

# Assembly of the Ligand-binding Conformation of $M_r$ 46,000 Mannose 6-Phosphate-specific Receptor Takes Place before Reaching the Golgi Complex

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**Abstract.** The early steps in the biosynthesis of  $M_r$  46,000 mannose 6-phosphate-specific receptor (MPR 46) have been studied by *in vivo* labeling of transfected BHK cells. The acquisition of phosphomannan-binding activity was compared with changes in protein structure and posttranslational modifications of MPR 46. Intramolecular disulfide bonds were formed before MPR 46 acquired a ligand-binding conformation. A conformational change that resulted in increased trypsin resistance, formation of highly immunogenic epitopes and assembly to noncovalently linked homodimers was observed almost simultaneously with the acquisition of ligand-binding activity. MPR 46 was

shown to acquire ligand-binding activity before N-linked oligosaccharides were processed to complex-type forms. Maturation of the ligand-binding conformation was observed under conditions where transport to the Golgi was blocked by lowering the temperature to 16°C, or by addition of brefeldin A or dinitrophenol to the medium at 37°C. This suggests that receptor maturation and assembly take place before reaching the Golgi complex. The affinity towards phosphomannan-containing ligands was shown to be similar for the high-mannose and complex-glycosylated forms of MPR 46.

**S**ORTING and transport of newly synthesized lysosomal enzymes from the Golgi apparatus to prelysosomes are mediated by the mannose 6-phosphate recognition system (for review see von Figura and Hasilik, 1986 and Kornfeld, 1987). Lysosomal enzymes are synthesized as glycoproteins at the rough ER. Their N-linked, high-mannose oligosaccharides are modified by transfer of phosphodiester groups, which are hydrolyzed to mannose 6-phosphate monoesters in the *trans*-Golgi (Lazarino and Gabel, 1988). Binding of lysosomal proteins to mannose 6-phosphate-specific receptors, the first step for sorting from secretory proteins, occurs most likely in the *trans* cisternae of the Golgi stack (Duncan and Kornfeld, 1988). After transport to prelysosomes, the receptor-ligand complexes dissociate due to the acidic pH, and the receptors recycle back to the *trans*-Golgi.

Two mannose 6-phosphate-specific receptors (MPR 46 and MPR 215)<sup>1</sup> are known (for review see von Figura and Hasilik, 1986 and Kornfeld, 1987). Both receptors are integral membrane glycoproteins. The NH<sub>2</sub>-terminal, luminal domain of MPR 46, which contains the mannose 6-phosphate-binding domain (Wendland et al., 1989), was reported to be homologous to each of the 15 repetitive domains of MPR 215 (Dahms et al., 1987). As a first attempt to characterize the mannose 6-phosphate-binding domain of MPR 46,

we have recently investigated the tertiary and quaternary structure of MPR 46 using purified and *in vitro* synthesized receptor (Hille et al., 1989). We have shown that N-linked core glycosylation and formation of intramolecular disulfide bonds are prerequisites for the ligand-binding conformation of MPR 46. In addition, the ligand-binding conformation of MPR 46 differed from nonbinding conformations with regard to the presence of highly immunogenic epitopes, increased resistance towards tryptic digestion, and assembly to noncovalently linked homodimers.

Here we have used pulse-chase labeling of BHK-MPR 46 cells to investigate the time course of structural changes that occur during maturation of MPR 46 *in vivo*, and to identify the subcellular compartment where folding and assembly of MPR 46 take place.

## Materials and Methods

### Cell Culture, Metabolic Labeling and Preparation of Cell Extracts

Transfected BHK cells overexpressing human MPR 46 (BHK-MPR 46 cells) have been described (Wendland et al., 1989). Labeling with [<sup>35</sup>S]methionine (3.7 or 37 MBq per 6-cm Petri dish for 30 or 5 min pulse, respectively) was performed as described (Hasilik and Neufeld, 1980). At the end of the labeling, the medium was removed and immediately replaced by chase medium that contained 1.5 mM methionine and was equilibrated to 37°C. Cells were harvested by scraping. A detergent extract of the membranes,

1. *Abbreviations used in this paper:* MPR 46,  $M_r$  46,000 mannose 6-phosphate-specific receptor; MPR 215,  $M_r$  215,000 mannose 6-phosphate-specific receptor.

referred to as membrane extract, was prepared as described in detail elsewhere (Wendland et al., 1989) and used for phosphomannan affinity chromatography or immunoprecipitation.

For chase at 16°C, cells were labeled for 5 min at 37°C and chased for up to 5 h at 16°C. For chase in the presence of dinitrophenol, cells were labeled for 5 min at 37°C and chased at 37°C in the presence of 3 mM dinitrophenol (stock solution 300 mM dinitrophenol in boiling ethanol). For pulse-chase in the presence of brefeldin A, brefeldin A (Sandoz Ltd., Basel, Switzerland) was added to the culture medium (final concentration 2.5–10 µg/ml; stock solution 1 mg/ml in methanol) during 1 h of preincubation, 30 min of labeling, and 1 h of chase.

To inhibit processing of *N*-linked high-mannose oligosaccharides, 1 mM deoxymannojirimycin (Bayer, Wuppertal, FRG) was added to the chase medium.

### **Phosphomannan Affinity Chromatography**

Affinity chromatography was performed with phosphomannan Sepharose 4B (Stein et al., 1987a) as described (Wendland et al., 1989). Unless otherwise indicated, the affinity column was washed with 5 mM glucose 6-phosphate and eluted with 5 mM mannose 6-phosphate. The results for the glucose 6-phosphate eluate, which did not contain radioactivity, are not shown in Figs. 1–5.

### **Trypsin Treatment of MPR 46**

The eluates of phosphomannan affinity chromatography (containing 0.5 mg/ml of BSA) were adjusted to 5 µg/ml of TPCK-trypsin (Sigma Chemie GmbH Deisenhofen, FRG) and incubated at 37°C. At the times indicated, an aliquot of the incubation mixture was removed and the reaction was stopped by adding 18% FCS, 16 µg/ml trypsin inhibitor from egg white (Boehringer, Mannheim, FRG) and 2 mM PMSF (final concentrations).

### **Cross-Linking with Disuccinimidylsuberate**

Chemical cross-linking with 0.06 mM disuccinimidylsuberate (Pierce Chemical Co., Oud Beijerland, The Netherlands) was performed as described (Wendland et al., 1989).

### **Immunoprecipitation**

Immunoprecipitation with antiserum against human MPR 46 was performed as described (Wendland et al., 1989). Unless otherwise indicated, incubation with antiserum was performed overnight.

### **Enzymatic Deglycosylation**

Metabolically labeled MPR 46 was isolated by immunoprecipitation. 30 µl of the solubilized immunoprecipitate were treated with 100 mU of endoglucosaminidase H for 15 h at 37°C (Stein et al., 1987b).

### **SDS-PAGE**

SDS-PAGE was performed as described (Laemmli, 1970). The sample buffer contained 5 mM EDTA and, for reducing gels, 10 mM DTT. <sup>14</sup>C-methylated protein standards were from New England Nuclear (Boston, MA). Fluorography was performed as described (Bonner and Laskey, 1974). [<sup>35</sup>S] radioactivity in SDS gels was quantitated by densitometric scanning of fluorograms.

## **Results**

### **MPR 46 Acquires a Ligand-binding Conformation *In Vivo* before *N*-linked High-Mannose Oligosaccharides Are Processed**

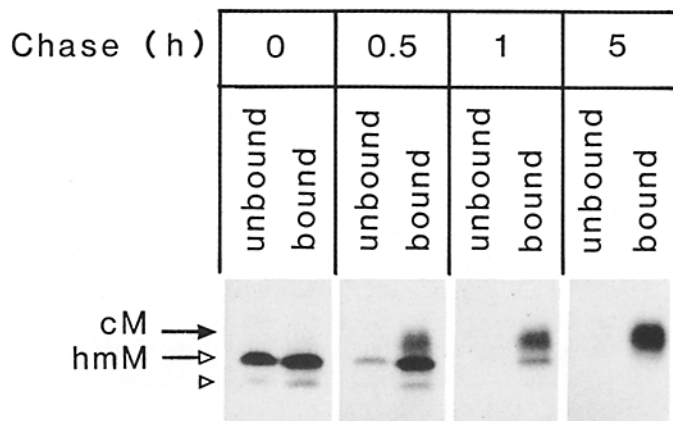
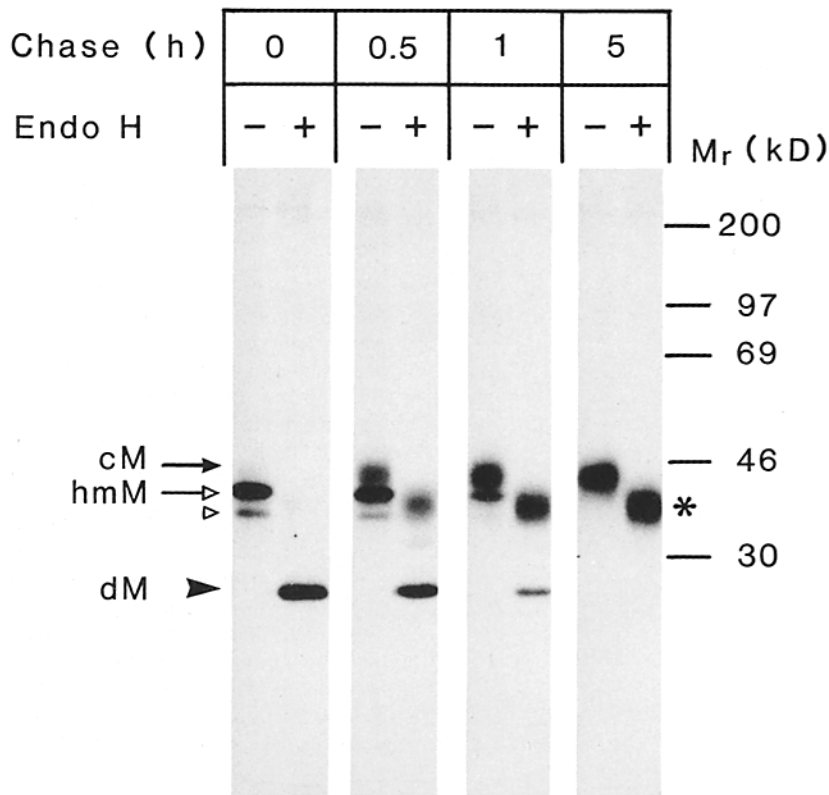
Cells were metabolically labeled for 30 min followed by up to 5 h chase, and membrane extracts were prepared from the cells to immunoprecipitate MPR 46. MPR 46 was found to be synthesized as a 37.5-kD protein that contained exclusively high-mannose type, endoglucosaminidase H-sensitive oligosaccharides (Fig. 1, *top*). During the 5-h chase, MPR 46 was completely converted to a 41-kD form that contained

mainly complex-type, endoglucosaminidase H-resistant oligosaccharides. To investigate the ligand-binding activity of newly synthesized MPR 46, the membrane extracts were subjected to phosphomannan affinity chromatography (Fig. 1, *bottom*). After 30 min pulse, about half of the high-mannose forms of MPR 46 bound to the phosphomannan affinity column in a mannose 6-phosphate-dependent manner. The amount of ligand-binding high-mannose forms increased during the chase, resulting in efficient binding after 30 min. The complex glycosylated forms of MPR 46 bound efficiently at any time of the chase.

To investigate in more detail the early steps in maturation of MPR 46, a short pulse of 5 min was performed, followed by up to 30 min chase. At the end of the chase, MPR 46 was recovered mostly in the high-mannose form, and only small amounts had been processed to complex-type forms (Fig. 2, *left*). Quantitation of newly synthesized MPR 46 (Fig. 3, *top*) showed an increase in the amount of total immunoprecipitable MPR 46 during the first 10 min of chase, which is most likely due to an increase in immunoreactivity after biosynthesis (see below). After 5 min of labeling, 15–30% of MPR 46 bound to phosphomannan (Fig. 3, *top*; see also Fig. 7). The amount of phosphomannan-binding MPR 46 increased to 80% after 30 min of chase. Half of the newly synthesized MPR 46 had acquired a phosphomannan-binding conformation after ~12 min of chase.

### **Acquisition of the Ligand-binding Conformation of MPR 46 Requires Formation of Intramolecular Disulfide Bonds**

To investigate the formation of disulfide bonds in newly synthesized MPR 46, the immunoprecipitates obtained after pulse labeling (5 min), chase (up to 30 min) and phosphomannan affinity chromatography were analyzed by SDS-PAGE under reducing and nonreducing conditions (Fig. 2). In the phosphomannan-binding fraction, the monomer of MPR 46 as well as the trace amounts of SDS-resistant dimer (see Stein et al., 1987a) showed higher electrophoretic mobilities under nonreducing than under reducing conditions (Fig. 2). This suggests that the phosphomannan-binding forms contained internal disulfide bonds as observed for MPR 215 (Sahagian and Neufeld, 1983). When MPR 46 from the unbound fraction was analyzed under reducing conditions, it showed the same electrophoretic mobility as the phosphomannan-binding forms. Under nonreducing conditions, however, MPR 46 from the unbound fraction showed a heterogenous electrophoretic mobility: part of it had acquired the increased electrophoretic mobility indicating that in this fraction MPR 46 contained intramolecular disulfide bonds. A second band exerted the same electrophoretic mobility as reduced MPR 46, suggesting that it represented still unfolded MPR 46. The fraction of unfolded MPR 46 decreased during the chase as did the immunoreactive material of heterogenous molecular weight in the high-molecular weight region of the gel. The latter is most likely attributed to the formation of intermolecular disulfide bonds between receptor molecules and other proteins. Together, these results show that formation of correct intramolecular disulfide bonds is a prerequisite for the phosphomannan-binding conformation. However, part of the newly synthesized MPR 46 that already contained intramolecular disulfide bonds failed to bind to the affinity column. This means that



**Figure 1.** Processing of N-linked carbohydrate and maturation of the phosphomannan-binding conformation of newly synthesized MPR 46. BHK-MPR46 cells were labeled for 30 min with [ $^{35}$ S]methionine followed by up to 5 h chase. A detergent extract of the cell membranes was analyzed. (*Top*) MPR 46 was immunoprecipitated, treated with Endo H (*Endo H*) and analyzed by reducing SDS-PAGE and fluorography. (*Bottom*) The membrane extract was subjected to phosphomannan affinity chromatography. MPR 46 was immunoprecipitated from the flow-through (*unbound*) and mannose 6-phosphate eluate (*bound*) and analyzed by reducing SDS-PAGE and fluorography. (*cM*) complex glycosylated monomeric MPR 46; (*hm*) high-mannose type monomeric MPR 46; (*dM*) deglycosylated MPR 46; (*asterisk*) partly deglycosylated MPR 46; (*open arrowhead*) proteolytic fragment of MPR 46 created during membrane preparation. The apparent molecular mass ( $M_r$ ) of protein standards is indicated.

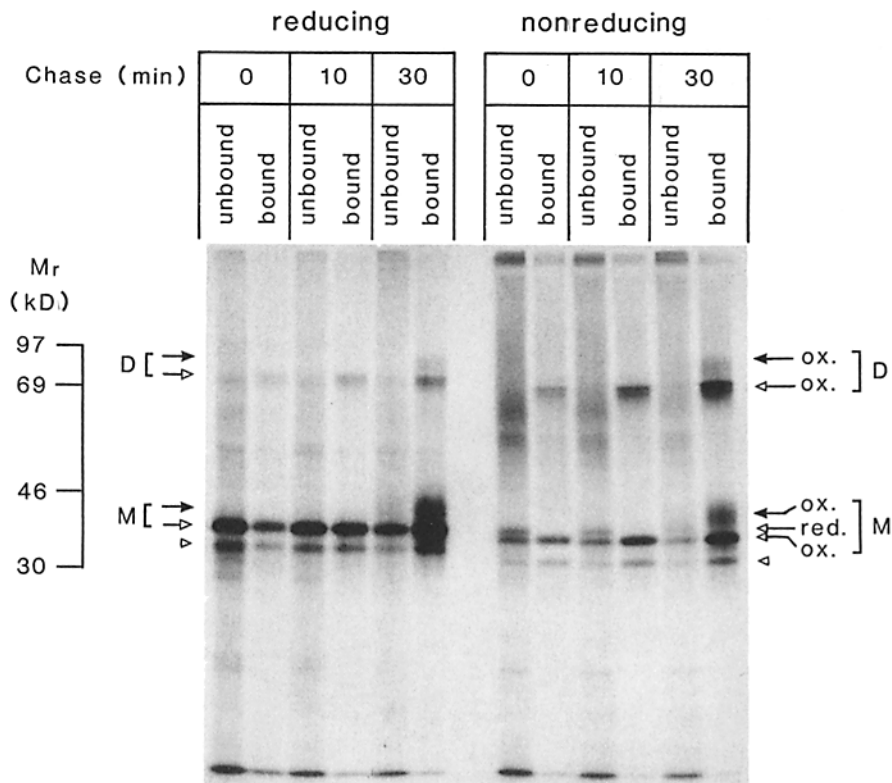
either rearrangement of disulfide bonds has to occur or formation of disulfide bonds is not sufficient for the acquisition of the phosphomannan-binding conformation.

#### **Acquisition of the Phosphomannan-binding Conformation of MPR 46 Occurs Simultaneously with a Conformational Change and Formation of Homodimers**

The conformation of newly synthesized MPR 46 was studied using an antiserum that precipitates the mature forms of MPR 46 efficiently within 1 h, whereas precipitation of newly synthesized MPR 46 requires an overnight incubation with antiserum (Hille et al., 1989). After 5 min pulse-labeling and up to 30 min of chase, MPR 46 was sequentially immunoprecipitated for 1 h and overnight. The amount of

MPR 46 that could be immunoprecipitated within 1 h increased continuously during the chase suggesting that additional immunogenic epitopes had been formed (Fig. 3, *bottom*). The increase in immunogenicity was exactly parallel to the increase in phosphomannan-binding activity with a half time of  $\sim 12$  min (compare *top* and *bottom* of Fig. 3). When the eluates of phosphomannan affinity chromatography were analyzed separately, MPR 46 that had not bound to the affinity column was recovered mainly in the overnight immunoprecipitate (not shown), whereas the phosphomannan-binding forms of MPR 46 were efficiently immunoprecipitated within 1 h (Fig. 3, *bottom*).

The conformation of MPR 46 was further studied by limited digestion with trypsin. The treatment resulted in two major 34- and 30.5-kD polypeptides, as documented for the



**Figure 2.** Formation of disulfide bonds in newly synthesized MPR 46. BHK-MPR 46 cells were labeled for 5 min with [<sup>35</sup>S]methionine followed by up to 30 min chase. A detergent extract of the cell membranes was subjected to phosphomannan affinity chromatography. MPR 46 was immunoprecipitated from the flow-through (*unbound*) and manose 6-phosphate eluate (*bound*) followed by SDS-PAGE under reducing or nonreducing conditions and fluorography. (*M*) monomeric MPR 46; (*D*) SDS-resistant dimer; (*ox.*) disulfide bonds containing MPR 46; (*red.*) reduced MPR 46; (*arrows*) complex glycosylated forms of MPR 46; (*open arrows*) high-mannose forms of MPR 46; (*open arrowhead*) proteolytic fragment of MPR 46 created during membrane preparation. The apparent molecular mass (*M<sub>r</sub>*) of protein standards is indicated.

10-min chase in Fig. 4 A. Quantitation of the tryptic peptides showed that the yield of peptides was higher for the phosphomannan-binding forms of MPR 46 than for MPR 46 that did not bind to the affinity column (Fig. 4 B). Furthermore, at any time of the chase, the larger 34-kD polypeptide was found to be more abundant in the tryptic products of the phosphomannan-binding forms compared with the unbound fraction (shown for the 10-min chase in Fig. 4 A). Because the bound fraction only contained phosphomannan-binding proteins, whereas the unbound fraction contained all remaining proteins from the detergent extract, it was necessary to rule out the possibility that differences in the proteolytic pattern were due to endogenous protease activity in the unbound fraction. Therefore, both fractions were incubated for 1 h at 37°C without trypsin. This treatment did not cause a detectable decrease in the amount or molecular mass of MPR 46 compared to the starting material (data not shown). Thus, at any time point of the chase, the phosphomannan-binding forms of MPR 46 were more trypsin-resistant than those forms of MPR 46 that did not bind to the affinity column.

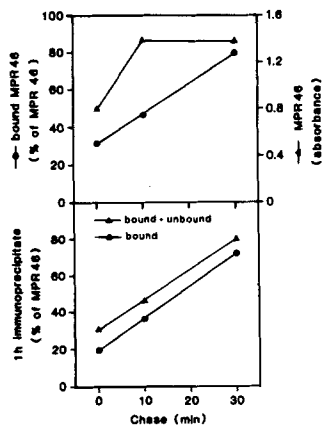
When newly synthesized forms of MPR 46, that had been separated by phosphomannan affinity chromatography, were subjected to chemical cross-linking with disuccinimidylsuberate, the phosphomannan-binding forms were efficiently cross-linked to dimers. In contrast, dimers were not detectable in the unbound fraction (Fig. 5).

Taken together, the correlation between the appearance of highly immunogenic epitopes, increased trypsin resistance, dimer formation and phosphomannan-binding activity suggests that all these properties are conferred to newly synthesized MPR 46 simultaneously as the result of a conformational change.

#### Maturation of MPR 46 Occurs before Reaching the Golgi Complex

The structural changes of MPR 46, which are involved in the maturation of the ligand-binding conformation, were observed while MPR 46 still contained high-mannose oligosaccharides. Therefore, we have investigated whether maturation of MPR 46 takes place before exit from the ER, or in the *cis*-Golgi just before processing of the N-linked oligosaccharides. Cells were metabolically labeled for 5 min at 37°C followed by a chase at 16°C to prevent the fusion of transport vesicles that deliver products of the ER to the Golgi apparatus (Saraste and Kuismanen, 1984). After the chase at 16°C for 5 h, MPR 46 was recovered exclusively in the high-mannose form which was sensitive to digestion with Endo H (Fig. 6, first and second lane from the left). In contrast, at 37°C processing to complex-type forms was observed after 30–45 min of chase already (Fig. 7, top; compare also Fig. 1). When cells were rewarmed to 37°C after the 5 h chase at 16°C, the carbohydrate chains of MPR 46 were processed to complex-type forms within 60 min (Fig. 6), i.e., with kinetics similar to MPR 46 from cells that had not been cooled to 16°C (compare with Fig. 1). These data suggest that transport to the Golgi had been efficiently blocked by lowering the temperature to 16°C and that the block of transport was reversible. The fraction of MPR 46 that bound to phosphomannan increased substantially during the chase at 16°C (Fig. 7, bottom), albeit more slowly than in control cells chased at 37°C.

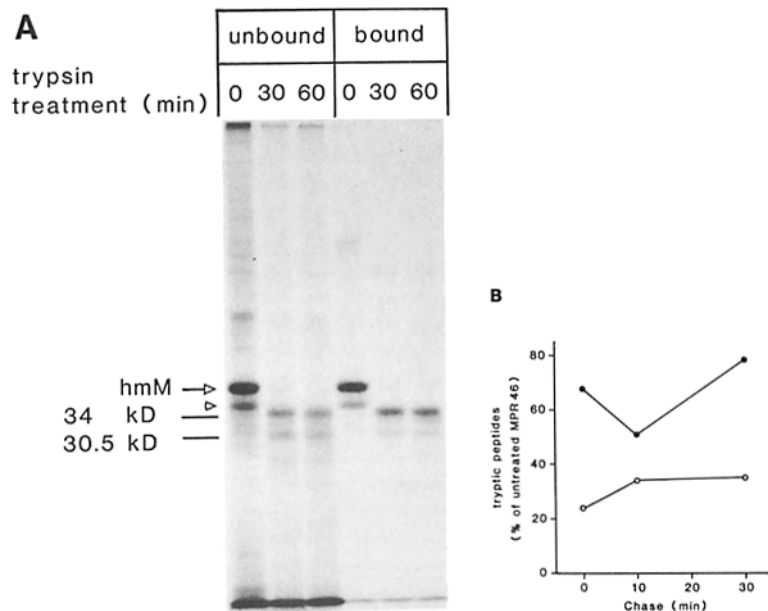
The exit of proteins from the ER into transport vesicles can be blocked by dinitrophenol (Balch et al., 1986; Copeland et al., 1988). To investigate maturation of MPR 46 under



**Figure 3.** Maturation of the phosphomannan-binding conformation and highly immunogenic epitopes of MPR 46. BHK-MPR46 cells were labeled for 5 min with [<sup>35</sup>S]methionine followed by up to 30 min chase. A detergent extract of the cell membranes was subjected to phosphomannan affinity chromatography. MPR 46 was immunoprecipitated from an aliquot of the membrane extract, the flow-through (*unbound*) and mannose 6-phosphate eluate (*bound*) for 1 h. The supernatant of the

1 h immunoprecipitation was incubated overnight with fresh anti-MPR46 antiserum. The immunoprecipitates were analyzed by reducing SDS-PAGE followed by fluorography. MPR 46 was quantitated by densitometric scanning of the fluorogram. **Top panel**, phosphomannan binding of MPR 46. (▲—▲) Total immunoprecipitable MPR 46; for each time point, MPR 46 recovered in the 1 h and overnight immunoprecipitates from the membrane extract is given as relative units of the absorbance determined by densitometric scanning. The increase in immunoprecipitable MPR 46 during the first 10 min of chase was most likely due to an increase in immunoreactivity of MPR 46, and/or by ongoing [<sup>35</sup>S]methionine incorporation using the pool of labeled methionyl-tRNA that had been formed during the pulse. (●—●) Bound MPR 46; for each time point, the sum of the 1 h and overnight immunoprecipitates obtained from the bound fraction was calculated and is expressed as percent of total immunoprecipitable MPR 46. **(Bottom)** immunogenicity of MPR 46. (▲—▲) 1 h immunoprecipitate of MPR 46 from the membrane extract; (●—●) 1 h immunoprecipitate of bound MPR 46; values are expressed as percent of total immunoprecipitable MPR 46 at each time point.

these conditions, cells were metabolically labeled for 5 min and chased at 37°C in the presence of 3 mM dinitrophenol. At the end of a 60-min chase, ~93% of the oligosaccharide chains of MPR 46 were recovered in the high-mannose form



(Fig. 7, top). The amount of complex glycosylated MPR 46 increased to 10 and 13% after 90 and 120 min of chase, respectively (data not shown). On the other hand, 87% of MPR 46 had achieved a ligand-binding conformation after 60 min of chase in the presence of dinitrophenol (Fig. 7, bottom).

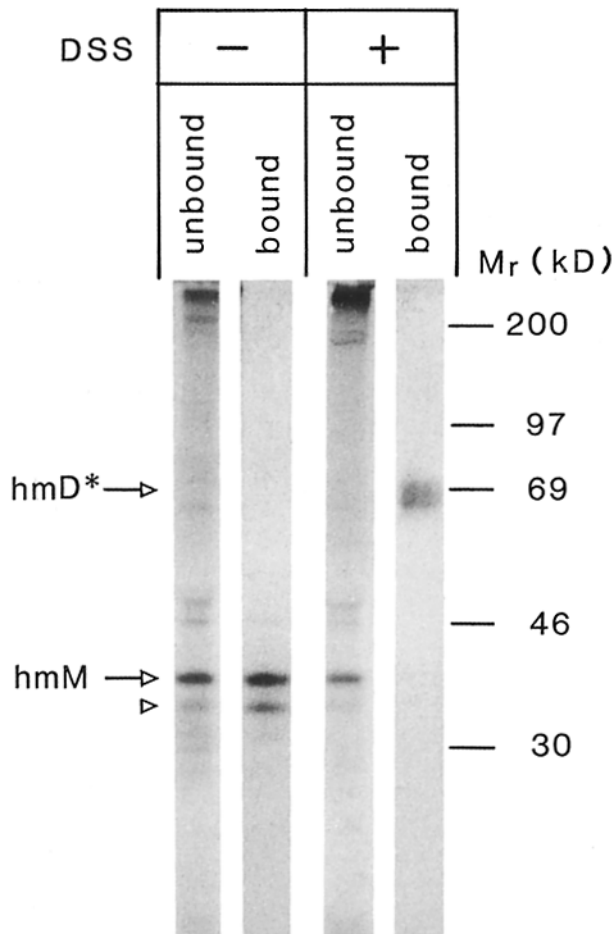
Another inhibitor of transport from the ER to the Golgi is the drug brefeldin A (Fujiwara et al., 1988). To investigate the effect of brefeldin A on maturation of MPR 46, cells were metabolically labeled for 30 min and chased for 1 h in the presence or absence of brefeldin A (2.5 μg/ml). In contrast to controls, the oligosaccharides of MPR 46 from brefeldin A-treated cells remained completely sensitive to endoglucosaminidase H (data not shown). The endo H-sensitive forms of MPR 46 from brefeldin A-treated cells efficiently bound to the phosphomannan affinity column in a mannose 6-phosphate-dependent manner (Fig. 8). The same results were obtained, when BHK-MPR 46 cells were treated with 5 or 10 μg/ml brefeldin A (data not shown).

Together, these results suggest that maturation of phosphomannan-binding MPR 46 takes place before the receptor is delivered to the Golgi complex.

#### The Affinity of MPR 46 to Phosphomannan-containing Ligands Is Not Changed by Oligosaccharide Processing

Because we have shown above that the high-mannose forms of MPR 46 bind to phosphomannan-containing ligands, carbohydrate processing does not seem to be required for binding under the conditions of affinity chromatography routinely used for these experiments. To find out whether carbohydrate processing might have a more subtle effect on phosphomannan binding of MPR 46 by changing its affinity to the ligands, we have compared MPR 46 with high-mannose and complex-type oligosaccharides in more detail. Cells were metabolically labeled for 30 min and, where indicated, a chase of 5 h was performed in the absence or presence of deoxymannojirimycin. Membrane extracts were prepared from the cells and subjected to phosphomannan affinity chromatography. In all cases, MPR 46 was eluted as

**Figure 4.** Trypsin treatment of newly synthesized MPR 46. BHK-MPR46 cells were labeled for 5 min with [<sup>35</sup>S]methionine followed by up to 30 min chase. A detergent extract of the cell membranes was subjected to phosphomannan affinity chromatography. The flow-through (*unbound*) and mannose 6-phosphate eluate (*bound*) were treated with trypsin for 30 or 60 min. MPR 46 was immunoprecipitated overnight followed by reducing SDS-PAGE and fluorography. **(A)** Fluorogram of the tryptic patterns obtained for the 10-min chase; (*hmM*) high-mannose type monomeric MPR 46; (*open arrowhead*) proteolytic fragment of MPR 46 created during membrane preparation; the position of the 34- and 30.5-kD tryptic peptides is indicated. **(B)** Trypsin resistance of MPR 46 from the bound and unbound fraction. The tryptic peptides were quantitated by densitometric scanning of fluorograms. For each time point, the sum of the 34- and 30.5-kD tryptic peptides was calculated in the bound (●—●) and unbound (○—○) fraction. Values are expressed as percent of untreated MPR 46 in the bound or unbound fraction.



**Figure 5.** Dimerization of newly synthesized MPR 46. BHK-MPR 46 cells were labeled for 5 min with [<sup>35</sup>S]methionine followed by a 10 min chase. A detergent extract of the cell membranes was subjected to phosphomannan affinity chromatography. Half of the flow-through (*unbound*) and mannose 6-phosphate eluate (*bound*) were used for cross-linking with disuccinimidylsuberate (*DSS*). MPR 46 was immunoprecipitated and analyzed by reducing SDS-PAGE and fluorography. (*hmM*) high-mannose type monomeric MPR 46; (*hmD\**) chemically cross-linked dimer; (*open arrowhead*) proteolytic fragment of MPR 46 created during membrane preparation. The apparent molecular mass (*M<sub>r</sub>*) of protein standards is indicated.

a broad peak with a maximum at 2 mM mannose 6-phosphate (Fig. 9). No significant difference in the elution profiles was observed suggesting that high-mannose and complex-type forms of MPR 46 have similar affinities towards immobilized phosphomannan.

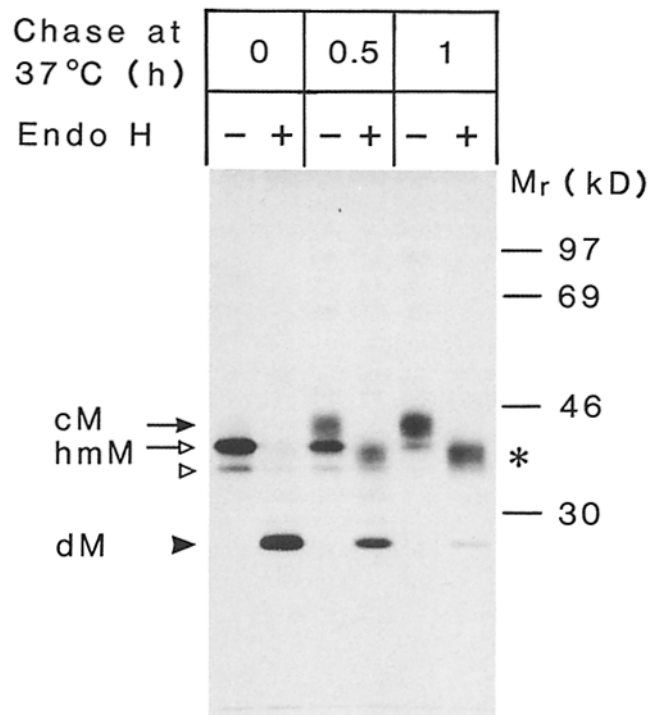
### Discussion

The time course of structural and conformational changes observed after biosynthesis of MPR 46 is summarized in Table I. The sequence of events derived from the *in vivo* studies presented here coincides with the results obtained recently using an *in vitro* translation-translocation system (Hille et al., 1989). After translation and core glycosylation of MPR 46 *in vitro* and *in vivo*, acquisition of the phosphomannan-binding conformation required two steps: formation of in-

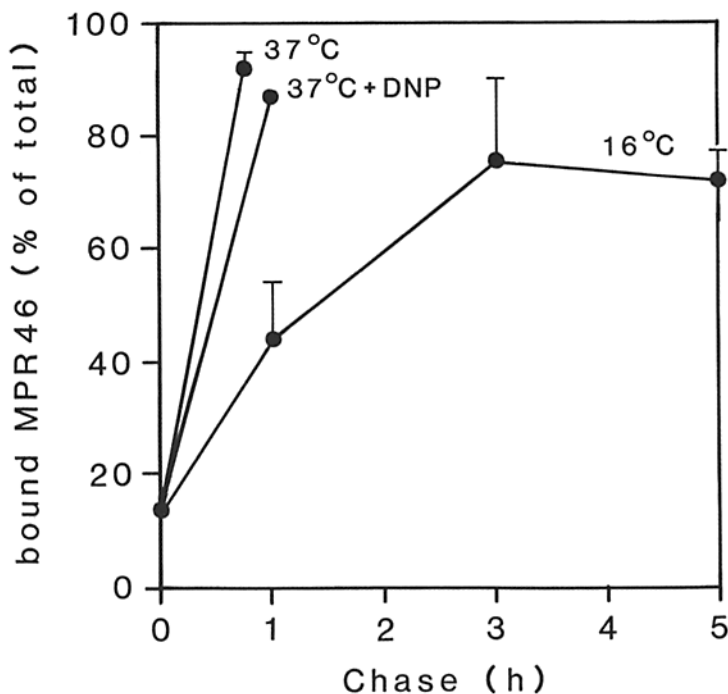
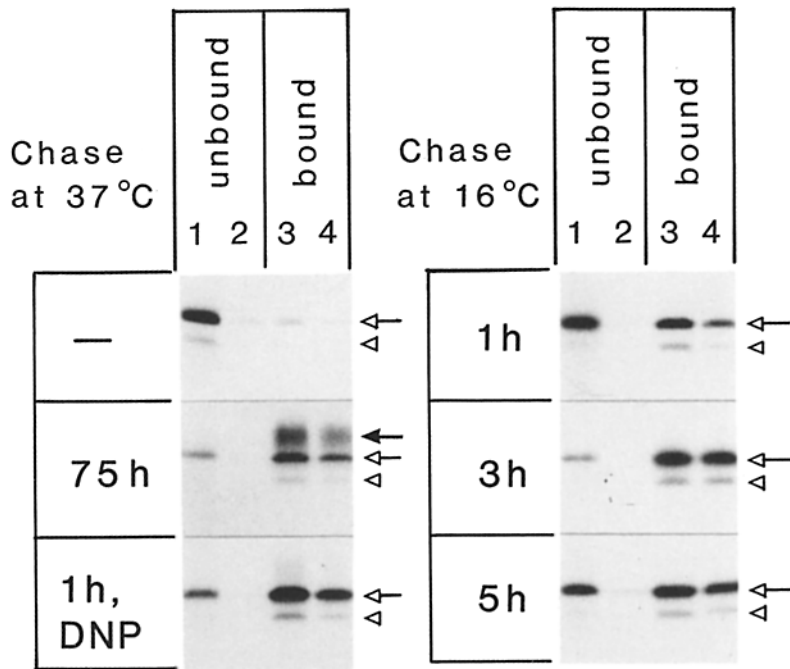
tramolecular disulfide bonds and a conformational change which resulted in increased trypsin resistance, formation of highly immunogenic epitopes and dimerization. While only ~25% of *in vitro* synthesized MPR 46 had acquired a ligand-binding conformation even after prolonged chase, *in vivo* synthesized MPR 46 was efficiently converted to phosphomannan-binding forms within 30 min of chase.

In the BHK-MPR 46 cells used here, maturation of the phosphomannan-binding conformation occurred with a half time of about 12 min. Since acquisition of the ligand-binding conformation of MPR 46 requires oligomerization, which has been shown to be concentration dependent (Waheed et al., 1989), and the transfected BHK-MPR 46 cells express at least 40 times more human MPR 46 than endogenous MPR 46 (Wendland et al., 1989), we cannot exclude that in cells expressing only endogenous MPR 46 maturation of the ligand-binding conformation takes longer than in transfected BHK-MPR 46 cells. For the M6P-IGF II receptor it has been also shown that acquisition of ligand-binding activity after biosynthesis is preceded by a lag phase (Sahagian and Neufeld, 1983).

Previous studies have shown the existence of tetramers of MPR 46, as demonstrated by cross-linking of purified MPR



**Figure 6.** Reversible inhibition of transport by lowering the temperature to 16°C. BHK-MPR 46 cells were labeled for 5 min with [<sup>35</sup>S]methionine, chased for 5 h at 16°C, and recultured at 37°C for the times indicated. At the end of the chase, a detergent extract of the cell membranes was prepared and used for immunoprecipitation of MPR 46. The immunoprecipitate was treated with endo H (*Endo H*) and analyzed by reducing SDS-PAGE and fluorography. (*cM*) complex glycosylated monomeric MPR 46; (*hmM*) high-mannose type monomeric MPR 46; (*dM*) deglycosylated MPR 46; (*asterisk*, partly deglycosylated MPR 46; (*open arrowhead*) proteolytic fragment of MPR 46 created during membrane preparation. The apparent molecular mass (*M<sub>r</sub>*) of protein standards is indicated.

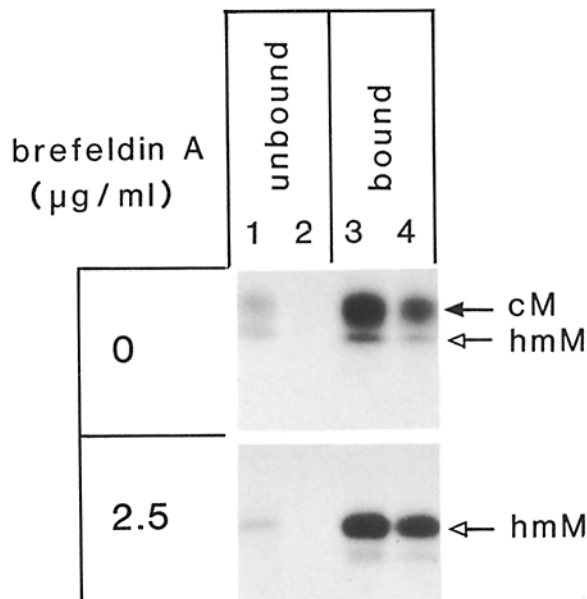


**Figure 7.** Maturation of MPR 46 under conditions that block transport to the Golgi apparatus. BHK-MPR46 cells were labeled for 5 min with [<sup>35</sup>S]methionine at 37°C. Where indicated, an 1 h chase at 37°C in the presence or absence of 3 mM dinitrophenol (DNP), or an up to 5 h chase at 16°C was performed. A detergent extract of the cell membranes was subjected to phosphomannan affinity chromatography using 10 mM glucose 6-phosphate and 10 mM mannose 6-phosphate for elution. MPR 46 was immunoprecipitated from the flow-through (lane 1), the glucose 6-phosphate eluate (lane 2) and the mannose 6-phosphate eluates (lanes 3 and 4) and analyzed by reducing SDS-PAGE and fluorography. (Top) Fluorogram of the gel section containing MPR 46; (open arrows) high-mannose type monomeric MPR 46; (arrows) complex glycosylated monomeric MPR 46; (open arrowheads) proteolytic fragment of MPR 46 created during membrane preparation. Bottom, quantitation of bound MPR 46. For each time point, the values are expressed as percent of total MPR 46 recovered from the affinity column. Except for the chase in the presence of DNP, all values represent the mean of two independent experiments. The bars indicate the values of individual experiments.

46 with higher concentrations of DSS (Stein et al., 1987b; Waheed et al., 1989) or by cross-linking of MPR 46 in intact cells using a membrane-permeable cross-linker (Hille, A., unpublished observations). With the membrane extracts used here, it was not possible to investigate the presence of tetramers of MPR 46, because treatment of the extracts with DSS at concentrations higher than 0.06 mM resulted in the formation of a heterogeneous population of receptor-related aggregates (100–300 kD *M*.) obscuring the detection of tetramers in SDS-PAGE (not shown). Therefore, we cannot

decide whether formation of tetramers occurs simultaneously with the acquisition of ligand-binding activity of MPR 46, or whether dimers are assembled to tetramers only later during intracellular transport of MPR 46.

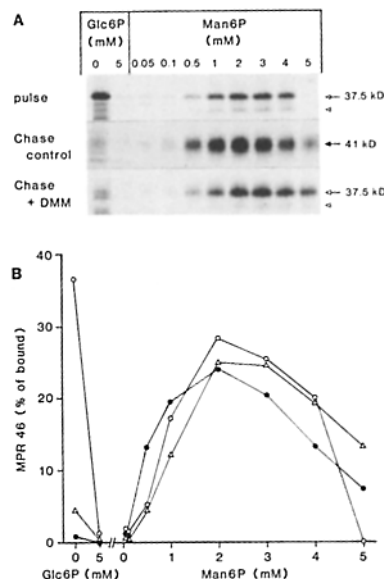
We have shown recently that transfer of N-linked high-mannose oligosaccharide cores is a prerequisite for the acquisition of the ligand-binding conformation of MPR 46 (Hille et al., 1989). Here we show that processing of the high-mannose oligosaccharides in the *trans*-Golgi did not significantly change the binding affinity for immobilized



**Figure 8.** Maturation of MPR 46 in cells treated with brefeldin A. BHK-MPR 46 cells were treated with or without 2.5 µg/ml of brefeldin A during a 1 h preincubation, 30 min labeling with [<sup>35</sup>S]methionine, and 1 h chase at 37°C. A detergent extract of the cell membranes was subjected to phosphomannan affinity chromatography. MPR 46 was immunoprecipitated from the flow-through (lane 1), the glucose 6-phosphate eluate (lane 2), and the mannose 6-phosphate eluates (lanes 3 and 4), and analyzed by reducing SDS-PAGE. The fluorogram of the gel section containing MPR 46 is shown. (*cM*) complex glycosylated monomeric MPR 46; (*hmM*) high-mannose type monomeric MPR 46; (*open arrowhead*) proteolytic fragment of MPR 46 created during membrane preparation.

phosphomannan. It remains to be established which properties of MPR 46, e.g., intracellular transport or stability against proteases, are regulated by terminal glycosylation.

Several lines of evidence suggest that MPR 46 acquires a phosphomannan-binding conformation in a pre-Golgi compartment. First, in pulse-chase experiments, ligand-binding activity was observed before core-glycosylated MPR 46 was processed to complex glycosylated forms. Second, MPR 46 acquired a ligand-binding conformation under conditions which have been reported to block transport from the ER to the Golgi complex. At 16°C, transport seemed to be efficiently blocked for at least 5 h of chase, whereas >65% of MPR 46 had achieved a ligand-binding conformation during this time. Also when transport was blocked by the drug brefeldin A (Fujiwara et al., 1988), MPR 46 was efficiently converted to a ligand-binding conformation during a 1-h chase. A recent report showing that treatment of cells with brefeldin A may cause transport of enzymes from the cis and middle Golgi back to the ER (Lippincott-Schwartz et al., 1989) raises the question whether maturation of MPR 46 in the presence of brefeldin A was an ER-specific process, or whether it might have been caused by enzymes recycling from the Golgi to the ER. To address this question, we have investigated the carbohydrate processing of MPR 46 after brefeldin A treatment of cells. Under the conditions used here, the oligosaccharides of MPR 46 were found to remain completely sensitive to endo H indicating that they had not been trimmed by Golgi-mannosidase II (data not shown).



**Figure 9.** Binding affinity of high-mannose forms and complex glycosylated MPR 46 to phosphomannan. BHK-MPR46 cells were labeled for 30 min with [<sup>35</sup>S]methionine followed by a 5 h chase in the presence or absence of deoxymannojirimycin (*DMM*). A detergent extract of the cell membranes was subjected to phosphomannan affinity chromatography. After washing the column with 5 mM glucose 6-phosphate, MPR 46 was eluted stepwise with 0.05–5 mM mannose 6-phosphate. MPR 46 was immunoprecipitated from the eluates and analyzed by reducing SDS-PAGE and fluorography. (*Top*) fluorogram of the part of the gels containing MPR 46. (*Arrow*) complex glycosylated monomeric MPR 46; (*open arrow*) high-mannose type monomeric MPR 46; (*open arrowhead*) proteolytic fragment created during membrane preparation. (*Bottom*) quantitation of MPR 46 in the column eluates. After immunoprecipitation and SDS-PAGE, MPR 46 was quantitated by densitometric scanning of fluorograms. (○—○) 30 min pulse; (●—●) 5 h chase; (△—△) 5 h chase in the presence of deoxymannojirimycin.

Thus, MPR 46 has not been transported out of the ER in the presence of brefeldin A. If Golgi-mannosidase II has been recycled to the ER due to the presence of brefeldin A, it did not act on MPR 46 under the conditions used. Another inhibitor of transport, dinitrophenol, acts by depleting ATP that is required for the formation of transport vesicles (Balch et al., 1986; Copeland et al., 1988). As with the other inhibitors, MPR 46 was converted efficiently to a ligand-binding conformation in the presence of dinitrophenol, but transport

**Table 1. Time Course of Assembly and Maturation of MPR 46 In Vivo**

Translocation	}	Cotranslational
Core glycosylation		
Disulfide bond formation		<5 min after translation
Conformational change	}	≈5 min after translation, <i>t</i> <sub>1/2</sub> ≈ 12 min
→ Trypsin resistance		
→ Highly immunogenic epitopes		
Dimer formation		
Phosphomannan binding		
Carbohydrate processing		≈30 min after translation



was not completely blocked. 6–7% of MPR 46 were found to be transported to the *trans*-Golgi per hour of chase. Therefore, it was difficult to evaluate whether the core-glycosylated forms of MPR 46 found after treatment with dinitrophenol were in fact retained in the ER, or whether they had reached the *cis*-Golgi but trimming to endo H-resistant forms by Golgi-mannosidase II had not yet been performed. The latter possibility seems unlikely, however, since exit from the ER has been reported to be the rate-limiting step for transport through the ER-Golgi pathway, and no transport block in the *cis* or middle Golgi has been reported so far (for review see Lodish, 1988). Additional evidence obtained recently by *in vitro* translation and translocation of MPR 46 into dog pancreas microsomes supports the conclusion that maturation of MPR 46 occurs in the ER, although maturation of MPR 46 *in vitro* was incomplete (at most 25% of MPR 46 translated *in vitro* achieved a ligand-binding conformation) (Hille et al., 1989). Together, our results suggest that MPR 46 achieves its ligand-binding conformation in a pre-Golgi compartment. Due to the leakiness of the block by dinitrophenol, we cannot rule out that maturation of the ligand-binding conformation takes place in intermediate elements that are involved in transport between ER and Golgi.

That MPR 46 seems to acquire its phosphomannan-binding conformation before reaching the Golgi complex is remarkable, as phosphomonoesters of the high-mannose oligosaccharides of lysosomal enzymes, which are recognized by MPR 46, are formed only in the *trans*-Golgi (Lazarino and Gabel, 1988). Therefore, the presence of phosphomannan-binding MPR 46 in a pre-Golgi compartment most likely does not allow the conclusion that the receptor fulfils a transport function at this early stage in the secretory pathway. On the other hand, the results reported here for MPR 46 are in line with previous reports on other oligomeric membrane proteins that exert their physiological functions at the plasma membrane, but are folded and assembled in the ER (Copeland et al., 1986; Copeland et al., 1988; Doms et al., 1987; Gething et al., 1986; Kvist et al., 1982; Olson et al., 1988; Smith et al., 1987). Therefore, a general function to control the folding and assembly of newly synthesized proteins of the secretory pathway has been ascribed to the ER (Gething et al., 1986). This hypothesis has received further support by the discovery of a proteolytic pathway that takes place in a compartment closely related to the ER and may serve to dispose misfolded or incompletely assembled newly synthesized proteins (Chen et al., 1988; Lippincott-Schwartz et al., 1988).

The structural characteristics reported here for the phosphomannan-binding conformation of MPR 46 may be useful to study several biochemical or cell biological issues. First, in the characterization of MPR 46 mutants that show altered binding properties or altered intracellular transport, the structural analysis may help to discriminate mutations that affect directly the ligand-binding site or signals for intracellular transport from mutants that disturb these functions indirectly by altering the protein conformation of the receptor. Second, to find out why MPR 46, unlike MPR 215, is not involved in ligand binding at the cell surface (Stein et al., 1987c), it will be interesting to investigate whether MPR 46 in the plasma membrane differs from the intracellular pool of MPR 46 with respect to its conformation or quaternary structure.

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