triton X-100 disrupted the interaction of AE1md with calnexin, as solubilization with 1% Triton X-100 resulted in reduced efficiency of co-immunoprecipitation of AE1 (12 versus 46%).

| Mutant       | Non-glycosylated | Mono-glycosylated | Diglycosylated | No. |
|--------------|------------------|-------------------|----------------|-----|
| Asn-642 (wild-type) | 20 ± 6*          | 46 ± 9            | NA             | 7   |
| N642D        | 12 ± 5           | 35 ± 0            | 44 ± 9         | 13  |
| 639          | 29 ± 1           | 77 ± 1            | 69 ± 13        | 4   |
| 639 + 642    | 20 ± 9           | NA                | 63 ± 16        | 3   |
| 556          | 15 ± 4           | 47 ± 7            | 63 ± 16        | 5   |
| 556 + 642    | 19 ± 7           | 56 ± 18           | 63 ± 16        | 3   |
| ins.EC1      | 12 ± 4           | 39 ± 19           | 54 ± 7         | 5   |
| ins.EC7      | 15 ± 4           | 39 ± 8            | 56 ± 18        | 3   |
| ins.EC2a     | 16 ± 5           | 47 ± 8            | 56 ± 18        | 3   |
| ins.EC2b     | 15 ± 6           | 47 ± 8            | 54 ± 7         | 4   |
| ins.EC2c     | 21 ± 3           | 50 ± 7            | 54 ± 7         | 4   |
| ins.EC2d     | 21 ± 6           | 64 ± 16           | 71 ± 10        | 4   |

* Results are represented as percent of AE1md co-immunoprecipitated ± S.D.
* Number of determinants.
* NA, insufficient monoglycosylated 639 + 642 mutant was produced to allow quantification.

The percentage of N-glycosylated AE1 mutants that could be immunoprecipitated with calnexin was in the range of 40–55% (Table I). The mutant with a single acceptor site at 639 (Fig. 3B) co-immunoprecipitated with calnexin with high efficiency (77%) (Table I). The interactions were N-glycosylation-dependent because the nonglycosylated bands, in the same translation reaction, were precipitated with lower efficiencies (10–20%) (Table I).
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ins.EC2a mutant, which was not N-glycosylated, co-immunoprecipitated with efficiency of 16% (Table I).

Multiple N-Glycosylation Enhances Calnexin Binding—Introduction of two N-glycosylation acceptor sites into AE1 allows double glycosylation to occur (52). We tested the effect of the additional glycosylation site on calnexin interaction with AE1md. Two types of doubly glycosylated mutants were made in the AE1md construct: (i) a mutant with two oligosaccharides in the same loop EC4 (639 + 642) and (ii) mutants with oligosaccharide chains in different EC loops, with combinations EC2 + EC4 (ins.EC2d) and EC3 + EC4 (556 + 642) (Fig. 1). Translation of mutants with two N-glycosylation sites resulted in a doubly glycosylated form of the protein (Fig. 3B, lanes 4, 6, and 9). The efficiency of double N-glycosylation matched the lower efficiency of the two individual sites. This pattern was observed whether the two N-glycosylation sites were positioned in the same or in different EC loops.

Calnexin co-immunoprecipitation of doubly N-glycosylated mutant 556 + 642 (Fig. 3B, lane 15) was more efficient (63%) compared with the individual efficiencies of co-immunoprecipitation of the 556 mutant (Fig. 3B, lane 14) (47%) or the wild-type Asn-642 (Fig. 3B, lane 11) (46%) (Table I). The co-immunoprecipitation efficiency was also higher for the doubly N-glycosylated ins.EC2d (Fig. 3B, lane 18) (Table I). In the case of the 639-642 mutant (Fig. 3B, lane 13 versus lane 12), the co-immunoprecipitation efficiency did not increase (Table I), probably because it already reached the maximum level. In secreted proteins, it has been shown that increasing the number of oligosaccharide chains from one to two increases the efficiency of co-immunoprecipitation with calnexin severalfold (30, 38). Here, we demonstrate that calnexin could interact with oligosaccharides located on different EC loops in a polytopic membrane protein and that increasing the number of oligosaccharide chains from one to two led to a modest increase in the efficiency of co-immunoprecipitation.

Calnexin Interaction with Truncated AE1md Constructs—To test whether the interaction of calnexin with AE1md occurs co-translationally, restriction enzymes were used to linearize the AE1md cDNA to produce truncated mRNAs that lacked a stop codon. During translation, this results in the production of translocation-arrested intermediates at defined positions in the AE1md sequence that retained the final tRNA (60). These intermediates can be N-glycosylated if the distance from the P-site in the ribosome to the active site on the oligosaccharyltransferase is greater than 67 residues (61). This number of residues increased to 71 if an intervening transmembrane segment was present (61). The intermediates can be released from their ribosomes upon the addition of puromycin, which allows the polypeptide chain to move from the translocon into the lipid bilayer (62, 63) and allows N-glycosylation of short fragments to occur (61). The truncated mRNAs at amino acid positions 696, 716, 754, 820, and 854 were constructed in order to vary the number of amino acid residues following the endogenous N-glycosylation acceptor site at Asn-642 (Fig. 1). The resulting polypeptide chain lengths, following Asn-642, were 54, 74, 112, 178, and 212 residues. Another construct was made by truncating the insertion mutant EC1 at position 854, 444 amino acid residues after the acceptor site in the expanded EC1. The constructs were translated in a cell-free system in the presence or absence of Ac-NYT-NH2.

The cell-free translation results are shown in Fig. 4A, including the full-length AE1md (Asn-642), which contained the original stop codon (Fig. 4A, lanes 1–3). Inclusion of Ac-NYT-NH2 in the translation reaction produced the nonglycosylated protein (Fig. 4A, lane 2, open circle). The posttranslational addition of puromycin did not change the level of N-glycosylation of the full-length AE1md (Fig. 4A, lane 1 versus lane 3, closed circles).

When truncation 54 was translated in the presence of microsomes less than 10% of the protein was N-glycosylated (Fig. 4A, lane 4, closed circle). The presence of a small amount of a glycosylated band indicated that under the experimental conditions 10% of the ribosomes were not firmly attached to the translocon complex, resulting in the release and N-glycosylation of nascent chains. The addition of puromycin after 30 min resulted in the release of the nascent chain and increased N-glycosylation of truncation 54 (Fig. 4A, lane 6). The chain length of truncation 54 was too short to be N-glycosylated co-translationally. Translation of truncation mutant 74 also resulted in some co-translationally N-glycosylation (Fig. 4A, lane 7), but the level of glycosylation increased after the addition of puromycin (Fig. 4A, lane 9). Truncations 112, 178, 212, and 444 resulted in co-translationally glycosylation (Fig. 4A, lanes 10, 13, 16, and 19). In these truncations, the level of glycosylation did not increase upon the addition of puromycin (Fig. 4A, lanes 12, 15, 18, and 21). This indicates that the nascent chains of these truncations were of sufficient length for the acceptor site to reach the oligosaccharyltransferase in the ER lumen. Fig. 4B shows that 112 residues were sufficient for efficient co-translationally N-glycosylation of AE1. Short constructs (truncations 54 and 74) were efficiently N-glycosylated after their release from the ribosome.

Anti-calnexin immunoprecipitation with the antibody directed against the carboxyl terminus of calnexin showed that calnexin interaction with the nascent chains of the truncated constructs could not be detected (Fig. 4C, lanes 10, 16, 22, and 28). This was as expected for the short constructs (truncations 54 and 74), which were poorly N-glycosylated. The longer constructs were efficiently N-glycosylated, yet they were not co-immunoprecipitated with the anti-calnexin antibody. Because this antibody is directed against the cytoplasmic tail of calnexin, the ribosome may sterically block the epitope. We therefore also performed co-immunoprecipitation with a polyclonal anti-calnexin antibody directed against the soluble, luminal region of calnexin. Although this antibody efficiently co-immunoprecipitated the full-length AE1md similar to the anti-carboxyl terminus calnexin antibody (Fig. 4C, lane 5), it could not co-immunoprecipitate any of the truncated constructs (Fig. 4C, lanes 11, 17, 23, and 29).

The interaction of N-glycosylated truncated chains with calnexin could be detected using the amino-terminal antibody after the nascent chains had been released from the ribosomes with puromycin (Fig. 4C, lanes 12, 18, 24, and 30). Similar results were obtained using the carboxyl-terminal antibody (data not shown). The immunoprecipitates were enriched in the N-glycosylated forms over nonglycosylated, consistent with a glycosylation-dependent interaction. Although truncations 112, 178, 212, and 444 were efficiently glycosylated co-translationally, the interaction with calnexin was not detected until their release from the ribosome. This suggested that even the extension of the nascent chain by 444 amino acid residues after the utilized N-glycosylation site was not sufficient to allow co-translational interaction with calnexin. The interaction was detected after incubation with puromycin, showing that the most prominent interaction of AE1md with calnexin was posttranslational.

Calnexin Interacts with AE1 in Transfected Cells—To test whether the AE1 interaction with calnexin also occurs in vivo, we transiently transfected HEK293 cells with full-length AE1, the nonglycosylated N642D mutant, and the Asn-555 mutant with a single novel N-glycosylation site at position 555. HEK293 cells endogenously express calnexin and were previ-
Microsomes were alkali-extracted (lanes 1–3, 7–9, 13–15, 19–21, and translocation arrested constructs lacking the stop codon (numbers 54, 74, 212, and 444 indicate number of residues after is indicated as

Antibody directed against the carboxyl terminus of calnexin is indicated as

of molecular mass markers are indicated to the left position of the glycosylated band, and open circles indicate the nonglycosylated band. The positions of molecular mass markers are indicated to the left of the figure in kDa. B, percentage of N-glycosylation for translocation arrested constructs lacking the stop codon and AE1md (N642) as indicated, without (black bars) or with (hashed bars) added puromycin. C, autoradiograph of cell-free translation products of the AE1md (N642) and translocation arrested constructs lacking the stop codon (numbers 54, 74, 212, and 444 indicate number of residues after N-glycosylation site). Microsomes were alkali-extracted (lanes 1–3, 7–9, 13–15, 19–21, and 25–27) or subjected to anti-calnexin immunoprecipitation, as indicated. Antibody directed against the carboxyl terminus of calnexin is indicated as C; antibody directed against the soluble, ER lumenal region of calnexin is indicated as N. Closed circles indicate the position of the glycosylated band, and open circles indicate the nonglycosylated band. The positions of molecular mass markers are indicated to the left of the figure in kDa.

Fig. 4. A, autoradiograph of cell-free translation products of the AE1md (N642) and translocation arrested constructs lacking the stop codon (numbers 54, 74, 112, 178, 212, and 444 indicate number of residues after N-glycosylation site). Cell-free translation was carried out in the presence of microsomes, and Ac-NYT-NH₂ (35 μM) was included in the translation reaction as indicated. Addition of puromycin (1.5 mM) was 30 min after the start of the translation reaction. Microsomes were alkali-extracted and resolved on SDS-polyacrylamide gels. Closed circles indicate the position of the glycosylated band, and open circles indicate the nonglycosylated band. The positions of molecular mass markers are indicated to the left of the figure in kDa. B, percentage of N-glycosylation for translocation arrested constructs lacking the stop codon and AE1md (N642) as indicated, without (black bars) or with (hashed bars) added puromycin. C, autoradiograph of cell-free translation products of the AE1md (N642) and translocation arrested constructs lacking the stop codon (numbers 54, 74, 212, and 444 indicate number of residues after N-glycosylation site). Microsomes were alkali-extracted (lanes 1–3, 7–9, 13–15, 19–21, and 25–27) or subjected to anti-calnexin immunoprecipitation, as indicated. Antibody directed against the carboxyl terminus of calnexin is indicated as C; antibody directed against the soluble, ER lumenal region of calnexin is indicated as N. Closed circles indicate the position of the glycosylated band, and open circles indicate the nonglycosylated band. The positions of molecular mass markers are indicated to the left of the figure in kDa.

uously used in studies with other membrane glycoproteins (35, 45, 46). Mock transfected cells were used as a control (Fig. 5, lanes C). Cells labeled with [35S]Met for 20 min were lysed in digitonin and subjected to immunoprecipitation. Immunoprecipitation with anti-AE1 antibody is shown in Fig. 5 (lanes 1–4). Transfected cells expressed comparable amounts of the AE1 protein that corresponds to the expected size of full-length AE1 (95 kDa) (50). Immunoprecipitation with anti-calnexin antibody detected a number of labeled proteins (Fig. 5, lanes 5–8). This is likely residual calnexin, which has a molecular mass (90 kDa) similar to that of deglycosylated AE1. Double immunoprecipitation detected the two N-glycosylated forms, AE1 and Asn-555. The nonglycosylated mutant of AE1, N642D, interacted poorly with calnexin (lane 11). Some of the protein band in this double immunoprecipitation may be contributed by calnexin as in the control sample (lane 9). This N642D-calnexin interaction was consistently weaker than the AE1-calnexin interaction in four independent transfections. Therefore, we conclude that AE1-calnexin interaction in vivo is glycosylation-dependent. Pulse-chase experiments show that AE1-calnexin interaction is transient (data not shown). The interaction of AE1 and Asn-555 mutant with calnexin was diminished after a 1-h chase and completely lost after a 5-h chase, similar to findings with other membrane glycoproteins (5, 44, 64).

**DISCUSSION**

*N*-Glycosylation-dependent Interaction of Calnexin with AE1—Although calnexin acts primarily as a lectin, glycosylation-independent interactions of calnexin with a number of membrane proteins (6, 35, 37, 65) and some secretory proteins (36) have been reported. The significance of these interactions is controversial. The glycosylation-independent interaction may be an additional mode of interaction (23) or alternatively an interaction with accumulated aggregates of the protein (38). In our *in vitro* and *in vivo* experiments, interaction of AE1 with calnexin was glycosylation-dependent and glycosylation-independent interactions were at least 3-fold lower. Major changes to the AE1 structure introduced by insertion into EC loop 1, 2, or 7 did not increase the glycosylation-independent interactions.

In previously described interactions of calnexin with membrane proteins, the glycosylated region of the membrane protein was restricted to a single EC domain. In a polytopic membrane protein, various EC domains may not be equally accessible to interact with calnexin. Here, we systematically
introduced N-glycosylation sites into EC loops in AE1, a protein with 12–14 TM segments. Our study shows that there is no spatial restriction for calnexin to approach any EC loop in AE1. The dimer of the AE1md of AE1 is 110 Å long and 60 Å wide, as viewed by electron microscopy of two-dimensional crystals (66). Also, it is known that oligosaccharide positioned very close (12 residues) to the membrane were not restricted in their interaction with calnexin (31). Together with our results, we suggest that calnexin can interact with different glycosylated luminal regions of polytopic membrane proteins.

In secreted proteins, multiple oligosaccharide chains increase the amount of protein co-immunoprecipitated with calnexin (30, 38). The presence of two oligosaccharide chains in AE1 also increased the proportion of protein complexed with calnexin, regardless of the positions of the oligosaccharide chains. There are reports that calnexin exists as a homotetramer/pentamer (11); therefore, multiple oligosaccharides on a glycoprotein could increase the avidity of binding. Alternatively, calnexin may be monomeric (7). In this case, multiple calnexin molecules could bind simultaneously to several oligosaccharides. It is also possible that multiple lectin domains within calnexin could interact simultaneously with several oligosaccharides on one glycoprotein. Our data suggest that several calnexin molecules are required to interact with multiple oligosaccharide chains placed in AE1 because the interaction is enhanced regardless of the relative locations of the two N-glycosylated sites in the protein.

Posttranslational Interaction of Calnexin with AE1—Our results indicate that calnexin-AE1 interactions occur after release of the nascent chain from the ribosome. Most calnexin interactions with newly synthesized proteins were characterized using full-length proteins, i.e. posttranslationally, where even multiple rounds of interaction with substrate proteins occur (9, 21, 67, 68). There are few reports in which calnexin was shown to interact with nascent chains co-translationally (43, 69, 70). Two papers (69, 70) describe the calnexin interaction with a truncated construct of glucose transporter Glut 1 (GT1). However, the yield of the calnexin-nascent chain complex immunoprecipitated with the anti-calnexin antibody was low compared with the total amount of nascent chain (70). In the other experiment, in which calnexin interacted with a population of incomplete type I membrane protein chains of influenza hemagglutinin, the level of calnexin interaction was estimated to be about 20% (43). An interaction with nascent chains was first detected – 250 residues after the first glycosylation site. This length of soluble hemagglutinin fragment could be sufficient to span the distance between the P-site in the ribosome and the binding site on calnexin. In our experiments on a polytopic membrane protein, AE1, a length of 444 residues was not sufficient to detect the calnexin-nascent chain interaction. Our results show that although calnexin can interact with any EC region of AE1 posttranslationally, it may not be positioned to interact with the translocating chains of this polytopic membrane protein.

Sec61p, a subunit of translocon-associated protein, and oligosaccharyl transferase complexes are directly associated with ribosomes (71). A high molecular weight form of calnexin has been reported to associate with ribosomes and Sec61p (72), the core component of the translocon apparatus. Calnexin has also been co-purified with the translocon-associated protein α (42). This suggests that a fraction of calnexin may be associated with the protein translocation complex in the ER. Calnexin in the vicinity of the translocon may be available to interact with N-glycosylated proteins upon their release from the translocon.

The journey of a nascent chain from the translocon to be a fully integrated membrane protein can be viewed through the fate of a oligosaccharide chain. After its co-translational attachment to nascent chains, the Glc3Man9GlcNAc2 oligosaccharide meets two α-glucosidases. The removal of glucose residues has been shown to occur co-translationally on a nascent vesicular stomatitis virus G protein (16). Removal of the outer two glucose residues primes the glycoprotein for the subsequent rendezvous with calnexin. It has been shown that the re-glucosylation reaction by UDP-glucose glycosyltransferase occurs postranslationally (20, 73); therefore, calnexin must interact with re-glucosylated unfolded glycoproteins postranslationally. Our results suggest that calnexin interacts with newly synthesized membrane glycoproteins postranslationally, after their release from the ribosome and movement into the lipid bilayer. Once in the bilayer, polytopic membrane proteins such as AE1 are free to interact with calnexin. We have shown that calnexin can bind to oligosaccharides on different loops in AE1, consistent with the interaction occurring within the bilayer. This is in agreement with findings in which multiple TM segments can be accommodated within the translocon before being released into the lipid bilayer (54). Also, in the experiments with single TM constructs, the TM segment was not released to the lipid bilayer until the stop codon was reached, regardless of the length of the carboxyl-terminal tail (62, 63). These results suggest a sequential and regulated movement of newly synthesized membrane proteins from Sec61p and translating-chain-associating membrane proteins to chaperones such as calnexin. Functional expression of complementary fragments of polytopic membrane proteins, including AE1 (55, 74–79), suggests that the final folding likely occurs in the lipid bilayer and supports the “two-stage model” of membrane proteins assembly (33). In this process, the transient interaction with chaperones may be a required step to prevent aggregation and nonspecific interactions between the incompletely folded membrane proteins that are released from the translocon.

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