CHAPTER - IV

Interaction of purified goat and chicken MPR 300 proteins with human IGF-II
INTRODUCTION:

Among the two receptors characterized from mammals, only MPR 300 has so far been shown to be a multifunctional protein which in addition to binding mannose 6-phosphate containing lysosomal enzymes, also binds IGF-II and other ligands such as retinoic acid and thyroglobulin (Hille-Rehfeld, 1995; Kang et al, 1998). In humans it has been shown that this receptor plays an important role in controlling the extracellular level of the insulin-like growth factor II (IGF-II) by mediating its binding at the cell surface and delivery to lysosomes (Kornfeld 1992). The luminal ligand-binding domain of MPR 300 contains 15 internal repeats, which are homologous to the luminal domain of MPR 46. It is therefore of interest to study the evolution and functions of these receptor proteins in order to analyze their biochemical and ligand binding properties. Receptors purified from rat, bovine, human and some other mammalian species such as the opossum exhibited IGF-II binding abilities (Schmidt et al, 1995) and the IGF-II binding domain in mammals has been localized to repeat 11 of this multifunctional receptor. The biochemical and immunological properties of the purified MPR 300 protein from goat, chicken, garden lizard, fish and Unio resemble those of the mammalian receptor (Siva Kumar et al, 1997; 1999; Udaya Lakshmi e al, 1999). Further a partial cDNA clone for the fish MPR 300 has been shown to contain the highly conserved mannose 6-phosphate binding region in the third domain similar to other vertebrate receptors (Udaya Lakshmi et al., 2000). When purified MPR 300 from chicken and Xenopus (non-mammalian vertebrates) was tested for its binding to human IGF-II, the receptors failed to bind IGF-II, under the conditions used and it was suggested that possibly acquisition of IGF-II binding site by the receptor is a late event in evolution (Canfield and Kornfeld 1989; Clairmont and Czech 1989). In a
recent study it has been shown that the MPR 300 from the earliest non-mammalian vertebrate, fish, binds human IGF-II under specific conditions (Mendez et al., 2001). This recent observation led us to believe that possibly the IGF-II binding property of the MPR 300 is also conserved in all non-mammalian vertebrates as our earlier studies demonstrated that the arginine residue in domain 3 of the MPR 300 that is responsible for ligand binding is evolutionarily conserved (Udaya Lakshmi et al., 2000). The objective of the present study was to investigate whether the MPR 300 receptors purified by us from goat and chicken possess IGF-II binding ability. To address this question four different assays were done. i) a cross-linking assay using $^{125}$I-IGF-II ii) a ligand blot assay using biotinylated IGF-II. iii) a filter binding assay using $^{125}$I-IGF-II and iv) an internalization assay employing CEF cells and $^{125}$I-IGF-II as the CEF cells have been shown to contain MPR 300 protein (Matzner et al., 1996).
**MATERIALS:**

Biotinylated IGF-II : Generously provided by Prof. Dr. Thomas Braulke, Hamburg, Germany.

BSA : Sigma

DMEM medium : Sigma

DSS (Disuccinimidyl suberate) : Sigma

ECL (Enhanced chemiluminescence) reagent: Pierce

HEPES : Sigma

Nitocellulose filters : Millipore

Polyethylene glycol : Sigma

Tris : Qualigens

Triton X 100 : Sigma

\(\gamma\)-globulin : Sigma

**Reagents:**

**Krebs Ringer phosphate buffer:**

(A) 0.15M NaCl

(B) 0.15M KCl

(C) 0.11M CaCl₂

(D) 0.15M MgSO₄·7H₂O

(E) 10mM Na₂HPO₄ pH 7.4

To prepare the Krebs Ringer phosphate buffer above solutions were mixed in the following proportions:

100 parts of A + 4 parts of B + 3 parts of C + 1 part of D + 20 parts of E.

**Equipment**

\(\gamma\)-ray counter : ECIL, India.
METHODS:

**Affinity cross-linking of $^{125}$I IGF-II with purified MPR 300 from goat and chicken**

Crosslinking studies were carried out as described by Causin et al., (1989). About 5 μg of purified receptors were acetone precipitated and the precipitate was solubilized in 37ul of 50mM-sodium phosphate, pH 7.4, containing 150 mM NaCl, 0.1% Triton X-100 and 0.1% bovine serum albumin and incubated for 20 min at 4°C with or without 200nM or 400nM unlabelled IGF-II. 13ul of $^{125}$I-IGF-II, (2,50,000 cpm) was added and incubated for 3h at 4°C. Crosslinking of bound IGF II with receptor was performed with 1mM-DSS by incubating the reaction mixture for 15 min. at 4°C. The reaction was stopped with 5 ul of 1 M-Tris/HCl pH 7.4, 2.75 ul of 20% SDS and 3 ul of glycerol (100%). The reaction mixture was heated at 95°C for 5 min. and the samples were subjected to 7.5% SDS-PAGE. The radiolabeled bands were visualized by autoradiography using a Kodak X-ray film with light intensifying screens.

**Immunoprecipitation**

Immunoprecipitation was carried out as described by Mendez et al., (2001). About 6 μg of purified mannose 6-phosphate receptor proteins from both goat and chicken were taken and incubated for 16 h at 4°C with $^{125}$I-IGF-II (25 pM) in HEPES buffer pH 7.6. After the incubation, cross-linking was carried out with 1mM DSS on ice for 15 min. The reaction was stopped by adding 10mM Tris-HCl buffer pH 7.4. Subsequently, receptor preparations were incubated with or without 10 μg of affinity purified IgG against the goat MPR 300 protein for 8h at 4°C with constant agitation. The immune complexes were then
collected using Protein A-agarose and washed with PBS containing 0.05% Tween 20. The complexes were solubilized by boiling at $95^\circ C$ for 5 min. in SDS sample buffer and subjected to 7.5% SDS-PAGE. The radiolabeled bands were visualized by autoradiography as described above.

**Binding of biotinylated IGF-II to the purified MPR 300 from goat and chicken:**

Binding was carried out as described by Shalamanova et al. (2000). About 1 ug of purified goat and chicken liver receptor (MPR 300) were electrophoresed on 7.5% SDS-PAGE under non-reducing conditions. The proteins were transferred on to a nitrocellulose membrane overnight at 4°C and the membrane was blocked for one hour at room temperature in PBS containing 0.1% Tween 20 and 1% BSA and incubated overnight at 4°C in monobiotinylated-IGF-II (20ng/ml) in PBS containing 0.1% Tween 20 and 1% BSA. The membrane was washed consecutively with PBS containing 0.1% Tween 20 (1 x 15 min, and 4 x 5 min) and incubated with peroxidase-conjugated streptavidin for one hour at room temperature in PBS containing 0.1% Tween 20 and 1% BSA. Finally the membrane was washed consecutively with PBS, 0.1% Tween 20 (1 x 15 min, and 4 x 5 min). The membrane was treated with ECL reagent (Pierce) for one minute and the blot analyzed using a Kodak X-ray film.

**Quantitation of $^{125}$I-IGF-II binding to the purified MPR 300 from goat and chicken using the Polyethylene Glycol Precipitation Method**

This was carried out as described previously (Clairmont and Czech 1989) with few modifications. 0.1ml of a stock solution of 4nM $^{125}$I-IGF-II and unlabeled IGF-II at 0, 10, 40, 100 and 400 nM were combined with an equal volume of a solution of purified receptors (goat and chicken MPR 300) containing 4 ug of protein. Following incubation
for 2h at 3°C, 0.5ml of 0.9mg/ml bovine γ-globulins in 0.1M sodium phosphate buffer, pH 7.4, and 0.5% polyethylene glycol were added to each sample, the sample was mixed and incubated at 0°C for 15 min. This mixture was then filtered through nitrocellulose filters (0.45μm) which were blocked by incubation in Krebs Ringer phosphate buffer containing 1% bovine serum albumin. The filters were washed three times with 8% polyethylene glycol and the γ-radiation from the filters measured in a γ-ray counter.

**Cell culture studies**

Chicken embryonic fibroblast (CEF) cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal calf serum. Cells were grown in standard flasks as well as in 3cm petri plates in an incubator at 37°C in an atmosphere containing 5% CO2 as described Matzner et al., (1996).

**IGF-II binding assays in CEF cells**

Internalization assay was carried out as described recently for fish receptor (Mendez et al., 2001). Cells grown in 3cm petriplates (2 million cells) as confluent monolayers were washed twice for over 2h in 0.1 M HEPES containing 0.12M NaCl, 5mM KCl, 1.2mM MgSO4, 8mM glucose and 0.5% BSA (binding buffer, pH 7.6) and were incubated for 4h at 4°C in 0.5ml of the same buffer containing 125I-IGF-II (25 pM) in the presence or absence of unlabelled IGF-II (200nM), insulin (100nM), mannose 6-phosphate (5mM) and affinity purified IgG for MPR 300 protein (15 μg). Subsequently, the cells were washed twice with the binding buffer and burst open with 0.5N NaOH for the determination of radioactivity.
RESULTS:

Purification of the MPR 300 protein by affinity chromatography:

The total membrane protein extracts obtained from the liver tissues of mammalian (goat) and non-mammalian vertebrate (chicken) were passed through two separate PM gels, and the gels were washed extensively with the column buffer. The bound protein was eluted from both gels with 5mM mannose 6-phosphate in the column buffer. The eluted fractions were concentrated and aliquots subjected to SDS-PAGE under reducing conditions. Consistent with our earlier findings (Siva Kumar et al., 1996) in both the species the purified MPR 300 behaved as a single protein band corresponding to molecular mass of about 300 kDa (Figure 18). The yield of the MPR 300 was comparable with earlier data.

Affinity crosslinking of $^{125}$I-IGF-II to MPR 300 from goat and chicken

Purified receptors were subjected to affinity labeling using $^{125}$I-IGF-II and disuccinimidyl suberate (DSS) as the cross-linker in the presence of 200nM and 400nM of unlabeled IGF-II. Purified goat and chicken MPR 300 protein showed efficient affinity labeling with $^{125}$I-IGF-II under the conditions used, as is evident from the autoradiogram suggesting that both goat and chicken MPR 300 proteins bind human IGF-II. In the presence of unlabeled IGF-II (200nM) (Figure 19A), there was a partial inhibition of affinity labeling and in the presence of unlabeled IGF-II (400nM) (Figure 19B), there was almost complete inhibition of affinity labeling.

In a separate experiment the identity of this cross-linked product was further confirmed by immunoprecipitation using affinity purified goat MPR 300 IgG (Figure 19C). The results obtained clearly demonstrate that the cross-linked product seen in both species is the MPR 300 protein.
Figure 18: 10% SDS-PAGE of the purified Mannose 6-phosphate receptor proteins (MPR 300) under reducing conditions. Lane 1, Purified goat receptor (3μg), and Lane 2, purified chicken (2μg). Arrow indicates position of MPR 300.
Figure 19:  (A). Affinity labeling of the purified receptors with $^{125}$IGF-II.

Lanes 1, 2 goat, 3, 4 chicken. Lanes 1, 3, in presence of 200nm of unlabeled IGF-II, lanes 2 and 4 without unlabeled IGF-II.

(B). Affinity labeling of the purified receptors with $^{125}$IGF-II.

Lanes 1, 2 goat, 3, 4 chicken. Lanes 1, 3, in presence of 400nm of unlabeled IGF-II, lanes 2 and 4 without unlabeled IGF-II.

(C). Immunoprecipitation of the IGF-II-MPR 300 cross-linked product with affinity purified goat MIR 300 IgG. Lane 1, goat, Lane 2, chicken.
Figure 19

(A) 200nM of Unlabeled IGF-II

(B) 400nM Unlabeled IGF-II

(C)
**Binding of biotinylated IGF-II to purified MPR 300 from goat and chicken**

The ability of the purified receptors from goat and chicken to bind *monobiotinylated* IGF-II was tested as described under methods. Both goat and chicken MPR 300 showed binding to IGF-II (Figure 20).

**Affinity of **$^{125}$I-IGF-II** to MPR 300 from goat and chicken:**

To analyze the binding property of the purified receptors to $^{125}$I-IGF-II, $^{125}$I-IGF-II was incubated with 2nM $^{125}$I-IGF-II in a final volume of 200 $\mu l$ in the presence of increasing concentrations of unlabelled IGF-II as described under methods. The radioactivity bound to the nitrocellulose filter was measured using a γ-ray counter and the data was analyzed by Scatchard analysis (Figure 21). This suggests that both goat and chicken receptor show IGF-II binding.

**Internalization assay of the **$^{125}$I-IGF-II:**

In order to analyze if the Chicken Embryonic Fibroblast (CEF) MPR 300 protein can internalize $^{125}$I-IGF-II, various experiments were carried out as described under methods. The amount of radioactivity internalized by the CEF cells when incubated with 25pM of $^{125}$I-IGF-II was taken as 100% (control). The results are shown in Figure 22. When the cells were pre-incubated with either unlabeled IGF-II or affinity purified specific IgG for goat MPR 300 protein followed by incubation with $^{125}$I-IGF-II, internalization was inhibited. When the cells were pre-incubated either with insulin (100nM) or 5mM mannose 6-phosphate followed by incubation with $^{125}$I-IGF-II, internalization of the radioactivity could be seen. The data indicates that the MPR 300 from CEF cells has both mannose 6-phosphate as well as IGF-II binding sites and is able to internalize $^{125}$I-IGF-II.
**Figure 20:** Interaction of purified goat and chicken receptor with Biotinylated IGF-II. Details as given under text. Lane 1 goat and lane 2 chicken. Arrow indicates position of the receptor.

**Figure 21:** Scatchard analysis of IGF-II binding to affinity purified MPR 300 proteins from (a) goat and (b) chicken in presence of increasing concentrations of unlabeled IGF-II. Details as given in text. B, Bound IGF-II and B/F bound/free. Results are average of five and three experiments for goat and chicken respectively.

**Figure 22:** Internalization of labeled IGF-II by CEF cells. Internalization of labeled IGF-II assessed after preincubation of the cells with unlabeled IGF-II (A), affinity purified anti-goat IgG (B), insulin (C), mannose 6-phosphate (D) and control (E).
**DISCUSSION:**

Mannose 6-phosphate receptors, MPR 300 and MPR 46 have earlier been affinity purified employing phosphomannan Sepharose gel from different mammals and non-mammalian vertebrates (Siva Kumar *et al.*, 1997; 1999) and these proteins exhibited similar biochemical and immunological properties as that of the well studied mammalian receptors (Hille-Rehfeld, 1995). The luminal domain of the MPR 46 displays 14-37% similarity to the 15 internal cassettes of MPR 300 as shown for the bovine MPRs (Lobel *et al.*, 1998). This finding led to the assumption that MPR 300 has evolved from MPR 46 by repeated gene duplication events and raised the question at what stage in evolution MPR 300 has occurred for the first time. First evidence for the presence of both MPR proteins among the CEF cells came from the studies of Matzner *et al.*, (1996).

Earlier studies have shown that different mammalian MPR 300 proteins bind human IGF-II, (Schmidt *et al.*, 1995). Though it has been shown that the chicken and *Xenopus* MPR 300 lack the IGF-II binding site under the conditions used by the authors (Canfield and Kornfeld 1989; Clairmont and Czech 1989), in a recent study it has been well established that the fish MPR 300 has the IGF-II binding ability (Mendez *et al.*, 2001) and in this study the authors suggested that failure to detect IGF-II binding in chicken and *Xenopus* by earlier authors is possibly due to the different conditions that were employed in their study. In the light of this recent finding that fish receptor binds IGF-II (Mendez *et al.*, 2001), in the present study we have carried out detailed analysis on the ability of purified goat MPR 300 (which has not been studied earlier) as well as that of chicken MPR 300 protein to bind human IGF-II.
In order to conclusively establish that the IGF-II binds to purified MPR 300, we have made cross-linking studies where radioiodinated IGF-II was cross-linked with the purified receptors employing 1mM DSS. The results indicate that the goat and the chicken receptors showed affinity cross-linking with labeled IGF-II. When these experiments were conducted in presence of 200nM unlabeled IGF-II, inhibition of affinity labeling was observed in both species (Figure 19A, lane 1 and 3) suggesting that the interaction seen with goat and chicken receptors and labeled IGF-II is indeed specific. When the concentration of the unlabeled IGF-II was increased to 400nM, using goat receptor alone there was complete inhibition of affinity labeling (Figure 19B, lane 1 and 3). Similar observations were noticed with the purified rat and human MPR 300 protein (Clairmont and Czech 1989; Causin et al., 1989).

Further confirmatory evidence that the cross-linked product represents the MPR 300 and the labeled IGF-II in both species, was established by specific immunoprecipitation of the product with affinity purified goat MPR 300 IgG (Figure 19C). It has been shown that the receptors from different vertebrate species can be recognized by goat MPR 300 antiserum (Siva Kumar et al, 1997). Further, the fish MPR 300 protein has been shown to react specifically with anti-MPR 300 rat antiserum (Mendez et al., 2001).

In recent years biotinylated IGF-IIls have been employed for determining their binding abilities to their receptors in a dot blot analysis using simple and sensitive detection by streptavidin-coupled HRP and enhanced chemiluminiscence (ECL). The high reliability and sensitivity of the biotinylated IGF-II was exploited in this study to investigate the binding of goat and chicken MPR 300 to IGF-II. The efficient binding of both receptors to
biotinylated IGF-II in this assay further support the above experimental findings that the
MPR 300 from both species indeed binds human IGF-II.

The results of the filter-binding assay using purified goat and chicken MPR 300 proteins
suggest that both receptors bind human IGF-II under the conditions used. Scatchard
analysis indicates a Kd value of 12.5nM for goat MPR 300 and 11.1nM for the chicken
MPR 300. The Kd values of bovine and opossum were reported to be 0.2nM and
14.5nM respectively (Dahms et al., 1993) and that of fish was found to be 0.12nM (Mendez et al,
2001). The results obtained for goat receptor is consistent with the findings of other
workers who showed that mammalian MPR 300 has IGF-II binding ability (Schmidt et al,
1995). The only variance is with respect to the chicken receptor.

In order to support the data obtained for chicken receptor, we employed CEF cells in our
studies, as these cells have been already shown to contain the MPR 300 protein (Matzner
et al, 1996) and there is no published information on the use of these cells to analyze the
IGF-II binding and internalization.

Experimental results indicate that the internalization of the labeled IGF-II by the CEF cells
is mediated by the MPR 300 protein. When the cells were preincubated with cold
(unlabeled) IGF-II (200nm or 400nm) followed by labeled IGF-II, 86% inhibition of
internalization was observed. When cells were incubated with affinity purified MPR 300
IgG (15 μg), followed by labeled IGF-II 84%, inhibition of internalization was observed.
However, when cells were incubated with insulin (100nM), only 5% inhibition could be
seen. When the binding sugar, 5mM mannose 6-phosphate was used to preincubate the
cells, followed by labeled IGF-II only 2% inhibition was seen. It is already well
established that the mannose 6-phosphate binding site and the IGF-II binding site in mammalian MPR 300 are distinct (Schmidt et al., 1995) and hence no effect of internalization in presence of mannose 6-phosphate is seen. Similarly insulin which has no binding to the receptor also shows no specific effect. However, cold IGF-II either at 200nm or 40(nm concentration showed distinct inhibitory effect. Additionally, an affinity purified goat MPR 300 antibody that is known to recognize mammalian and chicken MPR 300 protein (Siva Kumar et al., 1999) shows significant inhibition. Taken together, these results support the involvement of MPR 300 in internalization of $^{125}$I-IGF-II in CEF cells.

In mammals, the IGF-II binding site of MPR 300 has been localized to amino acid residues from 1508-1566 in the amino terminal domain 11 (Schmidt et al., 1995). cDNA sequence obtained for chicken MPR 300 (Zhou et al., 1995) from domain 11 reveals that there is divergence in the amino acid sequence compared to human MPR 300. However, in the present study we found that the CEF cells which are known to contain MPR 300 protein, can internalize labeled IGF-II and the internalization is blocked by MPR 300 specific IgG but not by mannose 6-phosphate. These data suggest that possibly the CEF MPR 300 internalizes the labeled IGF-II. In view of the recent observation that the fish MPR 300 can bind IGF-II, the results obtained by us additionally confirms that the MPR 300 protein among the non-mammalian vertebrates also has the ability to bind IGF-II. Due to the non-availability of a reptilian cell line and a purified receptor from that species this was not tested.

Since, the complete fish MPR 300 sequence is not published and the evidence that chicken MPR 300 is able to bind labeled IGF-II, (though primary sequence suggests a change in the amino acid that is critical for binding IGF-II in chicken MPR 300), it is possible that
other regions of the receptor may be aiding in the binding of IGF-II. This can possibly be validated as in a recent study it has been shown that the IGF-II binding site of the mammalian IGF-II/MPR is bipartite: the primary determinants for binding reside in domain 11 while sequence elements within domain 13 contribute a ~5-10 fold enhancement of the binding affinity of the receptor for IGF-II (Devi et al., 1998; Grimme et al., 2000; Linnell et al., 2001).

Four lines of evidence suggest that the IGF-II binding property is exhibited by both the purified receptors. First, purified receptors show affinity cross-linking with labeled IGF-II and this is specific as evidenced by immunoprecipitation of these employing goat MPR 300 specific IgG that is known to immunoprecipitate goat, as well as, chicken MPR 300 proteins. Second, both the receptors show specific reactivity with biotinylated IGF-II. Third, both the receptors (goat and chicken MPR 300) bind radiolabeled IGF-II in a filter-binding assay. Fourth, the CEF cells show specific internalization of the labeled IGF-II which can be inhibited by unlabeled IGF-II and affinity purified goat MPR 300 IgG but not by insulin or mannose 6-phosphate. The fact that the goat receptor binds to phosphomannan Sepharose gel in a mannose 6-phosphate dependent manner and its ability to bind human IGF-II suggests that it possibly functions in a similar way as the human or rat liver MPR 300 protein. These observations are consistent with the recent finding on the imprinting of m6p/IGF2 receptor gene in mammals (Killian et al., 2000). The goat MPR 300 has been shown to contain repeating cassette structures in the extracellular domain similar to other known receptors (Udaya Lakshimi et al., 2000) discussed in chapter V. It remains to be established whether the goat receptor has any additional ligand binding
properties like the other mammalian receptors. Table 7 below summarizes the information on the IGF-II binding ability of known MPR 300 proteins.

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>IGF-II binding property</th>
<th>Reference</th>
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<tbody>
<tr>
<td>MAMMALIAN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Yes</td>
<td>Tong et al., 1988.</td>
</tr>
<tr>
<td>Bovine</td>
<td>Yes</td>
<td>Morgan et al., 1987.</td>
</tr>
<tr>
<td>Rat</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Goat</td>
<td>Yes</td>
<td>Present study</td>
</tr>
<tr>
<td>Opossum</td>
<td>Yes</td>
<td>Dahms et al., 1993.</td>
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<tr>
<td>Kangaroo</td>
<td>Yes</td>
<td>Yandell et al., 1999.</td>
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<td>NON-MAMMALIAN VERTEBRATES</td>
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<tr>
<td>Chicken and Frog</td>
<td>No</td>
<td>Canfield and Kornfeld, 1989; Clairmont and Czuch, 1989.</td>
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<tr>
<td>Chicken</td>
<td>Yes</td>
<td>Present study</td>
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<tr>
<td>Reptilian</td>
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<tr>
<td>Fish</td>
<td>Yes</td>
<td>Mender et al., 2001.</td>
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<tr>
<td>INVERTEBRATES</td>
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<tr>
<td>Mollusc</td>
<td>ND</td>
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ND: Not Determined

*Table 7: IGF-II binding ability of some of the known MPR 300 proteins.*