Site-directed removal of N-glycosylation sites in the bovine cation-dependent mannose 6-phosphate receptor: effects on ligand binding, intracellular targetting and association with binding immunoglobulin protein

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The bovine cation-dependent mannose 6-phosphate receptor (CD-MPR) contains five potential N-linked glycosylation sites, four of which are utilized. To evaluate the function of these oligosaccharides, site-directed mutagenesis was used to generate glycosylation-deficient CD-MPR mutants lacking various potential glycosylation sites. The mutants were constructed in both a full-length and a soluble truncated ($STOP^{155}$ construct) form of the receptor and their properties were examined in transfected COS-1 cells. The results showed that the presence of a single oligosaccharide chain, particularly at position 87, on the CD-MPR significantly enhanced its mannose 6-phosphate (Man-6-P)-binding ability when compared with non-glycosylated receptors. In addition, the presence of a single oligosaccharide chain at position 87, and to a lesser degree at position 31 or 81, promoted the secretion of the $STOP^{155}$ CD-MPR. Pulse-labelling of transfected COS-1 cells followed by immunoprecipitation with binding immunoglobulin protein (BiP)-specific and CD-MPR-specific antibodies indicated that BiP associated with the non-glycosylated forms of the receptor but not with the wild-type CD-MPR. Furthermore, the association of the various glycosylation-deficient forms of the CD-MPR with BiP correlated inversely with their ability to bind Man-6-P. From these results we conclude that N-glycosylation of the bovine CD-MPR facilitates the folding of the nascent polypeptide chain into a conformation that is conducive for intracellular transport and ligand binding.

INTRODUCTION

The cation-dependent mannose 6-phosphate receptor (CD-MPR) is one of two distinct MPRs which function to segregate and transport newly synthesized soluble lysosomal enzymes to lysosomes (for reviews, see refs. [1–4]). The bovine CD-MPR has an apparent molecular mass of approx. 46 kDa and consists of four structural domains: a 28-residue N-terminal signal sequence, a 159-residue extracytoplasmic domain, a single 25-residue transmembrane region, and a 67-residue C-terminal cytoplasmic domain. Sequence analysis of the bovine CD-MPR cDNA revealed that it contains five potential N-linked glycosylation sites (Asn-X-Ser/Thr), at positions 31, 57, 68, 81 and 87, in the N-terminal mannose 6-phosphate (Man-6-P)-binding domain [5]. Alignment of the bovine, human and mouse sequences demonstrated that the mature proteins are 93–95% identical and that the five glycosylation sites are conserved [5–7]. Previous studies have shown that both the bovine and human CD-MPR utilize four of their five glycosylation sites [5,8,9].

After its synthesis in the endoplasmic reticulum (ER), the CD-MPR is transported along the secretory pathway to the Golgi apparatus where it functions in the delivery of newly synthesized lysosomal enzymes to a prelysosomal compartment. However, recent studies have suggested that the receptor attains its ligand-binding conformation before reaching the Golgi compartment [10]. Pulse–chase labelling experiments as well as mutational analyses of individual cysteine residues of the human CD-MPR expressed in BHK cells indicated that the formation of intramolecular disulphide bonds is required for an active ligand-binding site and occurs in a pre-Golgi compartment [10,11]. Along with the formation of intramolecular disulphide bonds, the CD-MPR becomes co-translationally modified by the addition of N-linked oligosaccharide chains. Earlier studies suggested that N-linked glycosylation, which constitutes approx. 20% of the total mass of the receptor, was required for the CD-MPR to bind ligand [12,13]. However, studies by Wendland et al. [8] provided evidence that N-linked oligosaccharides were required for the stability of the CD-MPR, but not for the acquisition of Man-6-P-binding activity.

In the current study, oligonucleotide-directed mutagenesis was used to prepare mutants of the bovine CD-MPR that lacked selected N-linked glycosylation sites. The resulting mutants were expressed in COS-1 cells and analysed for ligand-binding activity, intracellular transport and association with binding immunoglobulin protein (BiP). Our results indicate that the oligosaccharides on the bovine CD-MPR assist in the folding of the nascent polypeptide into its native conformation. A preliminary report of these findings has been published [14].

EXPERIMENTAL

Materials

The following reagents were obtained commercially as indicated: Bluescript SK plasmid (Stratagene), bacteriophage T_7 RNA polymerase (Molecular Biology Resources), rabbit reticulocyte lysate and dog pancreatic microsomal membranes (Promega), Tran^{35}S-label (1100 Ci/mmol, ICN Biomedicals), EXPRE^{35}S^{35}S [^{35}S]protein labelling mix (1200 Ci/mmol, DuPont–New England Nuclear), Klenow fragment of DNA polymerase I and restriction endonucleases (New England Biolabs), Sequenase version 2 kit (U.S. Biochemical Corp.), pSVL expression vector (Pharmacia LKB Biotechnology), COS-1 cells (American Type Culture

Abbreviations used: Man-6-P, mannose 6-phosphate; CD-MPR, cation-dependent mannose 6-phosphate receptor; endo H, endo-β-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; BiP, binding immunoglobulin protein.

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Collection), Dulbecco's modified Eagle's medium (Gibco-BRL Life Technologies), fetal bovine serum (Hyclone Laboratories), endo-β-*N*-acetylglucosaminidase H (endo H) (Boehringer-Mannheim). Phosphomannan from *Hansenula holstii* was a generous gift from Dr. M. E. Slodki of the Northern Regional Research Center (Peoria, IL, U.S.A.). The monoclonal antibody against rat BiP was generously provided by Dr. David G. Bole of the University of Michigan (Ann Arbor, MI, U.S.A.).

In vitro transcription and translation

The Bluescript SK plasmids containing the cDNA sequences of STOP^{155} (see below) or the wild-type CD-MPR were linearized with *Eco*RI. After *in vitro* transcription using bacteriophage T_7 RNA polymerase, transcripts were translated in a reticulocyte lysate system as described previously [5] and modified as described by Hille et al. [13]. Briefly, mRNA was incubated with rabbit reticulocyte lysate, dog pancreatic microsomes, [^{35}S]methionine and 1 mM oxidized glutathione for 1 h followed by a 1 h chase in the presence of 10 mM methionine and 3 mM oxidized glutathione.

Microinjection and translation of mRNA in *Xenopus laevis* oocytes

In vitro transcribed STOP^{155} or wild-type CD-MPR was microinjected into *X. laevis* oocytes and labelled with [^{35}S]methionine as described by Dahms and Kornfeld [15].

Construction of the CD-MPR and STOP^{155} mutants

Full-length CD-MPR mutants containing glutamine substitutions for asparagine residues in the recognition signal for N-linked glycosylation, Asn-X-Ser/Thr [16,17], were generated by oligonucleotide-directed mutagenesis by the method of Kunkel et al. [18]. Replacement of asparagine with glutamine has rarely been shown to affect the tertiary structure of a polypeptide [19]. The mutagenic oligonucleotides used are as follows: 5′-CA CCG CTA TTT CAG AAA AGC-3′ converts Asn^{31} into Gln (underlined codon); 5′-A GAA GCT GGC CAG CAC TCC T-3′ converts Asn^{57} into Gln (underlined codon); 5′-G CAG ATC CAG AAA AGT AAC G-3′ converts Asn^{68} into Gln (underlined codon); 5′-TT GGG AGA TTC CAG GAG ACT-3′ converts Asn^{81} into Gln (underlined codon); 5′-CAG ATC TTC CAG GGA AGT AA-3′ converts Asn^{87} into Gln (underlined codon). The following constructs contain four amino acid substitutions: mutant Asn31, glutamines replace Asn^{57}, Asn^{68}, Asn^{81} and Asn^{87}; mutant Asn57, glutamines replace Asn^{31}, Asn^{68}, Asn^{81} and Asn^{87}; mutant Asn68, glutamines replace Asn^{31}, Asn^{57}, Asn^{81} and Asn^{87}; mutant Asn81, glutamines replace Asn^{31}, Asn^{57}, Asn^{68} and Asn^{87}; mutant Asn87, glutamines replace Asn^{31}, Asn^{57}, Asn^{68} and Asn^{81}. Mutant Asn- has all five glycosylation sites removed by substitution of the asparagines at positions 31, 57, 68, 81 and 87 with glutamines. For each construct, the region that was synthesized *in vitro* by the Klenow fragment of DNA polymerase I was subjected to double-stranded DNA sequence analysis [20] to confirm the predicted sequence and then was cloned into the pSVL expression vector that had been cleaved with *Xba*I and *Sma*I. STOP^{155}, which encodes only the signal sequence and extracytoplasmic region, was constructed previously by changing the proline (residue 155) codon in the wild-type bovine CD-MPR sequence (CCA, nucleotide 584–586) to a stop codon (TGA) [15]. To generate various glycosylation-deficient STOP^{155} mutants, the sequence containing the mutated glycosylation sites in each of the full-length mutant constructs was cleaved with *Xba*I and *Taq*I (nucleotides 0–481) and ligated to restriction fragments (*Taq*I–*Pst*I, nucleotides 481–1070; *Pst*I–*Bam*HI, nucleotides 1070–1321) from the STOP^{155} cDNA. The STOP^{155} mutants were then inserted into the *Xba*I and *Bam*HI sites of the pSVL expression vector.

Transfection of COS-1 cells

COS-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and maintained in a 5% CO_2 atmosphere. Transfection of plasmid pSVL or plasmid pSVL containing various mutant forms of the CD-MPR cDNA into COS-1 cells was performed by the DEAE-dextran technique [21] followed by exposure to chloroquine [22]. Then 48 h after transfection, the cells were labelled in methionine-free Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum and 25 μCi/ml Tran^{35}S-label or EXPRE^{35}S^{35}S [^{35}S]protein labelling mix for 24 h. After labelling, the medium was harvested and dialysed exhaustively at 4 °C against column buffer (50 mM imidazole (pH 6.5), 150 mM NaCl, 10 mM $MnCl_2$, 5 mM β-glycerophosphate, Triton X-100 (0.05%, v/v) and 2 μg/ml BSA). The cells were solubilized at 4 °C in column buffer containing Triton X-100 (1%, v/v), sodium deoxycholate (0.1%, w/v), aprotinin (1%, v/v), antipain (4 μg/ml), benzamidine (20 μg/ml) and 2 μg/ml each leupeptin, chymostatin and pepstatin. After centrifugation, the resulting cleared supernatant was subjected to pentamannosyl phosphate–agarose chromatography. In some experiments, tunicamycin was added to the medium at a final concentration of 5 μg/ml 6 h before the radiolabelling of transfected COS-1 cells. The same amount of tunicamycin was also present throughout the labelling process.

Pentamannosyl phosphate–agarose affinity chromatography

Pentamannosyl phosphate was prepared from *Hansenula holstii* phosphomannan as previously described [23] and was coupled to agarose by the procedure of Distler and Jourdian [24]. Detergent-solubilized cell lysate or dialysed medium was passed over a pentamannosyl phosphate–agarose column (0.9 cm × 1.0 cm) equilibrated in column buffer and maintained at 4 °C [12]. The column was washed in a total of 15 ml of column buffer and then eluted with 1 ml of column buffer containing 5 mM glucose 6-phosphate (non-specific ligand) followed by 3 ml of column buffer containing 5 mM Man-6-P (specific ligand). The flow-through fractions and the material eluted from the column were immunoprecipitated as described below.

Immunoprecipitation and gel electrophoresis

Antibodies against purified bovine liver CD-MPR were generated in New Zealand rabbits as described previously [12]. Samples in solubilization buffer, or fractions from the ligand-affinity columns, were incubated with rabbit polyclonal antisera specific for the CD-MPR plus Protein A–Sepharose at 4 °C for 16–24 h. The immunoprecipitated material was subjected to gel electrophoresis under reducing conditions on slab gels using the buffer systems of Laemmli [25]. Detection of the CD-MPR mutants co-immunoprecipitated with BiP was as described by Blount and Merlie [26]. Briefly, transfected COS-1 cells were solubilized with detergent in the presence of 10 units/ml hexokinase and 0.2 mM D-glucose to hydrolyse ATP in order to maintain BiP association [27]. The lysate was separated into equal aliquots to which

Protein A–Sepharose beads plus CD-MPR-specific antisera or BiP-specific antibody were added. After an incubation of 16 h, the beads were washed exhaustively and either eluted with SDS/PAGE sample buffer or incubated in buffer containing 5 mM ATP for 45 min at room temperature. After brief centrifugation, the supernatant after the ATP treatment was further immunoprecipitated with the anti-(CD-MPR) sera as described above and the Sepharose beads were eluted with SDS/PAGE sample buffer. The samples were analysed by SDS/PAGE, and radiolabelled bands were visualized by fluorography. The bands were quantified by counting the radioactivity in isolated gel slices which were rehydrated and solubilized with TS-1 tissue solubilizer (Research Products International) and/or by scanning the autoradiographs on an Ambis radioanalytical imaging system.

Endo H digestion

Immunoprecipitated samples were eluted from Protein A–Sepharose beads by incubating them with buffer containing 1% SDS and 10 mM Tris/HCl, pH 7.4, for 5 min at 90 °C. The eluates were precipitated with acetone. Endo H digestion was carried out in a buffer containing 0.1 M citrate, pH 6.0, 0.075% SDS, 0.5% 2-mercaptoethanol and 1 munit of endo H for 16 h at 30 °C. The samples were analysed by SDS/PAGE as described above.

RESULTS AND DISCUSSION

Ligand binding of glycosylation-deficient forms of the bovine CD-MPR

Previously, we observed that a truncated form of the bovine CD-MPR (STOP^{155} construct), consisting of the signal sequence and the extracellular ligand-binding domain, is inefficiently glycosylated when expressed in *X. laevis* oocytes [15]. Figure 1(a) shows that more than 95% of each glycosylated, as well as a non-glycosylated (generated by endo H digestion) form of the STOP^{155} CD-MPR, binds Man-6-P. However, when the wild-type and STOP^{155} CD-MPR transcripts were translated in a rabbit reticulocyte lysate system supplemented with microsomal membranes in which both glycosylated and non-glycosylated forms of the receptor were produced, no detectable Man-6-P binding was seen for either the non-glycosylated STOP^{155} receptor (results not shown) or the non-glycosylated wild-type CD-MPR (Figure 1b). Only the glycosylated forms, containing one, two, three or four carbohydrate chains, bound Man-6-P (Figure 1b and results not shown). A similar result was observed when CD-MPRs were expressed in COS-1 cells in the presence of tunicamycin. Only 30% and 25% respectively of the non-glycosylated form of the wild-type CD-MPR and the cell-associated STOP^{155} CD-MPR bound phosphomannosyl residues (Figure 1c). In contrast, more than 95% of the STOP^{155} receptor containing one oligosaccharide chain was retained on the ligand-affinity column (Figure 1c). Taken together, we interpret these results to suggest that, although N-linked oligosaccharides are not directly involved in Man-6-P binding, they may assist in the initial folding of the bovine CD-MPR into its biologically active conformation.

Expression and ligand binding of glycosylation-deficient CD-MPR mutants

To examine the functional importance of the individual glycosylation sites of the bovine CD-MPR, six glycosylation-deficient mutants were generated (Figure 2) and were transiently expressed in COS-1 cells. Digestion with endo H demonstrated that asparagine at position 68 is not glycosylated, which is consistent with the findings observed for the human CD-MPR expressed in BHK cells [8]. Asn31, Asn57 and Asn81 mutants were completely sensitive to endo H digestion whereas 75% of Asn87 mutant and 77% of the wild-type CD-MPR were resistant to treatment with endo H (Table 1), demonstrating the presence of complex-type oligosaccharides [28]. The mutants were assessed for their ability to bind phosphomannosyl residues by affinity chromatography and the results are summarized in Table 1. Of the mutant constructs, Asn87 exhibited the highest ligand-binding activity (42%) and was the most similar to the wild-type CD-MPR in which 73% bound to pentamannosyl phosphate–agarose. The non-glycosylated constructs, Asn68 and Asn-, bound poorly to the affinity matrix (< 10%) whereas 35%, 17% and 21% of Asn31, Asn57 and Asn81 constructs respectively bound Man-6-P. The CD-MPR has been shown to exist primarily as a dimer in the membrane [15,29]. As COS-1 cells express endogenous CD-MPRs, it is possible that the bovine CD-MPR mutants formed heterodimers with the endogenous COS-1 receptors, resulting in a species that could bind Man-6-P with a different affinity from the mutant homodimers. However, studies using homo-

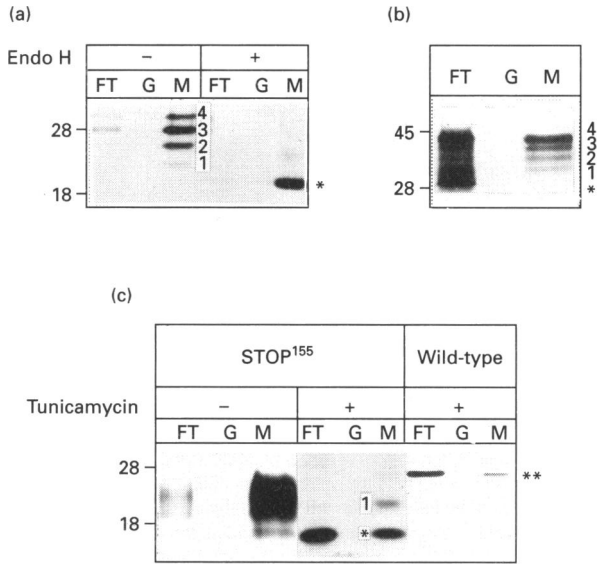

Figure 1 Ligand-affinity chromatography of glycosylation-deficient forms of wild-type and STOP^{155} CD-MPRs

(**a**) Affinity-purified [^{35}S]methionine-labelled STOP^{155} from *X. laevis* oocytes was incubated in the presence or absence of endo H and rechromatographed on pentamannosyl phosphate–agarose columns. The columns were eluted first with 5 mM glucose 6-phosphate and then with 5 mM Man-6-P. The flow-through fractions (FT), glucose 6-phosphate eluates (G) and Man-6-P eluates (M) were immunoprecipitated with CD-MPR-specific antisera and analysed by SDS/PAGE (14% gel) under reducing conditions. (**b**) The wild-type CD-MPR was expressed in a reticulocyte lysate system containing [^{35}S]methionine, microsomal membranes and oxidized glutathione (for details, see the Experimental section). The lysate was passed over a pentamannosyl phosphate–agarose column and analysed by SDS/PAGE as described above. (**c**) COS-1 cells transfected with STOP^{155} or wild-type CD-MPR cDNA were incubated in the presence or absence of 5 μg/ml tunicamycin plus [^{35}S]methionine. The cells were solubilized with detergent and passed over pentamannosyl phosphate–agarose columns as described above. After immunoprecipitation, the samples were analysed by SDS/PAGE. Identical results were obtained when the affinity chromatography was performed in the presence of 0.5% Triton X-100. *Non-glycosylated form of STOP^{155}. **Non-glycosylated form of the wild-type CD-MPR. The numbers 1–4 indicate the number of N-linked carbohydrate chains present on the receptor. The migration of molecular-mass standards is shown in kDa.

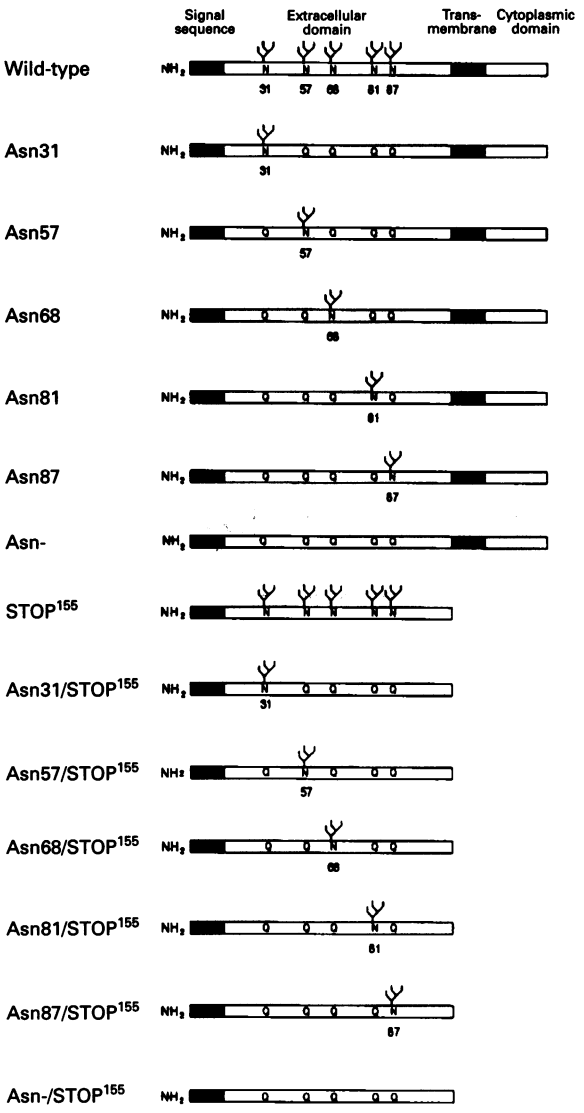

Figure 2 Schematic presentation of mutant forms of the CD-MPR

cDNA constructs encoding mutant forms of the bovine CD-MPR used in this study are depicted. STOP^{155} was generated by replacing the proline (residue 155) codon in the bovine CD-MPR sequence (CCA, nucleotides 584–586) with a stop codon (TGA) [15]. CD-MPR mutants lacking potential N-glycosylation sites were generated by replacing the asparagine residue with a glutamine residue in the consensus sequence Asn-X-Ser/Thr. The amino acid substitutions are defined using the following nomenclature: Asn31 is a mutant which preserves the asparagine (N) at position 31, while Asn^{57}, Asn^{68}, Asn^{81}, and Asn^{87} are all converted into glutamines (Q); Asn- is a mutant in which all five glycosylation sites have been removed by replacing Asn^{31}, Asn^{57}, Asn^{68}, Asn^{81} and Asn^{87} with glutamines. For details on the generation of these constructs, see the Experimental section.

bifunctional cross-linking agents showed that the majority of the mutant CD-MPRs formed homodimers (results not shown).

The differences seen in ligand binding between the various glycosylation-deficient mutants could be due to the presence of a carbohydrate chain at a particular position which could influence the conformation of the ligand-binding site. Alternatively, the replacement of asparagine residues with glutamine residues could affect Man-6-P binding by the receptor. To distinguish between these two possibilities, the CD-MPR mutants were expressed in COS-1 cells in the presence of tunicamycin so that only the

Table 1 Ligand binding, BiP association and endo H sensitivity of the CD-MPR mutants

The percentage of Man-6-P binding was calculated from the cell-associated material subjected to pentamannosyl phosphate–agarose chromatography. There were two independent transfections, and the values are means of the duplicates. In one set, CD-MPRs were purified from COS-1 cells grown in the presence of 5 μg/ml tunicamycin. The percentage of BiP association was calculated from the amount of CD-MPR that was co-immunoprecipitated with anti-BiP antibody (see the legend to Figure 3 for details). The percentage of the CD-MPR that is completely sensitive to endo H digestion is shown (two transfections). ND, not determined.

	Ligand binding (%)		BiP association (%)		
	Control	Tunicamycin	Control	Tunicamycin	Endo H sensitivity (%)
Asn31	35	6	10	ND	> 99
Asn57	17	2	16	ND	> 99
Asn68	8	4	53	ND	–
Asn81	21	1	19	ND	> 99
Asn87	42	2	6	ND	25
Asn-	9	3	52	ND	–
Wild-type	73	32	3	30	23

polypeptide chains of the mutant and wild-type CD-MPRs would be produced. Only 6% or less of the non-glycosylated form of each of the mutants produced in the presence of tunicamycin bound Man-6-P, whereas 32% of the non-glycosylated wild-type CD-MPR bound ligand (Table 1). The significantly lower binding exhibited by the non-glycosylated mutants than the non-glycosylated wild-type CD-MPR indicate that the amino acid substitutions in the mutants did have an effect on their ability to bind ligand. However, for each CD-MPR, the glycosylated form exhibited substantially higher binding than its corresponding non-glycosylated form (Table 1). These observations were further substantiated when the transfected COS-1 cells were grown in the presence of low concentrations (1 μg/ml) of tunicamycin. Under these conditions, both glycosylated and non-glycosylated species were expressed simultaneously. When the cell lysates were subjected to affinity chromatography, the glycosylated form of the wild-type and mutant CD-MPRs bound preferentially to the affinity matrix over the corresponding non-glycosylated form (results not shown), with percentages similar to those shown in Table 1. These observations support the hypothesis that N-linked oligosaccharides facilitate the folding of the CD-MPR into a functional conformation for ligand binding. The differences seen in ligand binding between the various mutant CD-MPRs is most likely due to a combination of effects caused by the amino acid substitutions and the placement of the sugar chain at a distinct glycosylation site. However, crystallographic analyses of the CD-MPR will be needed to demonstrate whether the asparagine residues of the glycosylation sites are involved in the formation of an active Man-6-P-binding site.

Association of CD-MPR mutants with BiP

To evaluate the conformation of the various mutant forms of the CD-MPR, the association of the receptors with the 78 kDa glucose-regulated protein, BiP, was measured. BiP is a soluble non-glycosylated resident protein of the ER which belongs to the 70 kDa heat shock protein family and exhibits weak intrinsic ATPase activity (for reviews, see refs. [30–32]). BiP has been shown to form stable non-covalent complexes with numerous misfolded proteins, resulting in their retention in the ER. After ATP hydrolysis, BiP dissociates from this complex [33]. This

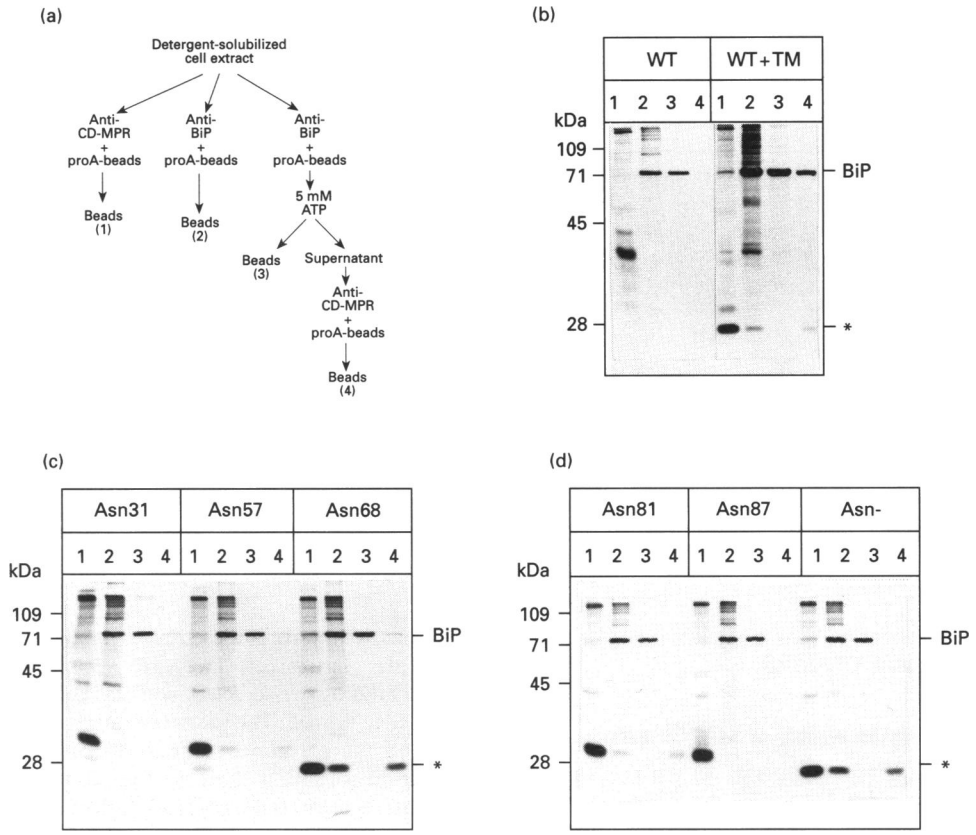

Figure 3 Association of BiP with glycosylation-deficient CD-MPR mutants

(**a**) COS-1 cells transfected with the CD-MPR mutants were solubilized with detergent. The lysate was separated into three equal aliquots to which Protein A–Sepharose beads (proA-beads) plus CD-MPR-specific antisera (anti-CD-MPR) or BiP-specific antisera (anti-BiP) were added. After an incubation of 16 h, the beads were washed exhaustively and then either eluted with SDS/PAGE sample buffer (1 and 2) or were incubated with 5 mM ATP to dissociate molecules stably associated with BiP. The Protein A–Sepharose beads (3) were eluted with SDS/PAGE sample buffer, and the supernatant containing 5 mM ATP was immunoprecipitated with CD-MPR-specific antisera plus Protein A–Sepharose (4). The samples were analysed on 12% polyacrylamide gels (**b–d**). (**b**) COS-1 cells transfected with wild-type (WT) CD-MPR in the presence or absence of 5 μg/ml tunicamycin (TM). (**c**) and (**d**) COS-1 cells transfected with glycosylation-deficient CD-MPR mutants. The migration of BiP and non-glycosylated receptor (*) are shown. The migration of molecular-mass standards are shown in kDa.

association is thought to be due to the ability of BiP to interact with exposed hydrophobic residues [27]. Detergent-solubilized COS-1 cells expressing the wild-type and mutant CD-MPRs were incubated with BiP-specific antisera (Figure 3a). The wild-type CD-MPR expressed in the presence of tunicamycin was co-immunoprecipitated by the BiP-specific antisera (Figure 3b and Table 1). As shown in Figures 3(c) and 3(d) and summarized in Table 1, a significant amount of the Asn31, Asn57, Asn68, Asn81 and Asn- mutants were also co-immunoprecipitated by the BiP-specific antisera. Furthermore, the ATP-dependent release from BiP indicated that the association between BiP and these altered receptors was specific and was not due to the recognition of a cross-reactive epitope(s) on the CD-MPR by the BiP-specific antisera (Figures 3b, 3c and 3d). In contrast, only 6% of the Asn87 mutant was found associated with BiP (Figure 3d and Table 1). The degree of BiP association varied among the different mutants, suggesting that the presence of an N-linked carbohydrate chain at a specific glycosylation site and/or the glutamine substitutions contributed to differences in the conformation of the receptor that was detected by BiP. Our interpretation that association with BiP is indicative of a non-native conformation is consistent with the finding that the level of BiP association correlates inversely with ligand binding (Table 1). For example, the two non-glycosylated mutant CD-MPRs, Asn68 and Asn-, exhibited the poorest binding to Man-6-P ($< 10\%$) but had the highest percentage (approx. 50%) of association with BiP (Table 1). To determine if the mutant CD-MPRs were degraded more rapidly than the wild-type CD-MPR, a pulse–chase labelling experiment was performed. The results showed that the various mutant CD-MPRs have a degradation rate similar to that of the wild-type receptor (results not shown). These results indicate that the mutant CD-MPRs associated with BiP are not targetted for rapid degradation.

Several studies have suggested that a transient BiP association is a normal route in the folding of some nascent polypeptide chains [26,34,35]. To evaluate the role of BiP in the biosynthesis of the wild-type CD-MPR, pulse-labelling followed by immunoprecipitation with anti-BiP and anti-(CD-MPR) antibodies was performed. We failed to co-immunoprecipitate any radiolabelled BiP molecules using the anti-(CD-MPR) antisera even when a brief pulse of 200 μCi/ml [^{35}S]methionine for 5 min was utilized (Figure 3b and results not shown). One possible explanation for this result is that there is a large intracellular pool of BiP with a slow turnover rate such that only a small percentage of BiP is labelled in this short pulse period. However, only 3% of the wild-type CD-MPR was precipitated using the BiP-specific antisera (Figure 3b and Table 1). These results suggest that BiP is not essential for the folding and oligomerization of the wild-type bovine CD-MPR. Thus, although BiP appears to be involved in the normal biosynthesis of some multimeric proteins

Table 2 Ligand binding, secretion and endo H sensitivity of STOP^{155} mutants

The percentage of Man-6-P binding was calculated from the cell-associated (Cells) or secreted (Media) material subjected to pentamannosyl phosphate–agarose chromatography. The values for the percentage of the total material expressed by transfected COS-1 cells that is found in the medium after a 24 h labelling period are shown. Samples isolated from the medium were subjected to endo H digestion. The percentage of the receptor that was completely sensitive to endo H digestion is shown. Wild-type and mutant STOP^{155} CD-MPRs were purified from COS-1 cells grown in the presence of 5 μg/ml tunicamycin. Two independent transfections were carried out in each case, and the values are means of duplicates.

	Man-6-P binding (%)						
	Control		Tunicamycin		Secretion (%)		
	Cells	Media	Cells	Media	Control	Tunicamycin	Endo H sensitivity (%)
Asn31/STOP^{155}	27	77	5	–	23	< 1	35
Asn57/STOP^{155}	29	71	3	–	7	< 1	74
Asn68/STOP^{155}	25	66	5	–	3	< 1	–
Asn81/STOP^{155}	42	76	2	–	38	< 1	78
Asn87/STOP^{155}	64	84	2	–	60	< 1	24
Asn-/STOP^{155}	18	28	1	–	5	< 1	–
STOP^{155}	47	97	27	81	96	11	10

[26,35–37], it does not appear to play a significant role in the assembly of the homodimeric CD-MPR.

Expression, secretion and ligand binding of STOP^{155} mutants

As many misfolded proteins are retained in the ER [38–40], one indication that a protein has acquired its native conformation is the efficiency with which it is targetted to its final destination. Because of the variabilities associated with the COS-1 transient expression system, the existence of endogenous COS-1 CD-MPR and the low amount of receptor present on the cell surface at steady state [29], it is difficult to quantify the levels of the various constructs that are transported out of the ER and delivered to the plasma membrane. Therefore, in order to monitor the mutants' exit from the ER easily, soluble forms of the glycosylation-deficient CD-MPRs were generated by placing the mutations in the STOP^{155} construct (Figure 2) and were measured for their presence in the medium at steady state (Table 2). Consistent with our previous studies on the STOP^{155} construct, which existed as a monomer and was efficiently secreted by *X. laevis* oocytes [15], the majority (96%) of the wild-type STOP^{155} CD-MPR was recovered from the medium of transfected COS-1 cells (Table 2). These results demonstrate that the STOP^{155} receptor efficiently traverses the secretory pathway of COS-1 cells. In contrast, the various glycosylation-deficient mutants differed in their levels of secretion. Of the mutants, the Asn87/STOP^{155} receptor was most efficiently secreted (60%), and 38% of Asn81/STOP^{155} and 23% of Asn31/STOP^{155} CD-MPRs were secreted (Table 2). Only 7% of the Asn57/STOP^{155} mutant and less than 6% of the two non-glycosylated constructs, Asn68/STOP^{155} and Asn-/STOP^{155}, were found in the medium (Table 2). These results indicate that the overall conformation of the Asn87/STOP^{155} mutant may be similar to that of the STOP^{155} CD-MPR, as it can be targetted for secretion with similar efficiency. These data also suggest that a single oligosaccharide chain at position 87, and to a lesser degree at position 81 or 31, can promote the secretion of the STOP^{155} CD-MPR. Ligand-affinity chromatography analyses showed that for each of the constructs, the receptor present in the medium exhibited a significantly higher ligand-binding ability than the corresponding cell-associated STOP^{155} mutant (Table 2). The majority (66–84%) of each of the secreted mutants, except for Asn-/STOP^{155}, was capable of binding Man-6-P (Table 2). Taken together, we interpret these results as suggesting that the receptor molecules that have folded into a conformation conducive to ligand binding are secreted, whereas the misfolded receptors are retained in the cell. The lower binding ability of the secreted Asn-/STOP^{155} mutant may be due to the presence of five amino acid substitutions, rather than the four found in the other STOP^{155} mutants, which could adversely affect the conformation of the binding site.

The relative Man-6-P-binding ability of the full-length and corresponding soluble forms of the CD-MPR mutants were found to agree well with each other (compare Tables 1 and 2). For example, consistent with the data obtained with the full-length CD-MPR mutants (see Table 1), the secreted Asn87/STOP^{155} mutant was the most similar to the secreted STOP^{155} receptor, with 84% and 97% respectively binding to Man-6-P (Table 2). However, the secreted Asn68/STOP^{155} mutant bound Man-6-P much more efficiently than the secreted Asn-/STOP^{155} mutant (66% and 28% respectively; see Table 2), whereas their corresponding full-length receptors, Asn68 and Asn-, bound ligand similarly (8% and 9% respectively; see Table 1). In addition, a significantly higher percentage of each of the secreted glycosylation-deficient STOP^{155} mutants, as well as the secreted wild-type STOP^{155} CD-MPR, bound to the ligand-affinity columns than their full-length counterpart (compare Tables 1 and 2). One possible explanation for this finding is that the conformation of the membrane-bound CD-MPR differs from that of the soluble forms of the receptor. An indication of this was seen in *X. laevis* oocytes where the wild-type CD-MPR was efficiently glycosylated, with virtually all of the receptor molecules having four oligosaccharide chains, whereas the STOP^{155} mutant was expressed as a mixed population of molecules containing one, two, three or four carbohydrate chains [15].

The various STOP^{155} mutants were synthesized in the presence of tunicamycin to prevent N-glycosylation and were analysed for ligand binding and secretion. In the presence of tunicamycin, the secretion of each STOP^{155} mutant was impaired to the extent that no receptor was detected in the medium and only 11% of the STOP^{155} CD-MPR was secreted (Table 2). In contrast, a significant amount of their glycosylated counterpart produced in the absence of tunicamycin was secreted (Table 2). A similar observation has been made for the VSV G protein [42] and the transferrin receptor [43]. When synthesized in the presence of tunicamycin, these non-glycosylated proteins did not reach the

cell surface. The authors concluded that N-linked oligosaccharides play an indirect role in the intracellular transport of these molecules [42,43]. In the presence of tunicamycin, 5% or less of the cell-associated STOP^{155} mutants bound Man-6-P whereas 27% of the cell-associated STOP^{155} CD-MPR bound ligand (Table 2). These results are similar to those seen for the full-length CD-MPRs synthesized in the presence of tunicamycin (Table 1) and indicate that the amino acid substitutions had some affect on the ligand-binding conformation of the receptor. However, the presence of an oligosaccharide chain greatly increased the ligand-binding ability of all the STOP^{155} mutant constructs (Table 2). These data suggest that the presence of a single carbohydrate chain on the CD-MPR influences the folding of the nascent polypeptide chain, which is required for departure out of the ER, and subsequent formation of the Man-6-P-binding site. In support of this hypothesis are earlier studies which showed that the lack of N-linked oligosaccharides on the Semliki Forest virus spike glycoproteins, E1 and P62 [44], influenza haemagglutinin [44] and VSV G [45] resulted in the aggregation and retention of these proteins in the ER. It was concluded from these studies that the addition of sugar chains to nascent polypeptide chains increases the overall solubility of the folding intermediates which prevents the formation of aggregates [44] and aberrant disulphide bonds [45]. Thus the carbohydrates on the CD-MPR may exert a global effect on the solubility properties of the receptor which, in turn, may promote the correct folding of the polypeptide.

To determine the type of oligosaccharide chain (complex or high-mannose) added to each glycosylation site of the bovine CD-MPR, the secreted STOP^{155} mutants were subjected to endo H digestion (Table 2). As the processing of N-linked carbohydrates to the complex type takes place in the Golgi [46], the STOP^{155} constructs recovered from the medium of COS-1 cells represent the fully processed forms of the receptors. The results show that Asn68/STOP^{155} is not glycosylated. Asn31/STOP^{155} and Asn87/STOP^{155} have predominantly complex-type oligosaccharides, whereas Asn57/STOP^{155} and Asn81/STOP^{155} contain primarily high-mannose-type oligosaccharides (Table 2). These results differ from those for the full-length CD-MPR mutants in that the oligosaccharide at position 31 is predominantly of the high mannose type (Table 1). This discrepancy may be due to differences in the conformation of the soluble versus membrane-associated forms of the Asn31 mutant which could affect oligosaccharide processing. The types of oligosaccharides found at specific glycosylation sites of the bovine CD-MPR expressed in COS-1 cells differ from those found for the human CD-MPR expressed in BHK cells [8]. This may be due to differences in the levels and types of glycosyltransferases present in the different cell types [46] and/or to the amino acid sequence differences between the bovine [5] and human [6] CD-MPRs which could influence oligosaccharide processing.

Studies by Li and Jourdian [9] have provided evidence that the type of carbohydrates on the CD-MPR can affect the affinity the receptor displays towards phosphomannosyl-containing ligands. Two glycosylation isoforms of the CD-MPR, which differed in their sialic acid and polylactosamine content, were isolated from bovine testis homogenates. Chromatography on pentamannosyl phosphate–agarose followed by elution with increasing concentrations of Man-6-P revealed that the isoform containing sialic acid and polylactosamine residues exhibited a lower affinity for phosphomannosyl residues than the isoform that lacked sialic acid and lactosamine units [9]. Thus these results [9] and the current studies indicate that oligosaccharides on the CD-MPR can influence both ligand-binding affinities and the folding of the nascent polypeptide chain.

In summary, the data presented in this report support a role for N-linked oligosaccharides in the folding of the nascent CD-MPR polypeptide into a conformation that is required for intracellular transport and ligand binding. Our results also demonstrate that the Asn87 and Asn87/STOP^{155} mutants, each of which contains a single sugar chain, exhibit similar ligand-binding properties to those of the wild-type and STOP^{155} CD-MPRs respectively. The availability of functional CD-MPRs with reduced carbohydrate content may prove to be useful in future structural studies in which the presence of heterogeneous oligosaccharides would be detrimental in analyses requiring a homogeneous protein preparation.

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